

Signal Transduction and Signal Modulation by Cell Adhesion Receptors: The Role of Integrins, Cadherins, Immunoglobulin-Cell Adhesion Molecules, and Selectins

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I. Introduction

Cell adhesion is critical for the genesis and maintenance of both three-dimensional structure and normal function in tissues. The biochemical entities mediating cell adhesion are multiprotein complexes comprising three broad classes of macromolecules: the adhesion receptors, the extracellular matrix molecules, and the adhesion plaque proteins (Gumbiner, 1996). Cell adhesion receptors are typically transmembrane glycoproteins that mediate binding to extracellular matrix (ECM)^b molecules or to counter-receptors on other cells; these molecules determine the specificity of cell-cell or cell-ECM interaction. The ECM proteins are usually fibrillar in nature and provide a complex structural and functional network that can interact simultaneously with multiple cell surface receptors. The intracellular plaque proteins (or peripheral membrane proteins) provide structural and functional linkages between adhesion receptors and the actin microfilaments, microtubules, and

^b Abbreviations: AA, arachidonic acid; APC, adenomatous polyposis coli; CAK β , cell adhesion kinase β ; CAS, Crk-associated substrate; CDI, cyclin-dependent kinase inhibitor protein; CEF, chicken embryo fibroblast; CHD, CAM-homology domain; CHO, Chinese hamster ovary; CIB, calcium- and integrin-binding protein; CSK, C-terminal Src kinase; ECM, extracellular matrix; EGF, epidermal growth factor; ERM, ezrin/radixin/moesin; FAK, focal adhesion kinase; FAT, focal adhesion targeting; FGF, fibroblast growth factor; FRNK, FAK-related nonkinase; GAP, GTPase-activating protein family; GDP, guanosine 5'-diphosphate; GNEF, guanine nucleotide exchange factor; GRAF, GTPase regulator associated with FAK; GRB2, growth factor receptor-binding protein 2; GSK3, glycogen synthase kinase 3; GTPase, guanosine 5'-triphosphatase; IAP, integrin-associated protein; IBP, integrin-binding protein; ICAM, intercellular adhesion molecule; ICAP, integrin cytoplasmic domain-associated protein; Ig-CAM, immunoglobulin-cell adhesion molecule; IL, interleukin; ILK, integrin-linked kinase; IP₃, inositol triphosphate; IRS-1, insulin receptor substrate 1; JNK, Jun N-terminal kinase; LIM, Lin-II, Islet I and Mec 3; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MEK, MAPK-ERK kinase; MEKK-1, MEK Kinase-1; MHC, major histocompatibility complex; MLC, myosin light chain; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; NCAM, neural cell adhesion molecule; NGF, nerve growth factor; PA, phosphatidic acid; PAF, platelet-activating factor; PDGF, platelet-derived growth factor; PECAM-1, platelet endothelial cell adhesion molecule-1; PH, plekstrin homology; pH_i, intracellular pH; PI-3K, phosphatidylinositol-3-kinase; PIP₂, PI(4,5)P₂, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLA₂, phospholipase A₂; PLC, phospholipase C; PLD, phospholipase D; PMN, polymorphonuclear leukocyte; PSGL-1, P-selectin glycoprotein ligand-1; PTP, phosphotyrosine phosphatase; PYK2, proline-rich tyrosine kinase 2; Rb, retinoblastoma; REF, rat embryo fibroblast; RPTP, receptor tyrosine kinase phosphatase; RTK, receptor tyrosine kinase; SAPK, stress-activated protein kinase; SH2, Src homology region 2; SH3, Src homology region 3; SRE, serum response element; SRF, serum response factor; SYK, spleen tyrosine kinase; TCF, ternary complex factor; TCR, T-cell receptor; TNF, tumor necrosis factor; uPAR, urokinase-type plasminogen activator receptor; WASP, Wiskott-Aldrich Syndrome protein.

intermediate filaments of the cytoskeleton. An exciting concept that has emerged from recent cell biological research is that cell adhesion complexes are not simply static architectural entities. Rather, they are dynamic units that are capable of capturing and integrating signals from the extracellular environment (Rosales *et al.*, 1995). Moreover, the functions of cell adhesion complexes are regulated precisely by biochemical events within the cell. Thus cell adhesion receptors are at a nexus of two-way signaling between the cell and its external environment. This review will examine current knowledge concerning signal transduction events initiated or modulated by cell adhesion receptors. The emphasis will be on the integrin family of receptors because their signaling functions have been studied more extensively than those of other adhesion receptor families. However, we also will examine signaling events involving the cadherin, immunoglobulin-cell adhesion molecule (Ig-CAM), and selectin families of adhesion receptors. Adhesion-mediated signaling influences several critical cellular processes including gene expression, cell cycle, and programmed cell death; these aspects will be explored. However, the major emphasis of this review will be on mechanisms of adhesion receptor signaling.

II. Families of Cell Adhesion Receptors

A. Overview

This section will review some of the key features of the integrin, cadherin, Ig-CAM, and selectin families of cell adhesion receptors. Because there are thousands of publications on these topics (Edelman, 1993), only a few research articles and reviews will be used here to introduce some key ideas about these molecules. A diagram of the structures of several types of adhesion receptors, their counter-receptors, and some of their associated cytoskeletal components is presented in fig. 1.

B. Integrins

The integrins are a family of cell-surface glycoproteins that act as receptors for ECM proteins, or for membrane-bound counter-receptors on other cells. Integrin-mediated cell-ECM adhesion sites are complex specialized structures termed focal contacts or focal adhesions (Jockush *et al.*, 1995) and are discussed in more detail in Section III. Each integrin is a heterodimer that contains an α and a β subunit with each subunit having a large extracellular domain, a single membrane-spanning region, and in most cases (other than $\beta 4$), a short cytoplasmic domain (Hynes, 1992; Rosales *et al.*, 1992; Ruoslahti, 1991). The integrin receptor family of vertebrates includes at least 16 distinct α subunits and 8 or more β subunits which can associate to form more than 20 dis-

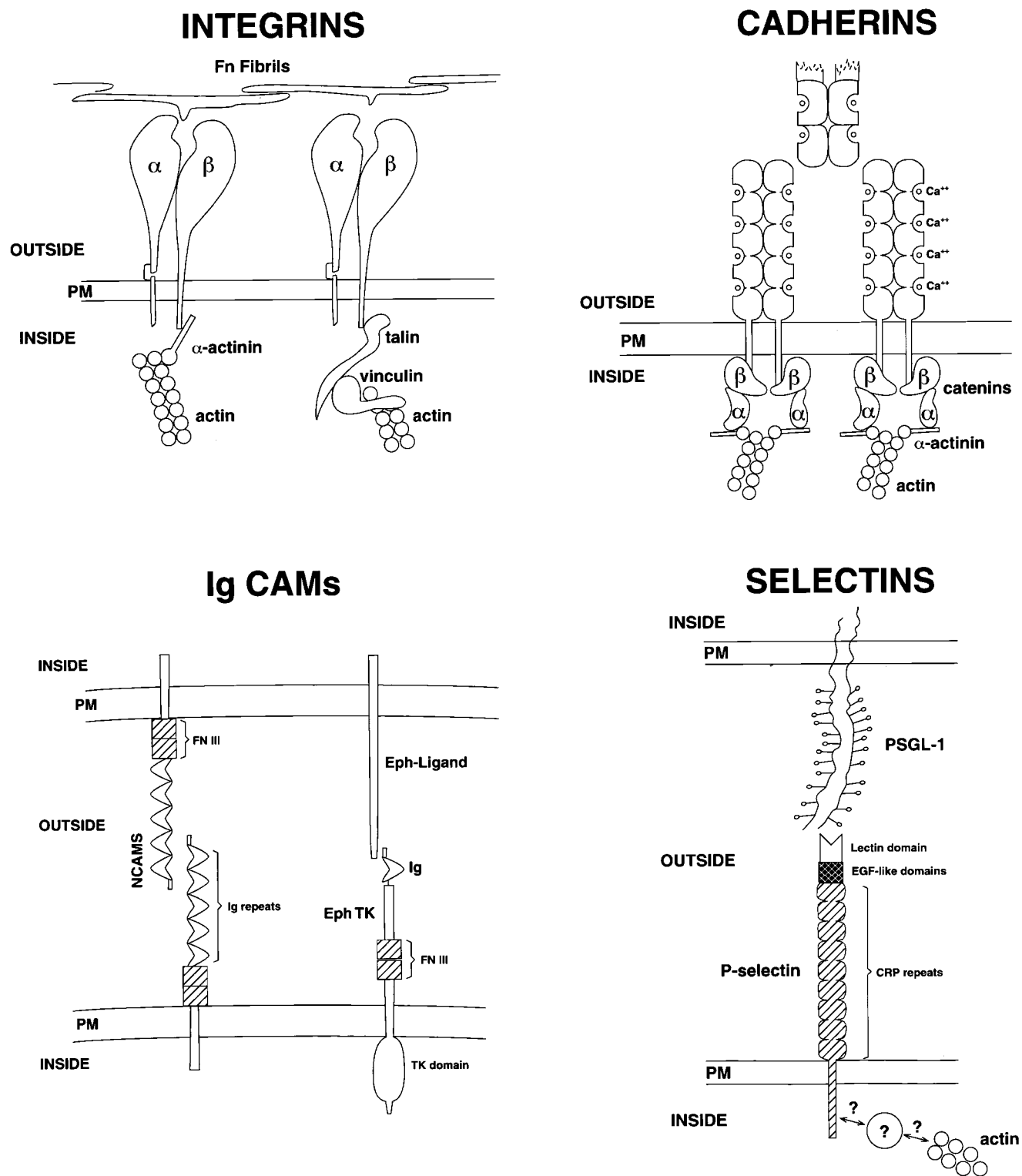


FIG. 1. Families of cell adhesion receptors. This diagram presents basic information about the integrin, cadherin, Ig-CAM, and selectin families of cell adhesion receptors. It illustrates their approximate structures, their ligands or counter-receptors, and some of their associated cytoskeletal proteins when they are known. A more detailed description is given in the text. The molecules illustrated here are not drawn to scale.

tinct integrins (Hynes, 1992; Rosales *et al.*, 1995). The α/β pairings specify the ligand-binding abilities of the integrin heterodimers. Although the ligands for integrins are often large ECM proteins such as collagen, laminin, vitronectin, or fibronectin, some integrins rec-

ognize rather short peptide sequences within the larger protein, for example, the RGD (Arg-Gly-Asp) sequence found in fibronectin and vitronectin. Because of this, there has been considerable interest in the pharmaceutical industry in developing short peptides or peptidomi-

metics that can interdict integrin functions in a variety of disease processes including coagulation disorders, inflammation, and cancer (Arap and Pasqualini, 1998; Ruoslahti, 1996). In other cases, however, integrin-ligand recognition depends on the overall conformation of the ligand protein (Kuhn and Eble, 1994). For example, some integrins interact with members of other adhesion receptor families, including Ig-CAMs and cadherins, in a manner that does not involve RGD motifs (Felding-Habermann *et al.*, 1997; Higgins *et al.*, 1998; Piali *et al.*, 1995). Some integrins, such as $\alpha 5 \beta 1$, the “classic” fibronectin receptor, bind to a single ECM protein. More generally, an individual integrin will recognize several distinct proteins (Hynes, 1992; Rosales and Juliano, 1995); for example, the $\alpha \nu \beta 3$ integrin has been reported to bind collagen VI, laminin, fibronectin, vitronectin, thrombospondin, Von Willebrand factor, and fibrinogen (Kuhn and Eble, 1994). Cells often display multiple integrins capable of interacting with a particular ECM protein, thus integrin expression is often apparently redundant, at least in terms of simple cell adhesion. Some integrin subunits undergo alternative splicing of their cytoplasmic domain regions in a tissue-type specific and developmentally regulated manner; this suggests that there are discrete intracellular functions for individual integrins, as we will discuss in much more detail below.

The relationships between integrin structure and the various functions of integrins are being actively investigated. In terms of ligand binding, it seems clear that both α and β subunit extracellular domains contribute to the formation of the binding site. The ligand binding regions of integrins have been explored with chemical cross-linking of ligands, monoclonal antibodies, mutation, and most recently, molecular modeling and X-ray crystallography (Loftus *et al.*, 1994; Qu and Leahy, 1995; Springer, 1997). Three regions apparently are particularly important: (a) a series of seven repeats of approximately 60 amino acids in the N-terminal portion of the α chain, each containing a putative Ca^{2+} binding site; (b) an inserted domain (I-domain) of approximately 200 amino acids found in several α chains and containing a nucleotide binding fold and a divalent cation coordination site; (c) an I-domain like region of approximately 250 amino acids found in the N-terminal region of the β subunit (Loftus and Liddington, 1997). The seven-repeat sequences of the α chain have been predicted by molecular modeling to form a β -propeller structure with the upper face of the propeller being the ligand binding region and the Ca^{2+} coordination motifs lying on the lower face (but probably essential to maintain the structure) (Loftus and Liddington, 1997; Springer, 1997). Propeller structures of this type have been found in a variety of other proteins, including the β subunit of trimeric G-proteins (Sondek *et al.*, 1996), and usually are involved in protein-protein interactions. The structure of the I-domain region has been determined by X-ray crys-

tallography (Lee *et al.*, 1995; Qu and Leahy, 1995); the divalent ion binding motif has been designated the “metal ion-dependent adhesion site” and seems to play a critical role in ligand coordination for integrins with I-domains. Less information is available about the I-domain-like region in the β subunit; however, molecular modeling and mutagenesis studies support its ligand binding role (Loftus and Liddington, 1997). Integrins likely undergo dynamic structural changes as part of the ligand binding process including relative movements of subunits and of domains, and conformational changes within domains (Loftus and Liddington, 1997). As we will discuss in more detail below, integrins can exist in various affinity states for their ligands; structural changes that occur in moving from low- to high-affinity states (or vice versa) can be detected by certain monoclonal antibodies (Hughes *et al.*, 1997).

Integrin cytoplasmic domains are a key nexus of interaction between the extracellular environment and intracellular structures and signaling cascades. Both the α and β subunit cytoplasmic domains make important contributions to various aspects of overall integrin function including cytoskeletal organization, cell motility, signal transduction, and modulation of integrin affinity for ligands (“activation”). Several cytoplasmic proteins including talin, α -actinin, and possibly focal adhesion kinase (FAK) bind directly to the $\beta 1$ cytoplasmic domain and contribute to integrin-cytoskeletal interactions (Burrige and Chrzanowska-Wodnicka, 1996; Yamada and Geiger, 1997). Studies using truncation, mutation, or “domain swaps” of cytoplasmic domains have delineated several important structure-function relationships for this region of integrins. The β cytoplasmic domain is critical for recruitment of integrins to focal contacts because its truncation/mutation impairs this process; in fact, the β cytoplasmic domain alone, expressed as a fusion chimera with another membrane protein, seems sufficient for focal contact localization (LaFlamme *et al.*, 1992; Reszka *et al.*, 1992; Rosales *et al.*, 1992). A widely accepted current model is that the α subunit cytoplasmic domain inhibits certain functions of the β cytoplasmic domain (e.g., focal contact recruitment) but that binding of a ligand to the integrin relieves this inhibition, possibly by allowing the subunits to swing apart like a hinge (Burrige and Chrzanowska-Wodnicka, 1996; Hughes *et al.*, 1996). The β cytoplasmic domain is also important in signal transduction, particularly integrin activation of FAK (discussed in detail below), whereas truncation/mutation of the α cytoplasmic domain has little effect on this process (Akiyama *et al.*, 1994; Bauer *et al.*, 1993; Tahiliani *et al.*, 1997). β cytoplasmic domains have played a critical role in a variety of cellular processes including endocytosis (Van Nhieu *et al.*, 1996), “cross-talk” between different integrins (Blystone *et al.*, 1995), assembly of fibronectin fibrils (Wu *et al.*, 1995), and cell motility (Pasqualini and Hemler, 1994), whereas α cytoplasmic domains also can

strongly influence cell motility (Bauer *et al.*, 1993; Chan *et al.*, 1992).

Like other receptors, integrins can exist in different affinity states with respect to their ligands. Integrins can enter a high-affinity (“activated”) state in response to certain agents that bind the extracellular domain and influence its conformation (divalent cations, antibodies); they also can respond to signals generated within the cell that presumably have an impact on the cytoplasmic domain (“inside-out signaling”) (Humphries, 1996; Keely *et al.*, 1998; O’Toole *et al.*, 1994). The activation status of a particular integrin is cell-type dependent and usually critically depends on cellular energy metabolism. The roles of the cytoplasmic domains in integrin activation have been studied extensively, and the findings are quite complex. For example, partial truncation of $\alpha 4$, $\alpha 2$, or $\beta 2$ cytoplasmic domains prevents integrin activation, whereas truncation of the α IIB subunit activates the fibrinogen-binding integrin α IIB β 3 (O’Toole *et al.*, 1994). Recently, a coherent model of how cytoplasmic domain structure relates to integrin activation has been presented (Hughes *et al.*, 1996). The membrane proximal regions of all α and β subunits are highly conserved, whereas the remainder of the subunits are quite divergent. The conserved α sequence is GFFKR, whereas the conserved β sequence is LLV-iHDR. Deletion of either of these conserved sequences activates the integrin, essentially “locking” it in a high-affinity conformation independent of cellular energy metabolism, whereas mutation at sites C-terminal to these conserved sequences can affect energy-dependent activation (Hughes *et al.*, 1996; O’Toole *et al.*, 1994). The conserved membrane proximal sequences may interact through salt bridges between the subunits, normally keeping the integrin in a low-affinity state. Cytoplasmic events, that is “inside-out signaling,” can disrupt the α/β association allowing the “hinge” to swing and opening up the extracellular ligand binding site (see fig. 2).

Recently, some of the biochemical pathways underlying “inside-out signaling” have begun to come to light. As first elucidated in platelets with regard to the α IIB β 3 integrin (Smyth *et al.*, 1993), several agonists (usually acting through heterotrimeric G-protein-coupled receptors) can activate integrins in various cell types. It also seems clear that signaling pathways coupled to Ras-related guanosine 5'-triphosphatases (GTPases) impinge on integrin activation and function. For example, chemokines such as formyl peptide or interleukin (IL)-8 which enhance β 1 and β 2 integrin-mediated adhesion in lymphoid cells seem to act via the Rho GTPase (Laudanna *et al.*, 1996); in this case, it is not entirely clear whether this represents a change in integrin affinity (interaction of a single ligand with its receptor) or in avidity (concerted interaction of several receptors with a polyvalent ligand). Transfection of myeloid cells with a constitutively activated form of the R-Ras GTPase clearly causes an increase in the affinity of several inte-

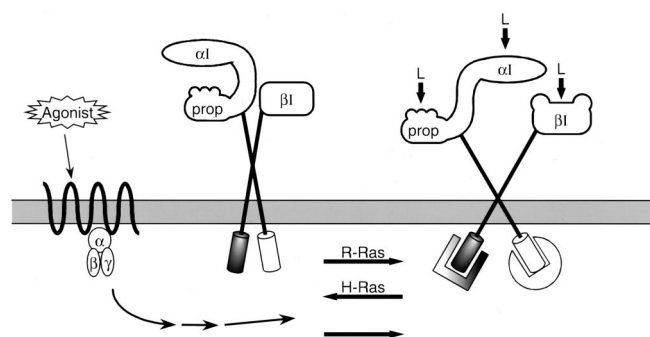


FIG. 2. Activation of integrins. Both high-affinity and low-affinity binding states exist for integrins. This may be because of conformational changes that occur when regulatory proteins bind to integrin cytoplasmic tails. The conformational changes are depicted here as integrin subunits swinging on a “hinge” and exposing ligand binding sites (α I, β I and propeller “prop”). The activating process is regulated by G-protein-coupled receptors and by small GTPases, where R-Ras activates and H-Ras inhibits high-affinity binding. Integrin ligands bind to the α and β subunits with the α domain and β I-like domain being particularly important. Adapted from Loftus and Liddington (1997).

grins, including α v β 3, α 4 β 1, and α 5 β 1, whereas coexpressed α IIB β 3 can be activated by R-Ras in Chinese hamster ovary (CHO) cells (Zhang *et al.*, 1996). By contrast, transfection of activated H-Ras, or of its downstream kinase Raf-1, inhibited the activation of coexpressed α IIB β 3 (Hughes *et al.*, 1997). Thus, different Ras-related small GTPases and their downstream effectors can either positively or negatively modulate the ligand binding affinity of integrins. Presumably integrin activation through inside-out signaling ultimately involves intracellular proteins that directly interact with integrin cytoplasmic domains; a discussion of proteins that interact directly with integrins is presented further on in this review (see Section III.).

In summary, integrins are a particularly complex family of cell adhesion receptors. These heterodimeric cell membrane glycoproteins can bind to a variety of ECM ligands or cellular counter-receptors. They engage with elements of the cytoskeleton and can thus influence cell shape, intracellular architecture, and cell motility. Integrins exist in either high- or low-affinity states with respect to their ligands, and they can engage in both “inside-out” and “outside-in” signal transduction via pathways that just now are being elucidated.

C. Cadherins

The cadherins comprise a family of transmembrane proteins that share an extracellular domain consisting of multiple repeats of a cadherin-specific motif (Suzuki, 1996) (see fig. 1). Members of the “classic” cadherin subfamily are calcium-dependent homotypic cell-cell adhesion molecules. This subfamily includes the N-, P-, R-, B-, and E-cadherins as well approximately 10 other members (Takeichi, 1995). These molecules localize in specialized sites of cell-to-cell adhesion that are termed adherence junctions; at these sites cadherins can establish linkages with the actin-containing cytoskeleton. The

classic cadherins play a key role in developmental processes. For example, “knockout” of the gene for E-cadherin (which mediates epithelial interactions) results in an embryonic lethal that arrests before blastocyst formation (Takeichi, 1995). Another important subfamily of cadherins involved in adhesion is represented by the desmogleins and desmocollins, a group of desmosome-associated cadherins that form intracellular linkages to intermediate filaments rather than actin filaments (Cowin and Burke, 1996).

The structure of a typical classic cadherin consists of an amino-terminal external domain having five tandem repeats, a single transmembrane segment, and a cytoplasmic carboxy-terminal domain of approximately 150 amino acids (fig. 1). The binding functions of the cadherin are localized in the amino-terminal tandem repeat, whereas the other repeats are bridged by calcium binding sites that impart rigidity to the molecule (Aberle *et al.*, 1996). A recent model based on the X-ray structure of N-cadherin (Shapiro *et al.*, 1995) suggests that cell-cell adhesion mediated by cadherins involves a “zipper” type of organization. Cadherins on one cell surface form a series of rigid dimers that presents the first (N-terminal) cadherin repeat to equivalent dimers on the opposing cells (Aberle *et al.*, 1996; Gumbiner, 1996). Lateral motion of these complexes allows the cell junction site to “zip up.”

The cytoplasmic domains of cadherins interact strongly with a group of intracellular proteins known as catenins; these proteins are essential for cadherin function, because truncation of the cadherin cytoplasmic domain to delete catenin binding sites leads to a loss of cadherin-mediated adhesion (Gumbiner, 1996; Takeichi, 1995). Because there is considerable homology among their cytoplasmic domains, different classic cadherins can compete for the same pool of catenins (Kinter, 1992). The catenins were described initially as a set of three proteins, α -, β -, and γ -catenin (also termed plakoglobin). β -Catenin binds directly to the cadherin cytoplasmic domain; subsequently, α -catenin binds to β -catenin and links the complex to the actin cytoskeleton by direct interaction with actin and by binding α -actinin, an actin-bundling protein (Cowin and Burke, 1996). The structure of α -catenin shows substantial homology to the protein vinculin, which binds α -actinin and talin and is critical for cytoskeletal assembly at integrin-mediated focal adhesion sites (see Section III. below) (Aberle *et al.*, 1996). In some cases plakoglobin replaces β -catenin in mediating cadherin-cytoskeletal complexes.

The cadherins play a critical role in the development of tissue organization during ontogeny and in maintenance of normal tissue structures in adult organisms (Huber *et al.*, 1996). For example, a variety of cadherins have been implicated in generating the complex architecture of the brain (Redies and Takeichi, 1996). In adult organisms, loss of cadherin expression or function can lead to serious pathophysiological consequences. Thus

progression toward an invasive, malignant phenotype in epithelial tumors is associated with loss or mutation of E-cadherin, or with the disruption of cadherin-catenin complexes. Phosphorylation of β -catenin by v-Src or other tyrosine kinases can lead to a breakdown of cadherin-cytoskeleton associations and loss of the adherence junctions that maintain normal epithelial organization (Behrens and Birchmeier, 1994; Birchmeier, 1995).

Recent evidence has indicated that cadherins and catenins also play an important role in signal transduction. As discussed in more detail below (Section VIII.), β -catenin not only interacts with cadherins but also with components of the wingless/Wnt signaling pathway (Peifer, 1996). Thus the cell-cell adherence junction, maintained by cadherins and their associated catenins, is a key element in both tissue organization and in regulation of signaling cascades.

D. Immunoglobulin-Cell Adhesion Molecule Superfamily

A diverse array of cell adhesion receptors are included in the immunoglobulin superfamily of cell adhesion molecules (Ig-CAMs). Proteins of this family are defined by the presence of one or more copies of the Ig fold, a compact structure with two cysteine residues separated by 55 to 75 amino acids arranged as two antiparallel β sheets (Vaughn and Bjorkman, 1996). In many (but not all) cases, CAMs in the Ig superfamily also contain one or more copies of a fibronectin type III repeat domain. Ig family adhesion receptors typically have a large amino-terminal extracellular domain, a single transmembrane helical segment, and a cytoplasmic tail (fig. 1). Members of the Ig-CAM family function in a wide variety of cell types and are involved in many different biological processes.

One of the most important contexts for Ig-CAMs is the developing nervous system, where many different members of this superfamily are involved in axon guidance and in the establishment and maintenance of neural connections (Baldwin *et al.*, 1996; Tessier-Lavigne and Goodman, 1996). The “classic” example of an Ig superfamily adhesion receptor is NCAM, which contains five Ig folds in its extracellular portion; there are three forms of NCAM, two with transmembrane domains and one having a glycosylphosphatidylinositol (GPI) link to the membrane (Edelman and Crossin, 1991). NCAM functions as a homotypic calcium-independent adhesion receptor and seems to be almost universally present in the nervous system. Adhesive interactions mediated by NCAMs are known to be regulated by both the abundance of receptor and its degree of polysialylation (Edelman and Crossin, 1991; Tessier-Lavigne and Goodman, 1996). Several other homotypic neural cell adhesion molecules belong to the Ig superfamily, including L1, TAG1, contactin, and *Drosophila* fasciculin II (Baldwin *et al.*, 1996; Tessier-Lavigne and Goodman, 1996). Another

group of Ig-CAMs important in neural development are the netrin receptors. Netrins are ECM matrix proteins, distantly related to laminins, that provide guidance cues to migrating axons (Tessier-Lavigne and Goodman, 1996). Vertebrate netrin receptors belong to the Ig family and include DCC, a protein originally identified as the product of a key tumor suppressor gene for colon cancer (Keino-Masu *et al.*, 1996). Yet another group of key adhesion receptors involved in neural development is the dozen or so members of the Eph subfamily of transmembrane tyrosine kinases; these proteins contain an Ig fold in their extracellular domains and are thus Ig-CAMs (Tessier-Lavigne and Goodman, 1996). The Eph kinases and their cell-bound ligands seem to be involved primarily in axon-target cell interactions. Thus neural Ig-CAMs can be involved in either homotypic (NCAM) or heterotypic (DCC, Eph kinases) adhesive interactions. There is relatively little known about the interactions of neural Ig-CAMs with cytoskeletal proteins. One report has suggested an association between the 180 kDa form of NCAM and spectrin (which can link to the actin cytoskeleton) (Pollerberg *et al.*, 1997). However, it seems likely that other interactions exist and contribute to the functions of neural Ig-CAMs. Presumably many of the interactions between neural adhesion molecules result in signal generation, as well as in establishment of adhesive contacts between cells; however, with a few exceptions (see Section VIII.), the mechanistic details of the signaling pathways are poorly understood at present.

Another important biological context for Ig-CAMs lies in the immune system. In fact, integrins, selectins, and Ig-CAMs are all critically involved in multiple aspects of immune function (Dustin and Springer, 1991; Rosales and Juliano, 1995; Springer, 1995). T lymphocytes express several Ig superfamily receptors including CD2, CD4, or CD8, ICAMs 1 and 2, and the T-cell receptor (TCR) itself. These receptors play important roles in antigen recognition, cytotoxic T-cell functions, and lymphocyte recirculation. In contrast to many of the neural Ig-CAMs, which are often homotypic receptors, Ig family proteins involved in the immune system primarily engage in heterotypic interactions. For example, CD2 on T cells interacts with LFA-3 (another Ig-CAM) expressed on target cells, the TCR interacts with major histocompatibility complex (MHC) class II proteins on antigen presenting cells (both Ig superfamily), whereas ICAMs on endothelial cells are recognized by $\beta 2$ integrins on leukocytes. Other Ig-CAM family adhesion receptors are found on vascular endothelial cells and play an important role in leukocyte trafficking to inflamed tissue sites. For example, vascular cell adhesion molecule-1 (VCAM-1) is an endothelial cell counter-receptor for the integrin $\alpha 4 \beta 1$ found on leukocytes. Platelet endothelial cell adhesion molecule-1 (PECAM-1) is an Ig-family cell-cell adhesion molecule that can engage in both homotypic and heterotypic interactions; one of its roles seems

to be maintaining tight contacts between adjacent vascular endothelial cells (DeLisser *et al.*, 1994).

Recently, X-ray crystal structure was obtained for the extracellular domains of several Ig-CAMs important in immune function including intercellular adhesion molecule (ICAM)-2, vascular cell adhesion molecule-1 and CD2 (Casanovas *et al.*, 1997). This has allowed a more detailed understanding of how these molecules recognize their ligands and support cell-cell contact. As with the cadherins, the intracellular domains of Ig-CAMs may also be important in regulating the adhesive functions of these receptors (DeLisser *et al.*, 1994; Doherty *et al.*, 1992).

Immune system Ig-CAMs are critically involved in the key signal transduction processes leading to activation of T cells and B cells by antigens (Weiss and Littman, 1994). A detailed account of these complex events is beyond the scope of this review. In essence, however, Ig-CAM and integrin-mediated contacts are established between the antigen-presenting cell and the T cell such that the TCR recognizes antigen bound to an MHC protein on the presenting cell. This triggers the activation of intracellular tyrosine kinases associated with the TCR and with accessory receptors (Crabtree and Clipstone, 1994). In B cells, the B-cell receptor (also an Ig-CAM) can recognize either soluble or particulate antigen, and it also can activate intracellular tyrosine kinases upon ligation. Both the Src family and the spleen tyrosine kinase (SYK)/ZAP-70 family of tyrosine kinases have been implicated in antigen-induced T- and B-lymphocyte signaling (Weiss and Littman, 1994). Further on in this review, we will return to the theme of adhesion receptors activating intracellular tyrosine kinases in great detail as we consider adhesion receptor signaling in fibroblasts, epithelial cells, and tumor cells.

Another important group of signaling proteins that overlaps the Ig-CAM superfamily are the receptor protein tyrosine phosphatases (RPTPs) (Neel and Tonks, 1997). Many of these transmembrane enzymes have extracellular domains that include Ig folds (and often fibronectin type III repeats as well). The RPTPs typically have a large external domain, a single transmembrane helix, and a cytoplasmic domain containing two signature tyrosine phosphatase domains flanked by a variety of noncatalytic sequences. RPTPs seem to function conversely to receptor tyrosine kinases (RTKs); that is, ligand binding results in dimerization of RPTPs but inhibits enzyme activity (rather than activating it as with RTKs). It has become clear recently that several RPTPs can engage in homotypic or heterotypic cell adhesion through their extracellular domains. For example, *Drosophila* LAR, a *Drosophila* Ig-CAM-RPTP, plays an important role in axon migration and innervation of muscle targets (Neel and Tonks, 1997).

Thus Ig-CAMs play multiple roles in the developing embryo and in the adult organism. In addition to mediating adhesive contacts that are important in tissue

organization, or in cellular trafficking in the immune system, many Ig-CAMs function in key signal transduction processes as well.

E. Selectins

The selectins are a small family of lectin-like adhesion receptors composed of three members, L-, E-, and P-selectin (Lasky, 1995; Rosen and Bertozzi, 1994; Tedder *et al.*, 1995). The structure of a selectin includes an amino-terminal domain that is homologous to calcium-dependent animal lectins, followed by an epidermal growth factor (EGF)-type domain, two to nine complement regulatory protein repeats, a transmembrane helical segment, and a short cytoplasmic tail (fig. 1). Selectins mediate heterotypic cell-cell interactions through calcium-dependent recognition of sialylated glycans. The best defined physiological role for selectins concerns leukocyte adherence to endothelial cells and platelets during inflammatory processes (Rosales and Juliano, 1995; Springer, 1995). The expression and function of selectins is tightly regulated so as to come into play only when leukocytes need to stick to the vessel wall as part of normal immune system cellular trafficking or during inflammation. Thus P-selectin is present in latent form in endothelial cells and platelets and it is rapidly translocated from secretory granules to the cell surface upon cell activation by thrombin or other agonists. E-selectin is synthesized and expressed on endothelial cells in response to inflammatory cytokines such as tumor necrosis factor (TNF) or IL-1. L-selectin is expressed constitutively on leukocytes, but its presentation at the cell surface may be regulated.

The precise identities of the ligands for the three currently known selectins are being pursued actively and are a matter of some controversy (Varki, 1997). For all three selectins, a key component of the binding ligand is tetrasaccharide residues of the sialyl-Lewis^x type (previously defined as a blood group antigen); such motifs appear on glycolipids as well as on glycoproteins. However, the binding affinities of selectins for isolated sialyl-Lewis^x saccharides are very poor, although selectins clearly are responsible for high-affinity cell-cell binding. Thus physiological high-affinity ligands for selectins likely must include sialyl-Lewis^x saccharides in the context of a macromolecular scaffold. The best documented high-affinity counter-receptor for a selectin is P-selectin glycoprotein ligand-1 (PSGL-1), an O-glycosylated mucin-like transmembrane glycoprotein found on leukocytes and lymphoid cells (Norman *et al.*, 1995). Glycoproteins of this type likely have a rigid, rod-like structure and may be able to present multiple copies of sialyl-Lewis^x to endothelial cell selectins. Two potential counter-receptors for L-selectin have been identified; GlyCAM-1 which is a small, secreted mucin-like glycoprotein, and CD34 a transmembrane mucin-like protein found on endothelial cells. A putative counter-receptor for E-selectin has been termed ESL-1 and is a glycopro-

tein bearing N-linked carbohydrate residues. However, the physiological importance of GlyCAM-1, CD34, and ESL-1 in leukocyte to endothelial adherence and leukocyte trafficking is still unclear (Varki, 1997).

There is much less known about selectin-mediated signal transduction than is the case for integrins, cadherins, and Ig-CAMs. However, it is rapidly becoming clear that selectins play an important role in signaling processes that regulate leukocyte-endothelial cell interactions (Zimmerman *et al.*, 1996b).

III. The Components of Adhesive Junctions

Signal transduction by cell adhesion receptors primarily occurs in the context of highly organized supramolecular complexes that are assembled at sites of cell-to-cell or cell-to-ECM adhesion. There are several distinct types of adhesive junctions. However, our present understanding of the role of adhesive junctions in signaling extends primarily to one type of junction, the integrin-containing focal contact (also termed focal adhesion) and thus we will discuss these structures in some detail. Another growing body of information concerns cadherin-containing adherence junctions, whereas less is known about the signaling properties of other types of adhesive junctions.

A. Integrin Containing Focal Contacts

Focal contacts are specialized sites where cells attach to the ECM (Burrige and Chrzanowska-Wodnicka, 1996; Jockush *et al.*, 1995). At focal adhesions, clusters of integrins bind externally to ECM proteins and internally to several specialized cytoplasmic proteins that in turn bind to actin filaments. It is becoming clear that focal adhesions are dynamic structures that change in size (and likely in composition) as the cell adhesion process progresses. Initially, rather small punctate structures are formed at sites of cell-to-substratum contact. As the focal contact matures, the actin filaments extend and bundle to form prominent structures termed stress fibers. Integrins can interact with numerous proteins at focal contacts and at other sites in the cell. In addition, there is a complex network of interactions among the specialized cytoplasmic focal contact proteins. In this section of the review we will introduce some of the key components of focal contacts. We also will discuss transmembrane proteins that directly interact with integrins. Finally, we will introduce several newly discovered cytoplasmic proteins that bind directly to integrin α or β subunit cytoplasmic tails and which may play important roles in integrin signaling. The known interactions of most of the proteins to be discussed below are depicted in fig. 3.

1. Cytoskeletal proteins The cytoplasmic structural proteins of the focal contact that directly bind to integrins include talin and α -actinin, which in turn bind to other structural proteins including vinculin, paxillin, and tensin, ultimately leading to the recruitment of ac-

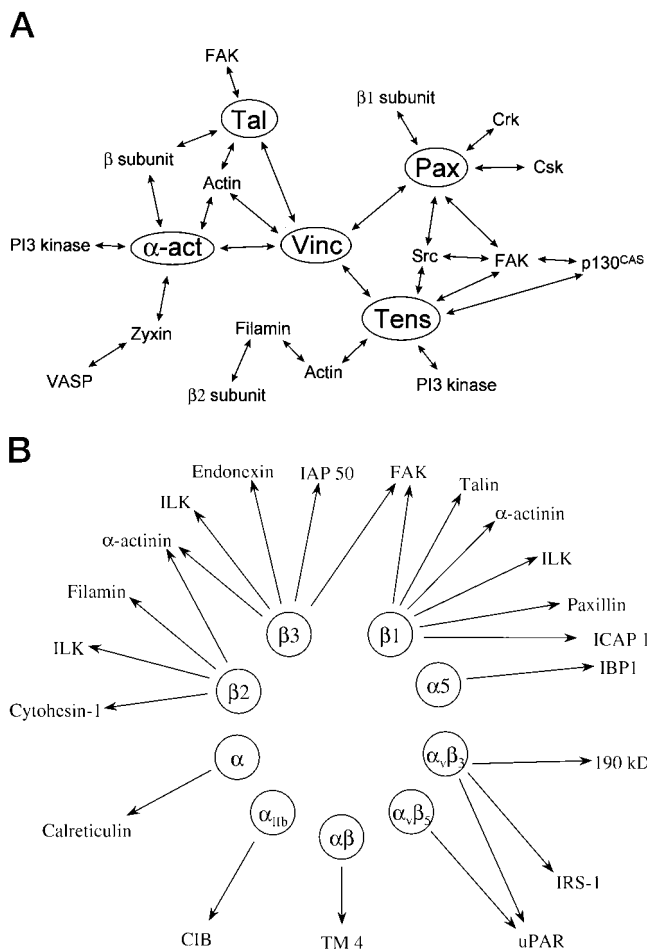


FIG. 3. Proteins associated with integrin-containing focal contacts. (A) Focal contact proteins and their interactions. This diagram illustrates the patterns of binding interactions of key structural proteins of the focal contact including talin (Tal), vinculin (Vinc), α -actinin (α -act), tensin (Tens), and paxillin (Pax). See text for additional details. (B) Proteins interacting directly with integrins. This diagram recapitulates current information concerning proteins that bind directly to integrins. In many cases it is clear that the interaction occurs with a particular α or β subunit or with an individual integrin heterodimer, whereas in other cases the interaction may involve binding to many different integrins. Clearly only some of these interactions will take place in any given type of cell, with the pattern of interaction determined by the expression of the various binding proteins, of various integrins, and by the physiological status of the cell.

tin filaments. Integrin-mediated phosphorylation of several cytoskeletal proteins seems to have a role in the organization of stress fibers at focal adhesions. It has been shown that several kinases, including FAK, play a role in the organization of cytoskeleton (see Section IV, below). The structure and function of several of the key structural proteins of the focal contact is described below (fig. 3A).

a. **TALIN.** Talin is a cytoskeletal protein that typically is localized in focal adhesions (Burrige and Connel, 1983). It is a homodimer of two 270 kDa polypeptides (Rees *et al.*, 1990) arranged in an antiparallel orientation. The N-terminal region interacts with phospholipids and membranes, whereas the C-terminal portion interacts with sites on the $\beta 1$ or $\beta 3$ integrin cytoplasmic

domain (Horwitz *et al.*, 1986; Jockush *et al.*, 1995; Niggli *et al.*, 1994). Talin has a role in connecting the actin cytoskeleton to focal adhesions and it is crucial in the initial formation of new focal adhesions (Nuckolls *et al.*, 1992; Pavalko *et al.*, 1991). In addition to binding integrins and actin, talin also binds to vinculin and to FAK (Burrige and Chrzanowska-Wodnicka, 1996; Chen *et al.*, 1995).

b. **α -ACTININ.** α -Actinin is an actin-bundling protein that comprises two identical polypeptides, each of which is approximately 104 kDa in size. The protein can be divided into three regions: the N-terminal actin binding site, a central region with four α -helical motifs, and a carboxy-terminal region (Jockush *et al.*, 1995; Noegel *et al.*, 1987). α -Actinin has been shown to interact with the cytoplasmic domains of the integrin $\beta 1$, $\beta 2$, and $\beta 3$ subunits (Burrige and Chrzanowska-Wodnicka, 1996; Otey and Burrige, 1990; Pavalko and LaRoche, 1993). The same region of α -actinin that binds to $\beta 1$ integrin, also binds to vinculin (Jockush *et al.*, 1995). α -Actinin also has a binding domain for zyxin within the actin binding region, and zyxin colocalizes with α -actinin at focal adhesions, at the ends of stress fibers, and in cell-cell junctions (Crawford *et al.*, 1992). Zyxin has Lin-II, Islet I, and Mec 3 (LIM) domains which are thought to play a role in signal transduction. α -Actinin has been shown to be necessary for the association of actin molecules to focal adhesions (Pavalko and Burrige, 1991).

c. **TENSIN.** This protein was so named because of its proposed role in connecting actin to focal adhesions and maintaining mechanical tension (Lo *et al.*, 1994; Wilkins and Lin, 1986). Tensin consists of two 200 kDa polypeptide chains and has three actin binding sites per chain. In addition, tensin has been reported to bind to vinculin, paxillin, Src, FAK, phosphatidylinositol-3-kinase (PI-3K), and CrK-associated substrate (CAS) p130^{CAS} (see below). Tensin has sequence homologies to several other structural proteins including spectrin, dystrophin, α -actinin, plasmin/fimbrin, phosphatase related to tensin (PTEN), and α -catenin (Lo *et al.*, 1994). The site with homology to α -catenin is predicted to be the vinculin binding site of tensin because both α -catenin and tensin bind vinculin (Nagafuchi *et al.*, 1991). In addition, tensin also has a Src homology type 2 (SH2) domain which binds to Src, paxillin, and other tyrosine-phosphorylated proteins in vitro (Davis *et al.*, 1991). Recently, it was shown that tensin forms complexes with PI-3K in a platelet-derived growth factor (PDGF)-dependent manner, suggesting that tensin might help localize the PI-3K to focal adhesions (Auger *et al.*, 1996). A tyrosine-phosphorylated 130 kDa protein (p130^{CAS}) associates with tensin via the tensin SH2 domain (Lo *et al.*, 1994).

As with certain integrins (Keely *et al.*, 1998), vinculin (Rodriguez-Fernandez *et al.*, 1992), and α -actinin (Gluck *et al.*, 1993), overexpression of tensin has been implicated in retarding tumor formation (Lo *et al.*, 1994). Recently, a novel tumor suppressor gene *PTEN* or

MMAC1 (Li *et al.*, 1997a; Steck *et al.*, 1997) was identified; this gene codes for a protein tyrosine phosphatase and also has strong homologies to the *tensin* gene. It is interesting to note that tensin also has a signature sequence for protein tyrosine phosphatases, although no phosphatase activity has been reported yet (Haynie and Ponting, 1996). Because *PTEN* has tensin homology regions, and has been proven to be a tumor suppressor, it is tempting to speculate that tensin might also function as a tumor suppressor.

d. **VINCULIN.** Vinculin is a 116 kDa polypeptide enriched in focal contacts. Vinculin binds to actin, α -actinin, talin, and paxillin (reviewed in Jockush *et al.*, 1995). Electron micrographs showed that vinculin has a globular head and a rod-like tail region (Molony and Burrige, 1985). The first 120 amino acids in the head region are essential for binding of talin (Burrige and Mageat, 1984; Gilmore *et al.*, 1992) and α -actinin (Kroemker *et al.*, 1994), whereas paxillin and actin bind to the rod-like tail region (Johnson and Craig, 1994, 1995). In addition, vinculin binds to acidic phospholipids (Iseneberg, 1991; Niggli *et al.*, 1986) including phosphatidylinositol 4,5-bisphosphate (PIP₂) (Fukami *et al.*, 1994). The head-tail association of vinculin masks its ability to bind other proteins. When PIP₂ binds to a region in the hinge between head and tail segments (Fukami *et al.*, 1994), the head-tail association of vinculin is disrupted allowing other interactions to occur (Gilmore and Burrige, 1996b). Thus PIP₂ regulation of vinculin may be an important way in which inositol lipids influence focal contact formation and stability.

e. **PAXILLIN.** The name paxillin is derived from Latin, meaning stake or peg because the 68 kDa protein seems tethered to the membrane at focal adhesions (Glenney and Zokas, 1989; Turner, 1994). The amino-terminal region of paxillin has a putative Src homology region 3 (SH3) domain and multiple putative SH2 binding sites, and the carboxy terminus has four LIM domains that are involved in protein-protein interactions (Turner and Miller, 1994). Paxillin binds to a region of 140 amino acid residues in the carboxy terminus of FAK, and it has been hypothesized that this interaction might coordinate the signals from focal adhesions to the cytoplasm or cytoskeleton (Hildebrand *et al.*, 1995). The paxillin binding domain of FAK overlaps with the focal adhesion targeting (FAT) domain (see Section IV. below) (Hanks and Polte, 1997). In addition to FAK, paxillin also associates with vinculin (Turner *et al.*, 1990), p47^{gag-crk} (Birge *et al.*, 1993), C-terminal Src Kinase (CSK) (Sabe *et al.*, 1994), and pp60^{src} (Weng *et al.*, 1993), which indicates that paxillin functions as an adapter protein involved in forming multiprotein complexes. There is evidence that integrin cytoplasmic peptides directly bind to FAK and paxillin in vitro (Schaller *et al.*, 1995; Tanaka *et al.*, 1996), but the biological significance of this is unclear.

f. **FILAMIN.** Filamin is an actin-binding protein that is involved in the formation of actin networks (Gorlin *et al.*, 1990; Stossel, 1993). Filamin exists as a homodimer with the two polypeptides associating only at the carboxy-terminal region, whereas the amino-terminal ends link with actin. This molecule has been referred to as "leaf spring," because of its flexible nature (Gorlin *et al.*, 1990). Recently filamin has been shown to associate with the β 2 integrin cytoplasmic domain and it has been suggested that the association of filamin with integrins might be important in spreading and extension of lamellipodia during cell movement and in phagocytosis (Sharma *et al.*, 1995).

In summary, the specialized focal contact proteins form an elaborate multiprotein complex. This complex serves as a structural and functional link between integrins and the actin-containing cytoskeleton.

2. *Transmembrane proteins: tetraspanners, integrin-associated protein 50, caveolin* As discussed above, integrins interact via their cytoplasmic tails with an assemblage of cytoplasmic proteins. However, the external and transmembrane domains of integrins also can participate in protein-protein interactions; in some cases these interactions may occur at sites away from focal contacts (fig. 3B).

Recently, it has been shown that certain proteins having four transmembrane α -helical regions, and thus known as tetraspan or TM4 proteins, can interact with integrins (Shaw *et al.*, 1995). These interactions seem to occur on the external surface of the cell and do not involve the integrin cytoplasmic domains. In general, integrin-TM4 complexes reside outside of focal adhesion sites. The precise function of TM4 proteins is unknown; however, they have been suggested to be involved in cell motility (Dong *et al.*, 1995), signaling (Shaw *et al.*, 1995; Wright and Tomlinson, 1994), and in functioning as ion channels (Wright and Tomlinson, 1994). Several groups have indicated that β 1 and β 3 integrins may interact with TM4 proteins such as CD9 and CD63 (Nakamura *et al.*, 1995; Slupsky *et al.*, 1989). Further evidence for CD9, CD63, and CD81 association with β 1 integrins has come from a study by Berditchevski *et al.* (1996). In this study, an interaction of integrins with CD81 was observed, and TM4 proteins also were found to interact with α 3 β 1 and α 6 β 1, but not with α 2 β 1, α 5 β 1, or α 6 β 4. This pattern of association also was detected in keratinocytes (Jones *et al.*, 1996). However, previous reports indicated that CD9 interacts with α 3 β 1 (Nakamura *et al.*, 1995), α 4 β 1, α 5 β 1 (Rubinstein *et al.*, 1994), and α IIb β 3 (Slupsky *et al.*, 1989) in B lymphocytes; the association between CD9 and α 5 β 1 thus was suggested to be cell type specific. An association of CD9 with α 3 and α 6 integrins also was shown in Schwann cells (Hadjiargyrou *et al.*, 1996). Recently, an interaction was demonstrated between phosphatidylinositol-4-kinase and α 3 β 1-CD63 complexes, suggesting a possible role of in-

tegrin-TM4 complexes in signaling cascades (Berdichevski *et al.*, 1997).

A novel protein of 50 kDa and having five transmembrane domains was shown to be associated with $\beta 3$ integrins (Lindberg *et al.*, 1993). The protein was named integrin-associated protein (IAP 50) and has been implicated in integrin-mediated phagocytosis in leukocytes. IAP 50 also is involved in the increase in calcium levels observed upon cell adhesion to fibronectin- or vitronectin-coated surfaces (Schwartz *et al.*, 1993). IAP 50 has a wide range of tissue expression (Campbell *et al.*, 1992; Reinhold *et al.*, 1995), whereas $\alpha v\beta 3$ has only limited tissue expression, suggesting that IAP 50 has functions in addition to cooperation with integrins.

Recently, evidence has been developed that certain integrins can interact with caveolin, a transmembrane protein found at specialized membrane sites rich in glycosphingolipids termed caveolae. Integrin-caveolin interactions may be quite important in signaling (Wary *et al.*, 1996), as discussed in more detail in Section V. below.

3. *Cytoplasmic integrin-binding proteins* The cytoplasmic domains of integrins have been implicated in the bi-directional transfer of information across the plasma membrane. Integrin cytoplasmic tails interact with several proteins that may participate in integrin-mediated functions including signal transduction. Novel proteins have been identified that interact with either the α or β cytoplasmic domains of integrins (fig. 3B).

a. β SUBUNIT-ASSOCIATED PROTEINS.

i. $\beta 3$ endonexin. A novel 111 amino acid protein, $\beta 3$ endonexin, was cloned by using the integrin $\beta 3$ cytoplasmic tail as bait in the yeast two-hybrid system (Shattil *et al.*, 1995). This interaction is very specific to $\beta 3$, because a mutation of serine to proline at position 752 in the $\beta 3$ cytoplasmic tail reduced the binding by 64%; this mutation is critical for $\alpha IIb\beta 3$ integrin-mediated function (Chen *et al.*, 1994b). Because this $\beta 3$ mutation abolishes binding to fibrinogen (Chen *et al.*, 1992b) and cell spreading (Chen *et al.*, 1994b), it suggests that $\beta 3$ endonexin is involved in the selective modulation of $\beta 3$ integrin function. In addition, it has been proposed that $\beta 3$ endonexin has a function independent of integrins because endonexin is expressed in a wide variety of tissues. An NITY motif at positions 756 to 759 of $\beta 3$ is critical for the interaction with endonexin (Eigenthaler *et al.*, 1997); this motif is highly conserved in $\beta 3$ integrins of different species. Another study from the same group (Kashiwagi *et al.*, 1997) demonstrated that $\beta 3$ endonexin bound to $\alpha IIb\beta 3$ in mammalian cells and was able to modulate the affinity state of $\alpha IIb\beta 3$, leading to fibrinogen-dependent aggregation. Only a weak staining of $\beta 3$ endonexin was observed in focal adhesions, suggesting that $\beta 3$ endonexin interacts with integrins transiently, so that other interactions with the integrin tails

can follow after endonexin dissociation (Kashiwagi *et al.*, 1997). A role of $\beta 3$ endonexin in outside-in signaling events has yet to be demonstrated.

ii. Integrin-linked kinase. A yeast two-hybrid approach also was used to identify another β subunit-binding protein termed integrin-linked kinase (ILK) (Hannigan *et al.*, 1996). ILK is a 59 kDa serine-threonine kinase that can associate with $\beta 1$, $\beta 2$, and $\beta 3$ cytoplasmic domains and has been shown to phosphorylate a $\beta 1$ integrin cytoplasmic domain peptide in vitro (Dedhar and Hannigan, 1996). Cell attachment to fibronectin reduced ILK kinase activity, whereas overexpression of ILK induced anchorage-independent growth and reduced cell adhesion to laminin, fibronectin, and vitronectin (Hannigan *et al.*, 1996). ILK and $\beta 1$ integrins colocalize in focal adhesions, suggesting a possible role in cytoskeletal assembly. Recently, it has been shown that ILK overexpression induces anchorage-independent cell cycle progression (Radeva *et al.*, 1997). As discussed in detail in Section X. below, upon integrin-mediated cell adhesion, levels of cyclins D1 and A are up-regulated and cyclin-dependent kinase (CDK) inhibitor levels are down-regulated. Normal cells placed in suspension have down-regulated expression of cyclin D1 and cyclin A and hypophosphorylated retinoblastoma (Rb), whereas suspension cells overexpressing ILK have higher expression of cyclin D1 and cyclin A and hyperphosphorylated Rb (Radeva *et al.*, 1997). Thus ILK may be a key link between integrins and downstream signaling events that influence the cell cycle. In addition, very recent evidence suggests that ILK is involved in epithelial cell differentiation and gene expression (see Section XI.).

iii. Cytohesin 1. Using the $\beta 2$ cytoplasmic domain as bait in the yeast two-hybrid system, Kolanus *et al.* (1996) identified a protein they termed cytohesin-1. In lymphoid cells cytohesin-1 binds to $\beta 2$; further, there seems to be a significant role for cytohesin-1 in the regulation of integrin activation, as measured by $\alpha L\beta 2$ adhesion to ICAM1. Cytohesin-1 has a plekstrin homology (PH) domain and a domain that resembles the yeast SEC7 protein. The SEC7 domain alone induces $\alpha L\beta 2$ binding to ICAM1, whereas the PH domain inhibits the process, seemingly acting in a dominant negative role. It has been postulated that PH domains function like SH2 and SH3 domains, mediating protein-protein associations in signal transduction pathways and/or localizing proteins to the plasma membrane (Pawson, 1995). Cytohesin-1 recently has been shown to function as a guanine nucleotide exchange factor (GNEF) probably via its SEC7 domain (Meacci *et al.*, 1997). Moreover, cytohesin-1 also binds via its PH domain to phosphoinositol 3,4,5-triphosphate, suggesting a link with PI-3K pathways (Klarlund *et al.*, 1997). Thus cytohesin-1 may play several important roles in integrin activation and in intracellular signaling.

iv. Integrin cytoplasmic domain-associated protein 1. Recently, another novel $\beta 1$ integrin cytoplasmic domain-associated protein (ICAP1) was isolated using a yeast two-hybrid approach. Two isoforms of ICAP1 that are derived from alternatively spliced mRNA have been reported; the longer isoform has 200 amino acids (ICAP1 α) and the shorter isoform has 150 amino acids (ICAP1 β). The interaction of ICAP1 α with $\beta 1$ integrins has been shown *in vitro* and *in vivo*. ICAP is a phosphoprotein, and its phosphorylation is regulated during cell-matrix interactions. Cell attachment to fibronectin resulted in enhancement of ICAP1 α phosphorylation, whereas expression of Rho A that disrupts cell-matrix interactions resulted in dephosphorylation of ICAP1 α . These data indicate that ICAP1 α has a role in integrin-mediated cell adhesion and spreading (Chang *et al.*, 1997).

b. α SUBUNIT INTERACTING PROTEINS.

i. Calreticulin. A conserved stretch of seven amino acids (KXGFFKR) exists in all integrins and subunits. To understand the role of such a highly conserved region, investigators tried to identify proteins that interact with this region. Rojiani *et al.* (1991) reported a 60 kDa protein that specifically binds to the KXGFFKR sequence. This protein had significant homologies to rabbit calreticulin (Fliegel *et al.*, 1989) and the Ro/SS-A antigen, its human equivalent (McCauliffe *et al.*, 1990). An *in vivo* interaction of calreticulin with the active form of the $\alpha 2\beta 1$ integrin has been demonstrated (Coppolino *et al.*, 1995). Further, inhibition of expression of calreticulin by antisense oligonucleotides resulted in inhibition of cell adhesion and less cell spreading, indicating a role for calreticulin in cell-ECM interactions (Leung-Hagesteijn *et al.*, 1994). Recently, a double knockout of calreticulin in embryonic stem cells suggested that calreticulin is essential for the function of integrins including "outside-in" signaling (Coppolino *et al.*, 1997).

ii. Calcium- and integrin-binding protein. A novel calcium- and integrin-binding protein (CIB) of 25 kDa that interacts with αIIb was identified using the yeast two-hybrid system. This protein specifically binds to αIIb and has similarities to the calcium-binding proteins calmodulin and calcineurin. CIB interacts with $\alpha IIb\beta 3$ *in vitro* and is expressed in platelets, which suggests that it may function as a regulatory molecule for $\alpha IIb\beta 3$ (Naik *et al.*, 1997).

iii. Integrin-binding protein 1. Using a yeast two-hybrid approach, we identified a novel protein that interacts with the $\alpha 5$ subunit cytoplasmic tail (Alahari and Juliano, unpublished results). This protein does not have significant homologies to known proteins, and henceforth was tentatively named integrin-binding protein 1 (IBP1). IBP1 binds to human $\alpha 5\beta 1$ *in vitro*, but its physiological role is currently unknown.

4. Interactions with Integrin Heterodimers. There have been several reports of proteins that associate with integrin heterodimers. In most cases it has not been

established clearly whether the interactions are direct or are mediated through other, as yet unidentified, proteins.

a. INSULIN RECEPTOR SUBSTRATE 1 AND 190 kDa. Two cytoplasmic proteins have been shown to associate with $\alpha v\beta 3$ integrin in response to growth factor stimulation. A 190 kDa tyrosine-phosphorylated protein associates with $\alpha v\beta 3$ in PDGF-stimulated fibroblasts (Bartfeld *et al.*, 1993). This protein is likely to be the PDGF receptor itself (Schneller *et al.*, 1997). Tyrosine-phosphorylated insulin receptor substrate-1 (IRS-1) associates with $\alpha v\beta 3$ upon insulin stimulation; IRS-1 mediates insulin signaling by binding to other signaling molecules including GRB2 and PI-3K (Vuori and Ruoslahti, 1994). The involvement of IRS-1 and 190 kDa with integrins is suggestive of a role in signal transduction, but the mechanistic details remain undetermined at this time (see Section IX.).

b. PROTEASES. Some recent observations have indicated the existence of interactions between integrins and proteases or protease receptors that may be very important in the regulation of inflammation and of tumor cell invasion and metastasis. Thus the $\alpha v\beta 3$ integrin has been shown to associate with both the matrix metallo protease (MMP2) (Brooks *et al.*, 1996) as well as with the urokinase-type plasminogen activator receptor (uPAR) (Wei *et al.*, 1996). In the first case, association of MMP2 and the integrin facilitated matrix protein degradation suggesting a role in invasion. In the second case, complex formation between uPAR and the integrin blocked the adhesive function of the integrin. These observations suggest a complex interplay between certain integrins and extracellular proteolytic cascades which may be of great biological importance.

B. Other Adhesive Junctions

In addition to integrin-containing focal adhesions, there are several other types of structures that link cells to each other or to the extracellular matrix. These include: cadherin-based cell-cell adherence junctions, and cell adhesion junctions containing certain proteoglycans and ezrin/radixin/moesin (ERM) proteins, both of which associate with the actin cytoskeleton; desmosomes and hemidesmosomes that interdigitate with intermediate filaments; tight junctions that serve a barrier function in epithelial sheets; gap junctions that play a role in cell-cell ionic communication; transient cell-cell junctions that occur in immune recognition; and specialized junctions such as nerve synapses (Staehlin and Hull, 1978). In most cases little is known about the role of junctional proteins in signal transduction. Thus we will mention only briefly two types of actin-based junctions where some understanding of signaling processes is beginning to emerge.

1. Cadherin-mediated adherence junctions As discussed in Section II. above, the classic cadherins bind to a group of proteins termed catenins which in turn can

link to the actin containing cytoskeleton forming a structure termed an adherence junction. Structures of this type are particularly important to the biology of epithelial cells, where "belts" of adherence junctions in neighboring cells are joined by dense bundles of actin filaments thus stabilizing the epithelial sheet. A more detailed picture of the structure of cadherin-based adherence junctions can be found elsewhere (Cowin and Burke, 1996; Geiger *et al.*, 1995); whereas the role of cadherin-based junctions in signaling will be described in detail in Section VIII.

2. *Junctions containing ezrin/radixin/moesin proteins* The ERM family of linker proteins seems to play a vital role in some aspects of cytoskeletal assembly (Tsukita *et al.*, 1997). These proteins share a highly homologous COOH-terminal actin binding domain and an equally well conserved N-terminal membrane binding domain. The membrane molecules that are the binding partners of ERM proteins have not been fully worked out; the polymorphic transmembrane glycoprotein/proteoglycan CD44 is clearly one of them, but the Na/K ATPase and the Ig-superfamily member ICAM-1 may also interact with ERM proteins. The ability of ERM proteins to link membrane molecules to actin seems to be closely regulated by the small GTPase Rho. However, evidence has been developed recently that moesin may in turn play a role in regulating the activity of Rho family GTPases (Takahashi *et al.*, 1997). In this manner, ERM proteins might affect the assembly of a variety of cytoskeletal structures thought to be regulated by Rho family proteins (see Section VI. for more detail). Thus, the precise biological role of ERM proteins has yet to be defined, but it seems likely that assembly/disassembly of adhesive junctions will be one aspect of their function.

IV. Direct Signal Transduction by Integrins: Activation of Tyrosine Kinases

A. Introduction

The concept that integrins function not merely as receptors for cell adhesion, but also elicit signal transduction events, emerged from several types of observations. For example, when $\alpha 3 \beta 1$ integrins were clustered on the surface of human carcinoma cells, the tyrosine phosphorylation level of a 115 to 130 kDa subset of proteins was enhanced (Kornberg *et al.*, 1991). Essentially the same subset of proteins was tyrosine phosphorylated in NIH3T3 and rat embryo fibroblasts (REFs) spreading on fibronectin-coated surfaces (Burrige *et al.*, 1992; Guan *et al.*, 1991). These effects were deemed to be integrin-specific through the use of antibodies to non-integrin cell surface molecules or by means of nonspecific attachment to polylysine-coated surfaces (Burrige *et al.*, 1992; Guan *et al.*, 1991; Kornberg *et al.*, 1991). Simultaneously, a similar pattern of tyrosine phosphorylation was observed in v-Src-transformed chicken embryo fibroblasts (CEFs) (Kanner *et al.*, 1990)

and upon treatment of quiescent Swiss 3T3 fibroblasts with the G-protein-coupled receptor agonists bombesin, vasopressin, and endothelin (Zachary *et al.*, 1991). These data helped to consolidate the idea that integrins have the ability to convey signals directly from the ECM to the inside of the cell. In this section and the next, we will review the identified key components, mechanistic models, and significance of these direct integrin-mediated effects on signaling, concentrating here on tyrosine phosphorylation events.

B. Focal Adhesion Kinase-Mediated Events

1. *Linkage to integrin signaling* Of the tyrosine phosphorylation events described above, the most prominent effect is observed in proteins in the molecular weight range of 110 to 130 kDa. It is now clear that the cytoplasmic tyrosine kinase, FAK accounts for a large proportion of the tyrosine phosphorylation in this region. Furthermore, tyrosine phosphorylation of FAK is regarded not only as an important event in integrin-mediated signaling, but as a common theme in multiple signal transduction pathways (see table 1) (Zachary *et al.*, 1992). FAK was cloned independently by the use of monoclonal antibodies raised to tyrosine-phosphorylated proteins in v-Src transformed CEFs (Schaller *et al.*, 1992) and by using a homology-based strategy to identify novel protein tyrosine kinases in a mouse cDNA library (Hanks *et al.*, 1992). FAK is a unique protein tyrosine kinase of approximately 125 kDa; it contains a central consensus kinase domain, a C-terminal domain having two proline-rich sequence, and a region required for focal adhesion targeting termed the "FAT" sequence. FAK has no transmembrane, SH2, or SH3 domains (fig. 4) (Hanks *et al.*, 1992; Hildebrand *et al.*, 1993; Schaller *et al.*, 1992). FAK now also has been cloned from humans (Andre and Becker, 1993) and *Xenopus* (Zhang *et al.*, 1995b) and exhibits a high degree of conservation between species.

An overabundance of evidence lends its weight to a critical role for FAK in integrin-mediated signaling. First, immunofluorescent staining shows that FAK colocalizes with proteins such as talin and tensin in focal adhesion sites of fibroblasts (Hanks *et al.*, 1992; Kornberg *et al.*, 1992; Schaller *et al.*, 1992). Immunoprecipitation studies demonstrate that FAK undergoes enhanced tyrosine phosphorylation upon adhesion to fibronectin or antibody-mediated integrin clustering (Burrige *et al.*, 1992; Guan and Shalloway, 1992; Kornberg *et al.*, 1992). Moreover, the tyrosine kinase activity of FAK in NIH 3T3 fibroblasts is increased two- to three-fold upon adhesion to fibronectin and activity is enhanced further in v-Src-transformed cells (Guan and Shalloway, 1992). FAK activity directly correlates with its phosphotyrosine content in several normal and tumor cell lines (Withers *et al.*, 1996). In platelets the FAK response is specific to a particular integrin, $\alpha \text{IIb} \beta 3$, the molecule that is required both for platelet aggregation

TABLE 1
Non-integrin-mediated tyrosine phosphorylation of focal adhesion kinase

Stimulus	Cell type	Observations	References
Anti-NCAM antibodies and soluble NCAM	NCAM-transfected COS-7 cells	Coprecipitates with NCAM140	(Beggs <i>et al.</i> , 1997)
PDGF	Quiescent Swiss 3T3 vascular smooth muscle cells	Bell-shaped dose response BB-homodimer only	(Abedi <i>et al.</i> , 1995; Rankin and Rozengurt, 1994)
Vascular endothelial growth factor	Human umbilical vein endothelial cells	Rapid, partially PKC-dependent response	(Abedi and Zachary, 1997)
Hepatocyte growth factor/scatter factor acting via the c-Met RTK	Human squamous carcinoma cells	Transient response, loss of phosphorylation corresponds with motile phenotype	(Matsumoto <i>et al.</i> , 1994)
Macrophage colony-stimulating factor via the M-CSF RTK	Human monocytes	Results in GRB2 binding to FAK	(Kharbanda <i>et al.</i> , 1995)
Bombesin, bradykinin, vasopressin, and endothelin acting via G-protein-coupled receptors	Quiescent Swiss 3T3	Rapid response (seconds)	(Leeb-Lundberg <i>et al.</i> , 1994; Zachary <i>et al.</i> , 1992)
Angiotensin II acting via G-protein-coupled receptor	Quiescent vascular smooth muscle cells	Rapid response	(Polte <i>et al.</i> , 1994; Turner <i>et al.</i> , 1995)
Prolactin acting via a cytokine family receptor	Human breast carcinoma cells	Also results in paxillin phosphorylation	(Canbay <i>et al.</i> , 1997)
Intracellular acting bacterial toxin	Quiescent Swiss 3T3	Response dissociated from PKC activation	(Lacerda <i>et al.</i> , 1996)
Bioactive lipids, e.g. sphingolipid metabolites and lysophosphatidic acid	Quiescent Swiss 3T3	Rapid, response, independent of PKC activation and Ca ²⁺ mobilization	(Seufferlein and Rozengurt, 1994a, b)
Aggregation of the FcεRI	Rat basophilic leukemia cells	Phosphorylation requires adherence	(Hamawy <i>et al.</i> , 1993)
Amyloid peptide	Differentiated neuroblastoma cultures	Effects with aggregated but not soluble peptide	(Zhang <i>et al.</i> , 1994)
Hyaluronan mediated by the HA receptor RHAMM	C-H-ras transformed 10T1/2 fibroblast cells	Rapid and transient response	(Hall <i>et al.</i> , 1994)

and platelet attachment to the blood vessel subendothelium (Kieffer and Phillips, 1990; Phillips *et al.*, 1991; Weiss *et al.*, 1986, 1989). Platelet α IIB β 3 undergoes a conformational change upon activation by thrombin or collagen enabling it to bind to fibrinogen (Siess, 1989). FAK shows increased tyrosine phosphorylation levels in platelets that have been stimulated with agonist and allowed to aggregate. In contrast, in platelets derived from patients with Glanzmann's thrombasthenia, which are deficient in α IIB β 3, FAK is not phosphorylated under the same experimental conditions (Lipfert *et al.*, 1992). FAK tyrosine phosphorylation in response to cellular adhesion to extracellular matrix proteins now has been shown in a range of cell types, which indicates that this pathway is used by a variety of integrins. In these cases, phosphorylation levels usually plateau 20 to 30 min after initiation of adhesion and remain persistently high for several hours (Burrige *et al.*, 1992; Hanks *et al.*, 1992; Lin *et al.*, 1997a). A central piece in the puzzle was provided by the development of FAK-deficient mice. These animals die early in development and the embryos show gastrulation abnormalities similar to those found in fibronectin-deficient animals, indicating that the same pathway is impacted in both models (George *et al.*, 1993; Ilic *et al.*, 1995).

More direct, albeit less physiological, evidence for an integrin-FAK connection is the finding that, under *in vitro* conditions, synthetic peptides based on β subunit cytoplasmic domains can bind to the N-terminal region of FAK (Schaller *et al.*, 1995); it should be noted that this putative integrin binding region is distinct from the

focal adhesion targeting sequence. Additionally, fibroblasts expressing chimeric receptors containing the cytoplasmic domain of various integrins linked to the extracellular domain of the IL-2 receptor show increased phosphorylation of FAK (Akiyama *et al.*, 1994). In this scenario, chimeras containing the β 1, β 3, and β 5 cytoplasmic domains, but not the α 5 or alternatively spliced β 3B cytoplasmic tail, enhance FAK tyrosine phosphorylation, indicating the participation only of specific integrin subunits in the process (Akiyama *et al.*, 1994). However, it is still controversial as to whether FAK interacts directly or indirectly with integrin cytoplasmic tails.

Besides integrin-mediated events, FAK tyrosine phosphorylation is increased through a variety of non-integrin cell surface receptors including growth factor receptor tyrosine kinases and G-protein-coupled receptors (see table 1). Many of these studies have been carried out using adherent quiescent Swiss 3T3 cells which, in contrast to NIH3T3 fibroblasts under similar conditions, exhibit low levels of focal adhesions and actin stress fibers. In this paradigm, effects on FAK are usually evident within seconds of treatment and are of a transient nature. The actions of G-protein-coupled receptor agonists, such as bombesin, sphingolipid metabolites, and lysophosphatidic acid (LPA), are likely to be mediated through the small GTP-binding protein Rho (Ridley and Hall, 1994; Wang *et al.*, 1997).

2. *Mechanism of activation* In growing fibroblasts, FAK is phosphorylated on both tyrosine and serine residues (Calalb *et al.*, 1995; Schlaepfer and Hunter, 1996).

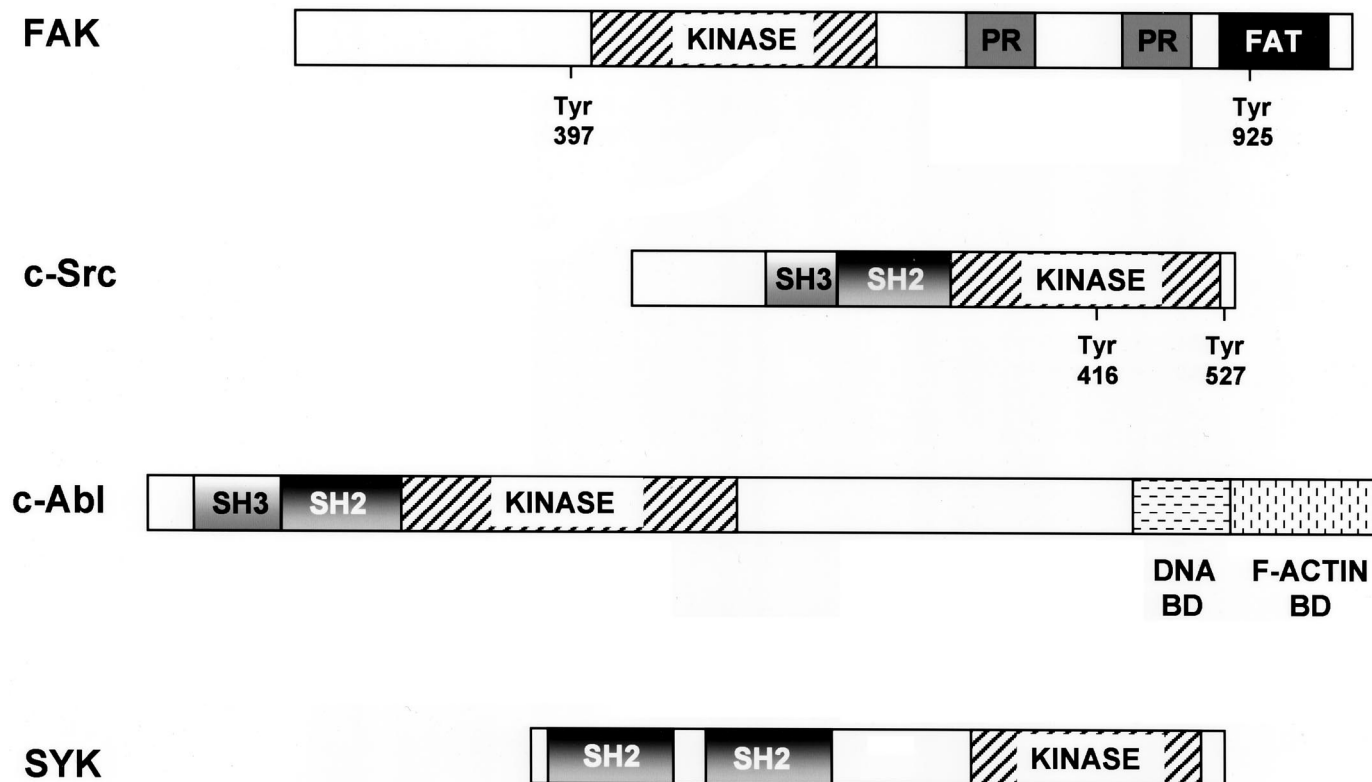


FIG. 4. The structural domains of integrin-responsive tyrosine kinases. The domain structure of FAK, c-Src, c-Abl, and Syk are shown. The position of the kinase domain and Src homology 2 (SH2) and 3 (SH3) domains, which mediate protein-protein interactions, are indicated. In FAK, the C-terminal proline-rich (PR) and focal adhesion targeting (FAT) domains are shown. Similarly, in c-Abl the position of the DNA and F-actin binding domains are illustrated.

All phosphotyrosine residues are dephosphorylated upon cell detachment, whereas phosphorylation at serine residues is largely unaffected (Calalb *et al.*, 1995). Presumably a protein tyrosine phosphatase is activated upon cell detachment, but the identity of this enzyme(s) remains elusive. Although it is worth remembering that serine phosphorylation can control protein function and protein-protein interactions (Muslin *et al.*, 1996), relatively little is known about the sites and function of FAK serine phosphorylation, and therefore this review will focus on the importance of tyrosine-phosphorylated residues. As observed for many receptor tyrosine kinases, it is the autophosphorylation of FAK that is the trigger for the regulation its downstream functional activities. FAK autophosphorylation within the sequence Y³⁹⁷AEI occurs *in vitro* in a bacterial-fusion protein and in immune complex kinase reactions of FAK expressed in fibroblasts (Calalb *et al.*, 1995; Schaller *et al.*, 1994). In some cell types, autophosphorylation of FAK *in trans* may also occur, as exogenously expressed kinase-dead FAK can be tyrosine phosphorylated (Cary *et al.*, 1996; Eide *et al.*, 1995; Schlaepfer and Hunter, 1996). FAK additionally is phosphorylated at Tyr407, Tyr576, Tyr577, Tyr861, and Tyr925 in growing fibroblasts (Calalb *et al.*, 1995, 1996; Schlaepfer *et al.*, 1994; Schlaepfer and Hunter, 1996). Phosphorylation at Tyr576 and Tyr577 in the catalytic domain of FAK is important for maximal kinase activity

(Calalb *et al.*, 1995). Tyr925 is within a consensus site, YENV, for binding the SH2 domain of the adapter protein, growth factor receptor-binding protein-2 (GRB2) (Schlaepfer *et al.*, 1994; Songyang *et al.*, 1993). The roles of P-Tyr407 and P-Tyr861 as yet remain elusive.

3. *The role of Src in focal adhesion kinase signaling*
Evidence of Src playing a pivotal role in FAK signaling events is manifest by augmented tyrosine phosphorylation of FAK in v-Src-transformed fibroblasts (Calalb *et al.*, 1995; Guan and Shalloway, 1992). The FAK autophosphorylation motif (YAEI) resembles the YEEI consensus site for Src-SH2 binding (Songyang *et al.*, 1993) and indeed Src binds FAK at this site after integrin-mediated FAK autophosphorylation in fibroblasts and in v-Src-transformed 3T3 fibroblasts (Schaller *et al.*, 1994; Schlaepfer *et al.*, 1994). This association is transient in adhering fibroblasts and is absent upon serum-starvation despite unaffected phosphorylation of FAK at Tyr397. However, in v-Src-transformed cells the association is stable and independent of serum and adhesion (Cobb *et al.*, 1994a; Schaller *et al.*, 1994; Schlaepfer *et al.*, 1994; Schlaepfer and Hunter, 1996). Thus, additional factors regulate the ability of Src to bind FAK, even when FAK is in a "primed" state. The FAK residues Tyr407, Tyr576, Tyr577, Tyr861, and Tyr925 all show enhanced phosphorylation upon v-Src transformation and can be phosphorylated directly by Src *in vitro* (Ca-

lalb *et al.*, 1995, 1996; Schlaepfer and Hunter, 1996). Src interaction with Tyr397 and subsequent phosphorylation of FAK results in GRB2 binding to Tyr925; GRB2 binding to FAK is a putative link between FAK tyrosine phosphorylation and the integrin-mediated activation of mitogen-activated protein kinase (MAPK) (see Section V.) (Schlaepfer and Hunter, 1996, 1997). FAK-Src interaction also enhances the association of the adapter protein, p130^{CAS} with FAK signaling complexes in cells adhering to fibronectin (Schlaepfer and Hunter, 1997).

In conclusion, these data point toward a model whereby the autophosphorylation of FAK leads to binding of the SH2 domain of Src and the resultant phosphorylation of FAK by Src on multiple residues. Phosphorylation of these residues directly initiates FAK-mediated signal transduction events (see fig. 5). It is noteworthy that FAK is capable of binding other Src-family kinases, including Fyn and Yes (Cobb *et al.*, 1994a; Polte and Hanks, 1995; Schlaepfer *et al.*, 1997), and hence, the relevant Src-family kinase may depend on cell-specific expression and/or regulation mechanisms.

4. Other focal adhesion kinase-binding proteins Formation of complexes through SH2 and SH3 domain-mediated protein-protein interactions is often essential for the instigation of a signaling cascade. FAK interacts with a variety of SH2/SH3 domain containing proteins

within the focal adhesion. Some of these interactions are constitutive, whereas some depend on the tyrosine phosphorylation state of FAK. Src-family kinases are not the only possible occupants of the Tyr397 binding site. The p85 α subunit of PI-3K interacts with Tyr397 upon cell adhesion or PDGF treatment of NIH3T3 cells, despite the fact that YAEI is not an optimal binding site for the p85 SH2 domain (Chen *et al.*, 1994a, 1996a; Songyang *et al.*, 1993). In thrombin-activated platelets, p85 binds FAK, but interactions are directed by the SH3 domain of p85 and the second proline-rich region of FAK (Guinebault *et al.*, 1995). Addition of the relevant FAK-derived peptides to p85 immunoprecipitates activates PI-3K activity in each case (Chen *et al.*, 1996a; Guinebault *et al.*, 1995).

Extensive studies show that the focal adhesion proteins p130^{CAS}, paxillin, tensin, and cortactin, in addition to FAK, show increased tyrosine phosphorylation levels upon integrin-mediated adhesion in fibroblasts (Bockholt and Burridge, 1993; Burridge *et al.*, 1992; Nojima *et al.*, 1995; Petch *et al.*, 1995; Vuori and Ruoslahti, 1995). These proteins account for some of the additional phosphotyrosine bands initially identified in extracts from cells adhering to the ECM and in v-Src-transformed fibroblasts. Two of these proteins, namely p130^{CAS} and paxillin, are able to interact with FAK; p130^{CAS} via SH3 domain interactions with the FAK proline-rich se-

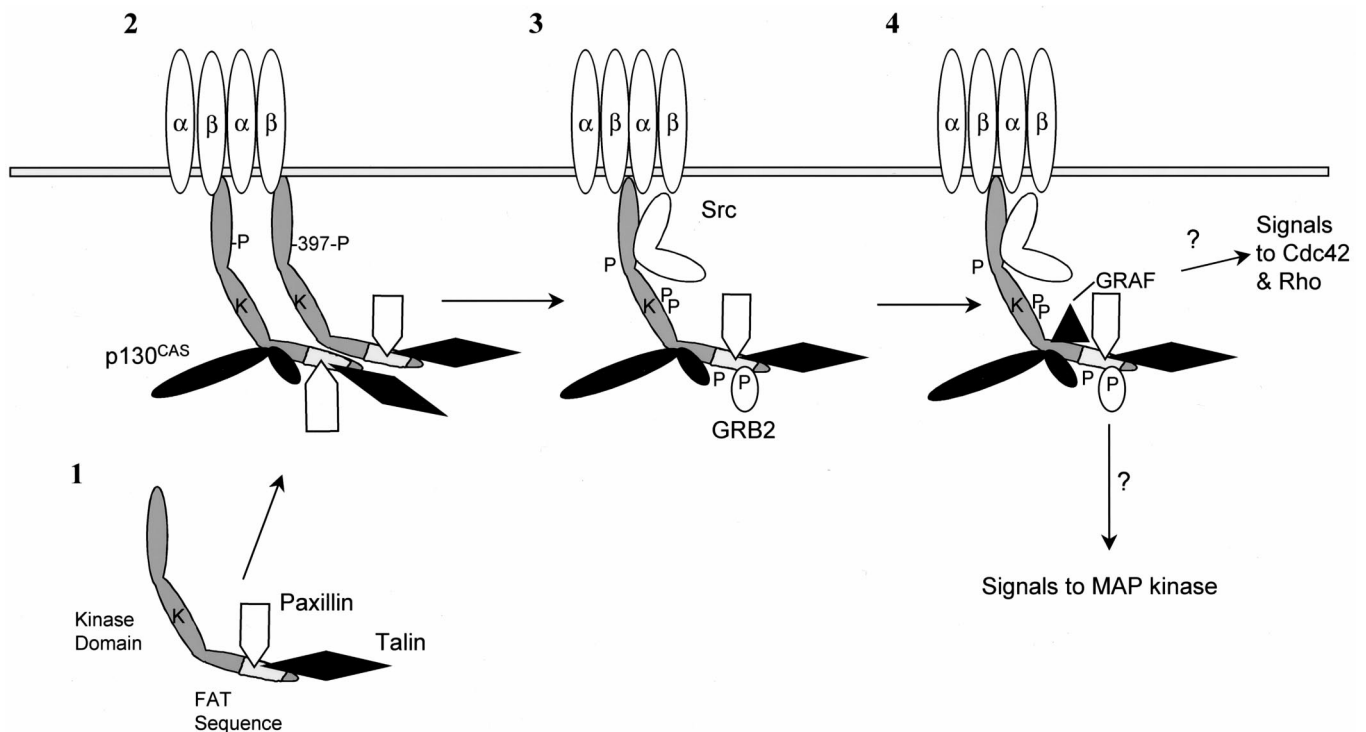


FIG. 5. Mechanism of integrin-mediated activation of FAK and signaling complex formation. Cytoplasmic FAK (1) is associated constitutively with paxillin and talin. Upon integrin engagement (2), FAK localizes to focal adhesions, possibly through an interaction with the integrin β subunit, resulting in autophosphorylation at Tyr397. This creates a binding site for the SH2 domain of c-Src, which on binding phosphorylates at the sites Tyr407, Tyr576, Tyr577, Tyr861, and Tyr925. Src phosphorylation of FAK enhances binding of p130^{CAS} and GRB2, the latter through binding to Tyr925 (3). Thus, a multimeric signaling complex may be formed (4), which can control downstream events and which contains a negative feedback mechanism through the binding of GRAF. The interaction of PI-3K with FAK has not been illustrated here.

quences and paxillin via the region spanning amino acids 904 to 1054 (Bellis *et al.*, 1995; Harte *et al.*, 1996; Hildebrand *et al.*, 1995; Polte and Hanks, 1995, 1997; Schaller *et al.*, 1993). p130^{CAS} originally was identified as a highly tyrosine-phosphorylated protein in v-Src- and v-Crk-transformed fibroblasts (Birge *et al.*, 1992; Kanner *et al.*, 1990, 1991; Matsuda *et al.*, 1990). Structurally, p130^{CAS} resembles a “docking protein” because it contains a SH3 domain followed by multiple SH2 binding motifs (Sakai *et al.*, 1994). The SH3 domain of p130^{CAS} is essential for focal adhesion targeting, a function that is independent of FAK binding (Nakamoto *et al.*, 1997). A pool of p130^{CAS} is associated constitutively with FAK, but upon adhesion, Src kinase activity may be responsible for stabilizing the FAK- p130^{CAS} complex (Nakamoto *et al.*, 1996, 1997; Polte and Hanks, 1997). Recently, p130^{CAS} was identified as an important mediator of FAK-promoted cell motility (Cary *et al.*, 1998).

Another integrin-regulated protein is the 68 kDa adapter molecule, paxillin (discussed in Section III. above). Paxillin includes a proline-rich sequence, four LIM domains and multiple putative SH2 binding sites (Salgia *et al.*, 1995; Sanchez-Garcia and Rabbitts, 1994; Turner and Miller, 1994). The paxillin binding site in FAK overlaps the C-terminal FAT sequence. Some groups report FAK localization to integrin clusters/focal adhesions in the absence of paxillin binding (Hildebrand *et al.*, 1995; Miyamoto *et al.*, 1995b), whereas others indicate that paxillin binding recruits FAK to focal adhesions (Tachibana *et al.*, 1995). Association between FAK and another focal adhesion protein, talin, also was observed by co-immunoprecipitation experiments, and the binding site was localized by *in vitro* experiments to amino acids 965 to 1012 in FAK (Chen *et al.*, 1995). The second proline-rich region in the C-terminus of FAK is capable of binding the SH3 domain of GRAF (GTPase regulator associated with FAK), a member of the GTPase-activating protein family (GAP) (Hildebrand *et al.*, 1996). This interaction may serve to turn off RhoA and Cdc42 activities after the formation of focal adhesions and actin stress fibers. Under *in vitro* conditions, phosphorylated FAK also can bind the SH2 domains of NCK, phospholipase C, and CSK (Sabe *et al.*, 1994; Schlaepfer *et al.*, 1994). As evident from the above data, FAK is capable of forming multiple associations with a variety of proteins and these interactions are likely to serve as connections to the actin cytoskeleton and to recruit other proteins to form complex signaling units (fig. 5). The relative importance of FAK kinase activity versus the ability of FAK to serve as a scaffolding molecule is under close scrutiny, and there is growing evidence that the scaffolding function is important (Cary *et al.*, 1996; Hamawy *et al.*, 1997).

5. Inhibition of focal adhesion kinase activity The adhesion-induced phosphorylation of FAK can be inhibited by agents that disrupt the cytoskeleton or delay cell spreading. Tyrosine phosphorylation of FAK can be po-

tently blocked by treatment with either the actin depolymerizing agent, cytochalasin, or the tyrosine kinase inhibitor, herbimycin (Burrige *et al.*, 1992; Sinnett-Smith *et al.*, 1993). Extracellular stimulation by insulin causes transient dephosphorylation of FAK and paxillin in rat 1 fibroblasts and in CHO cell lines overexpressing the insulin receptor (Knight *et al.*, 1995; Konstantopoulos and Clark, 1996; Ouwens *et al.*, 1996; Pillay *et al.*, 1995). This may be caused by insulin-mediated effects on the cytoskeleton or through phosphotyrosine phosphatase (PTP) 1D activity (Knight *et al.*, 1995; Ouwens *et al.*, 1996). FAK is dephosphorylated rapidly upon cell detachment (Calalb *et al.*, 1995), indicating a PTP is activated under these conditions. Recently the leukocyte common antigen related (LAR) PTP was found to localize partially to focal adhesions in human breast adenocarcinoma cells, although its specificity for proteins, such as FAK and paxillin, and therefore its role in focal adhesion disassembly, are presently unclear (Serra-Page *et al.*, 1995). Additionally, a proteolytic mechanism may be used in activated platelets. FAK, activated on α IIb β 3 engagement and platelet aggregation, can be proteolyzed by the protease calpain to produce fragments that exhibit reduced autokinase activity and dissociate from the cytoskeleton, an action that may be involved in cytoskeletal changes associated with blood clot retraction (Cooray *et al.*, 1996).

A regulatory mechanism that is likely to operate in some cell types is the autonomous expression of the C-terminus of FAK, referred to as FAK-related nonkinase (FRNK). FRNK contains the FAT sequence but is devoid of any kinase activity (Schaller *et al.*, 1993). Overexpression of FRNK in CEFs reduces the tyrosine phosphorylation state of FAK and the ability of these cells to spread on fibronectin by competing with FAK for sites in focal adhesions (Richardson and Parsons, 1996).

6. Focal adhesion kinase substrates Because of the complex associations between kinases and structural proteins, unveiling biologically relevant *in vivo* substrates is an arduous task. For example, paxillin, tensin, and p130^{CAS} tyrosine phosphorylation closely correlate with that of FAK upon cell adhesion. Additionally, FAK phosphorylates recombinant paxillin in immunoprecipitation kinase assays, whereas FRNK overexpression reduces the tyrosine phosphorylation of paxillin and tensin (Bellis *et al.*, 1995; Burrige *et al.*, 1992; Harte *et al.*, 1996; Richardson and Parsons, 1996); all this suggests that these proteins may be FAK substrates. On the other hand, Src is capable of interacting with paxillin (fig. 3A) (Salgia *et al.*, 1995; Weng *et al.*, 1993), and integrin-stimulated phosphorylation of p130^{CAS} likely is directed by c-Src (Bockholt and Burrige, 1995; Hamasaki *et al.*, 1996; Vuori *et al.*, 1996); further, phosphorylation of paxillin and tensin is not altered significantly in FAK $-/-$ fibroblasts (Ilic *et al.*, 1995). Thus the precise cause of tyrosine phosphorylation events in focal contacts remains obscure.

7. *Focal adhesion kinase function* Analysis of fibroblast lines derived from FAK $-/-$ mice downplays the importance of FAK in the assembly of focal adhesions during cell adhesion. FAK-deficient fibroblasts retain the ability to spread on a fibronectin substratum and have normal levels of tyrosine phosphorylation on paxillin and tensin (Ilic *et al.*, 1995). The cells, however, are morphologically dissimilar from wild-type controls in that they are more rounded, exhibit abundant ventral focal adhesions, and have numerous peripheral stress fibers and microspikes. Further, these cells show decreased mobility on fibronectin (Ilic *et al.*, 1995), which indicates a possible defect in filopodia formation/extension and focal adhesion turnover. Relevant to this concept is the knowledge that FAK tyrosine phosphorylation is increased during neurite outgrowth of differentiating neuroblastoma cells, a process that involves growth cone filopodia extension (Leventhal and Feldman, 1996).

Findings in a variety of other cell types support the view that FAK functions in cell migration. CHO cells overexpressing FAK exhibit enhanced migration on fibronectin, an effect dually mediated both by phosphorylation at Tyr397 and p130^{CAS} binding to the first proline-rich region of FAK, but independent of kinase activity (Cary *et al.*, 1996, 1998). Tyrosine phosphorylation of FAK is increased in migrating human umbilical vein endothelial cells (HUVECs) (Romer *et al.*, 1994), and both FAK localization to focal adhesion sites and cell migration in a wound healing assay are disrupted by microinjection of a FRNK-like protein (Gilmore and Romer, 1996). Furthermore, FAK is highly localized to the migrating edge of human keratinocytes (Gates *et al.*, 1994), and the migration rate of human melanoma cell lines correlates with the level of FAK expression (Akasaka *et al.*, 1995).

FAK is enriched particularly in the brain (Andre and Becker, 1993; Grant *et al.*, 1995; Hanks *et al.*, 1992) where alternatively spliced forms also are expressed (Andre and Becker, 1993; Burgaya and Girault, 1996). In primary developing rat neurons, FAK is localized to the growth cones of extending neurites and to the perikarya (Burgaya *et al.*, 1995). The FAK-Fyn interaction is likely to play a physiological role in the brain as FAK tyrosine phosphorylation is partially reduced in Fyn $-/-$ mice, which show impaired hippocampal development, spatial learning, and long-term potentiation defects (Grant *et al.*, 1992, 1995). Activation of neuronal cannabinoid receptors leading to inhibition of adenylyl cyclase regulates the tyrosine phosphorylation of a FAK isoform, termed FAK⁺, that contains a three amino acid insert within the FAT sequence (Burgaya and Girault, 1996; Derkinderen *et al.*, 1996). These studies implicate FAK in neuronal development and synaptic plasticity.

Evidence that FAK tyrosine phosphorylation may be delicately balanced inside the cell can be drawn from studies of adhesion-dependent cell survival. As will be

discussed in Section X., cell anchorage to ECM proteins suppresses apoptosis in epithelial and endothelial cells (Frisch *et al.*, 1996b). A variety of studies implicate FAK in protecting cells against apoptosis. For example, attenuation of high-level FAK expression in tumor cell lines causes cell rounding and apoptosis (Xu *et al.*, 1996) and FAK is cleaved by caspases during Fas- and Apo-2L-induced apoptosis of Jurkat cells (Wen *et al.*, 1997). Opposing effects on cell morphology are caused by tyrosine phosphorylation instigated by activation of integrins and by v-Src transformation. Despite the phosphorylation of a common set of proteins, v-Src-transformed cells exhibit reduced focal adhesions coinciding with a loss of cell-substrate adherence (Krueger *et al.*, 1983); therefore, hyperphosphorylation of FAK may have a deleterious effect on the cell.

8. *Focal adhesion kinase summary* Upon integrin-mediated cell adhesion FAK is rapidly tyrosine phosphorylated at Tyr397 leading to recruitment of Src and further phosphorylation events. This sets the stage for recruitment of additional SH2-domain proteins such as GRB2, whereas other moieties such as the adapter protein p130^{CAS} may be associated constitutively with FAK. Thus FAK is a key component in the assembly of focal contact structures that can influence the organization of the cytoskeleton and of associated signal transduction complexes. Although the overall relationship between integrin-mediated adhesion and FAK activation is quite clear, the underlying mechanisms are not. Thus, the question remains whether the FAK amino-terminal domain directly binds integrins, thus activating FAK, or whether FAK recruitment and activation involves other focal contact proteins. Cells are likely to highly regulate FAK tyrosine phosphorylation, and abnormally high levels of FAK tyrosine phosphorylation, such as those found in v-Src-transformed cells, may alter the normal function FAK. Likewise, tyrosine phosphatases are likely to be important in the regulation of FAK, but the identity and role of the relevant enzymes have yet to be defined.

There is a preponderance of evidence that FAK plays a critical role in cell migration and motility. Although FAK activation originally was thought to promote the establishment of focal contacts, newer insights suggest that it may regulate the disassembly of focal adhesions instead to permit cell movement. FAK also may have an important part to play in the regulation of apoptosis in certain cell types, as well as in aspects of neuronal development. The role of FAK in integrin signaling to the MAPK pathway remains controversial and will be discussed in detail in Section V. Thus, FAK has been implicated as a key structural component and upstream signal transducer in focal contacts; however, many aspects of its function await more precise definition.

C. Focal Adhesion Kinase Family Members

Recently, the identification of proline-rich tyrosine kinase 2 (PYK2), also known as cell adhesion kinase β

(CAK β), related adhesion focal tyrosine kinase (FAK), and calcium-dependent tyrosine kinase (CADTK), has established the existence of FAK family members (Avraham *et al.*, 1995; Lev *et al.*, 1995; Sasaki *et al.*, 1995; Yu *et al.*, 1996). PYK2 shows 60% identity to FAK in the kinase domain, with approximately 40% identity in the N- and C-terminal regions (Avraham *et al.*, 1995; Sasaki *et al.*, 1995). An autophosphorylation site (Tyr402) and the ability to associate with c-Src, p130^{CAS}, GRB2, and paxillin are retained in PYK2, suggesting that PYK2 and FAK may act through similar downstream mechanisms (Astier *et al.*, 1997; Dikic *et al.*, 1996; Lev *et al.*, 1995; Li and Earp, 1997). However, PYK2 shows a more limited tissue distribution than FAK (Avraham *et al.*, 1995; Sasaki *et al.*, 1995) and is regulated differently, becoming tyrosine phosphorylated in response to agents that increase intracellular calcium under conditions that do not influence FAK (Lev *et al.*, 1995; Siciliano *et al.*, 1996; Tokiwa *et al.*, 1996; Yu *et al.*, 1996). Further, recent studies have suggested that FAK and PYK2 have opposing functions in terms of cell survival (Xiong and Parsons, 1997). Thus the biology of FAK and PYK2 may be quite distinct even though the two proteins are structurally closely related. In certain cell types integrin signaling may impinge on the PYK2 activation. For example, PYK2 is tyrosine phosphorylated upon integrin-mediated adhesion of human B cells and megakaryocytic cells (Astier *et al.*, 1997; Li *et al.*, 1996), and when overexpressed in CEFs, is localized to focal adhesions in a fraction of the cells (Schaller and Terukatsu, 1997). However, PYK2 seems to show no response to ECM-mediated signals in other cell types (Sasaki *et al.*, 1995). Downstream signaling from PYK2 can lead to the activation of both the Jun N-terminal kinase (JNK) and ERK MAPK pathways (Dikic *et al.*, 1996; Tokiwa *et al.*, 1996). It is presently unclear whether PYK2 is capable of partially compensating for FAK in integrin signaling pathways. In particular, this may be relevant when studying adhesion-mediated signaling in fibroblasts derived from FAK $-/-$ mice.

D. Src Family Members

1. Activation of Src family members downstream of $\beta 1$ integrins Because of their ability to bind FAK and augment FAK signaling, the role of Src family kinases in integrin-mediated signaling events warrants further discussion. c-Src and its other family members (e.g., Yes, Fyn, Fgr, and Hck) share common structural features; an N-terminal myristylation and membrane localization signal, one SH2 and one SH3 domain, the catalytic domain, and a short C-terminal region containing a conserved critical tyrosine residue (see fig. 4), as described in a recent comprehensive review (Brown and Cooper, 1996). c-Src can be activated by many extracellular stimuli, including the ECM through integrins, growth factors acting via receptor tyrosine kinases (e.g., PDGF and EGF), G-protein coupled receptor agonists (e.g., thrombin and LPA), signals involved in T- and B-cell activation, and ultraviolet irradiation (Erpel and Courtneidge,

1995; Parsons and Parsons, 1997). c-Src acts as a molecular switch whereby activation, either by dephosphorylation of Tyr527 in the C-terminus or possibly through competition for the SH2 domain, results in a conformational change that breaks an intramolecular interaction between the SH2 domain and P-Tyr527 and in turn exposes the kinase domain (Matsuda *et al.*, 1990). Src autophosphorylation at Tyr416 within the kinase domain is necessary for full activity but is overridden by phosphorylation at Tyr527 (Stover *et al.*, 1994). The SH3 domain is also important in maintaining the inactive closed conformation of Src. This model recently was supported with structural evidence from a recombinant inactive fragment of human c-Src showing interaction of the SH2 domain with the phosphorylated Tyr527 residue and association of the SH3 domain with the linker region between the SH2 and kinase domains (Xu *et al.*, 1997).

The CSK is the major kinase responsible for phosphorylating Tyr527. Adhesion-mediated c-Src activation is transient and there is evidence that a proportion of cellular CSK also relocates to adhesion plaques upon Src activation (Bergman *et al.*, 1995; Howell and Cooper, 1994). The CSK SH2 domain is capable of binding both phosphorylated FAK and paxillin, and this interaction may serve to anchor CSK to focal adhesions (Sabe *et al.*, 1994; Schaller and Parsons, 1995). CSK overexpression in HeLa cells alters cell shape and decreases cell adhesion; therefore, CSK may be important in inactivating Src at focal adhesion sites as well as in the turnover of focal adhesions (Bergman *et al.*, 1995). The phosphatase responsible for activating Src through dephosphorylation at Tyr527 in vivo is still unknown. Putative candidates are the receptor PTP α (Zheng *et al.*, 1992), the ezrin-like PTP (Moller *et al.*, 1994), and the SH2 domain containing PTP, SHPTP2/Syp (Feng *et al.*, 1993; Peng and Cartwright, 1995). At present there is no evidence of localization of these PTPs to focal adhesions.

Upon fibroblast adhesion to fibronectin, c-Src becomes dephosphorylated at Tyr527, activated, and redistributes from a perinuclear localization to focal adhesions (Kaplan *et al.*, 1992, 1994, 1995). Mutational analyses implicate the myristylation sequence and the SH3 domain, but not catalytic activity, as Src FAT requirements (Kaplan *et al.*, 1994). Furthermore, this process may involve the GTP-binding protein Rho, because v-Src can be relocalized to focal adhesions upon microinjection of activated RhoA into quiescent Swiss 3T3 cells (Fincham *et al.*, 1996).

As mentioned above, Src plays a vital role in the formation of a scaffolding complex of signaling molecules at a focal adhesions. As for FAK, the intricacies of the interactions in vivo are still hazy at present. Both the SH2 and SH3 domains of Src are capable of interacting with p130^{CAS} (Nakamoto *et al.*, 1996). These interac-

tions may serve to target Src to focal adhesions or may enhance activation by stabilization of the open conformation once Src is located at focal adhesions. The tyrosine phosphorylation status of p130^{CAS} evidently is linked to interactions with Src family kinases, because p130^{CAS} tyrosine phosphorylation levels are decreased in all three Src-family $-/-$ cell types, most notably in Src $-/-$ fibroblasts (Bockholt and Burrige, 1995; Schlaepfer *et al.*, 1997; Vuori *et al.*, 1996). Furthermore, p130^{CAS} tyrosine phosphorylation levels are increased in CSK-deficient cells (Vuori *et al.*, 1996). p130^{CAS} localization to focal adhesions and subsequent tyrosine phosphorylation expands the potential signals emanating from focal adhesions via binding of the adapter proteins Crk and NCK (Hamasaki *et al.*, 1996; Schlaepfer *et al.*, 1997; Vuori *et al.*, 1996). A similar scenario is true of paxillin; Src is also capable of binding, through its SH2 and/or SH3 domain, and then phosphorylating paxillin (Schaller and Parsons, 1995; Weng *et al.*, 1993). This tethering may be important for localizing Src to focal adhesions, but more likely is required to increase the repertoire of SH2 domain binding sites. In this regard, phosphorylated paxillin, like p130^{CAS}, is capable of binding Crk at least in vitro (Schaller and Parsons, 1995). The importance of Src in this complex, in functions such as cell migration and motility, is evident from Src $-/-$ fibroblasts that show delayed spreading on fibronectin compared with cells re-expressing c-Src (Kaplan *et al.*, 1995). This effect requires the SH2 and SH3 domains of c-Src but, as with focal adhesion localization, is independent of kinase activity (Kaplan *et al.*, 1995). However, other studies in fibroblast that are negative for Src family members have not seen pronounced changes in cell spreading (Bockholt and Burrige, 1995). Thus, whereas Src seems to be implicated in cytoskeletal function, its quantitative importance still is undefined.

In summary, it is becoming apparent that FAK, Src, p130^{CAS}, and paxillin form a distinct quaternary signaling unit in adhering cells which is initiated by autophosphorylation of FAK (Schlaepfer and Hunter, 1997). This unit regulates cell spreading and mobility upon integrin engagement through the recruitment of additional signaling and adapter proteins. It is unclear why c-Src and v-Src seem to play paradoxically opposing roles in the focal adhesion; c-Src enhancing cell spreading, v-Src causing morphological transformation and reduced attachment. One possibility is that v-Src activity is refractory to inactivation by CSK and, hence, shows higher and more prolonged kinase activity within focal adhesions, thus disrupting the delicate balance involved in focal adhesion assembly.

2. Activation of Src family members downstream of $\beta 2$ integrins The studies mentioned thus far in this section mainly concern signaling through $\beta 1$ integrins. Interestingly, signaling through other β subunits shows discrete differences in the subset of proteins tyrosine phosphorylated compared with those proteins phosphorylated

upon $\beta 1$ engagement (Berton *et al.*, 1994; Graham *et al.*, 1994). Differential expression of alternative Src kinases may account for at least some of these observations. A pertinent example here is polymorphonuclear leukocytes (PMNs), in which $\beta 2$ -integrins are the dominant functional adhesion receptors important for attachment and migration into sites of inflammation, and in which the major expressed Src family kinases are Fgr, Hck, and Lyn (Tsygankov and Bolen, 1993). In PMNs, TNF activation of Fgr depends on the engagement of $\beta 2$ integrins (Berton *et al.*, 1994). Enhancement of Fgr activity may result in the tyrosine phosphorylation of other proteins, such as paxillin and the guanine nucleotide exchange factor, Vav, which also displays enhanced tyrosine phosphorylation levels in a $\beta 2$ -dependent manner in PMNs (Graham *et al.*, 1994; Zheng *et al.*, 1996a). These responses are specific to $\beta 2$ because PMNs derived from patients with leukocyte adhesion deficiency, which lack $\beta 2$ expression, do not activate Fgr or phosphorylate paxillin in response to TNF (Berton *et al.*, 1994; Graham *et al.*, 1994). Other studies implicate a role for Fgr and Hck in the spreading and respiratory bursts of PMNs adhering to ECM components (Lowell *et al.*, 1996). Interestingly, $\beta 2$ antibody-mediated clustering does not activate FAK, despite FAK expression in neutrophils (Fuortes *et al.*, 1994). Thus, several of the Src family kinases apparently perform physiological functions in integrin adhesion-dependent events in numerous cell types, and some degree of subunit specificity exists in the connections between integrins and cytoplasmic tyrosine kinases.

E. Other Tyrosine Kinases Activated by Integrins

1. C-Abl A more recently identified noncytoplasmic tyrosine kinase that is regulated by integrins is encoded by the c-Abl protooncogene. The kinase domain of c-Abl is flanked by N-terminal SH2 and SH3 domains, whereas the F-actin and DNA binding domains are at the extreme C-terminus of the protein (fig. 4) (Wang, 1993). Both the cytoplasmic and nuclear pools of c-Abl become activated upon adhesion of fibroblasts to fibronectin and a pool of the enzyme transiently localizes to focal adhesions (Lewis *et al.*, 1996). It is unclear at present where c-Abl activation is sequentially on the integrin signaling pathway. A constitutively active form of c-Abl, the Bcr-Abl found in human patients with chronic myelogenous leukemia, causes enhanced phosphorylation of paxillin in myeloid cells (Salgia *et al.*, 1995). Thus, c-Abl may participate in integrin-mediated events by phosphorylating focal adhesion proteins. Bcr-Abl also induces anchorage-independent, but growth factor-dependent, proliferation in fibroblasts, placing Abl downstream of integrins but upstream of the point of convergence with growth factor signaling pathways (Renshaw *et al.*, 1995). In addition, c-Abl, through interactions with the C-terminal F-actin and DNA binding domains, may function in integrin-dependent regulation

of the structure of actin filaments and in cell cycle progression.

2. *Spleen tyrosine kinase* In hematopoietic cell types such as monocytes, neutrophils, and platelets, tyrosine phosphorylation and activation of the 76 kDa SYK apparently are a common link in the integrin signaling chain. SYK is a member of a family, also containing ZAP-70, which is characterized by the presence of two tandem N-terminal SH2 domains and a C-terminal kinase domain (fig. 4) (Law *et al.*, 1994). The SH2 domains of SYK are capable of binding phosphotyrosine residues in B- and T-cell receptors, leading to phosphorylation of SYK and activation of SYK during T- and B-cell stimulation by antigens (Couture *et al.*, 1994; Qian and Weiss, 1997).

During inflammation, migration of monocytes into tissues requires adhesion to vascular endothelial cells and ECM components. As in other cell types, engagement of $\beta 1$ integrins in monocytes by ECM proteins, or with anti- $\beta 1$ antibodies, results in the enhanced tyrosine phosphorylation of several proteins, the most predominant of which is SYK (Lin *et al.*, 1994, 1995). These events in monocytes are independent of cytoskeletal integrity and correlate closely with NF κ B activation and the induction of immediate early genes, such as cytokines, that mediate the inflammatory response (Juliano and Haskill, 1993; Lin *et al.*, 1995). FAK is undetectable in human monocytes, although it is present in monocytic cell lines, such as THP1, and is tyrosine phosphorylated upon adhesion to fibronectin (Lin *et al.*, 1994, 1995). More recently, SYK has been shown to be activated on TNF α - or Mn²⁺-induced spreading of human neutrophils on a fibrinogen-coated surface (Yan *et al.*, 1997). Thus, integrin-mediated SYK activation is a common theme in leukocytic cell adhesion and transmigration into a site of inflammation.

Agonist activation of platelets, by thrombin or subendothelial collagen for example, triggers a cascade of events resulting in alterations in cell shape and cell aggregation. A pivotal stage in this response is the change in conformation of the α IIB β 3 integrin so that it will bind to fibrinogen (Siess, 1989). Activation of platelets involves several waves of tyrosine phosphorylation; the early wave is α IIB β 3-independent and results in Src activation, the second phase is provoked by α IIB β 3 engagement, and the late stage depends on aggregation. Collagen stimulation of platelets results in SYK activation within seconds, and a requirement for SYK activity in platelet aggregation has been demonstrated through the use of the SYK-selective inhibitor, piceatanol (Keely and Parise, 1996). SYK activation can be mimicked by the use of stimulatory antibodies against $\alpha 2\beta 1$ (Keely and Parise, 1996) and partially inhibited by use of $\alpha 2\beta 1$ function-blocking antibodies. $\alpha 2\beta 1$ -independent pathways leading to activation of SYK also add to the complexity of the response. Platelets react to thrombin stim-

ulation acting via G-protein-coupled receptors by rapidly activating SYK (Taniguchi *et al.*, 1993). This effect can be simulated by cross-linking $\beta 3$ integrins and partially blocked by $\beta 3$ function-blocking antibodies (Clark *et al.*, 1994). The response also partially depends on the presence of an intact cytoskeleton but is wholly independent of platelet aggregation (Clark *et al.*, 1994). Recent studies in an α IIB β 3-expressing CHO cell line show that SYK activation through α IIB β 3 may be mediated through Src activity (Gao *et al.*, 1997). Hence, it is evident that SYK activity is regulated at different points in the pathway leading to platelet activation and also by different integrins. It is noteworthy that SYK activation differs from FAK activation in the platelet response, in that it occurs earlier and does not require platelet aggregation (Lipfert *et al.*, 1992).

F. Summary

Activation of tyrosine kinases is a key proximal event for integrin-mediated signal transduction. In many cells FAK and Src are the kinases most directly responsive to integrin-dependent cell adhesion, whereas in hematopoietic cells SYK seems to have a special role. Despite extensive study, the precise mechanism by which integrins activate FAK or other tyrosine kinases remains obscure; for example, it is still uncertain whether FAK directly interacts in vivo with integrin cytoplasmic domains. The adhesion-triggered activation of cytoplasmic tyrosine kinases, the subsequent phosphorylation of several structural and adapter proteins, and the establishment of multiple SH2 domain-mediated interactions clearly play a vital role in the organization and function of focal adhesions. The formation of focal contacts involves a complex and seemingly redundant set of molecules, because "knockout" or inhibition of any one of several key components still permits assembly of the essential structure, at least by morphological criteria. However, it is clear that FAK and Src are important in regulating focal adhesion assembly and disassembly. FAK activation seems to be involved particularly in the promotion of cell motility, with several lines of evidence supporting this view. The potential role of FAK in linking integrins to downstream signaling events remains controversial, as will be described further in Section V. of this review. Perhaps the best defined role for FAK in downstream signaling is as an antiapoptotic factor in epithelial cells; this role is discussed in more detail in Section X. The functional roles of other integrin-activated tyrosine kinases, including c-Abl and SYK, are even less well defined. In summary, during the past few years there have been numerous studies concerning integrin-responsive tyrosine kinases. Despite this intense interest, major gaps remain in our understanding of the biological role of integrin-mediated tyrosine phosphorylation.

V. Direct Signal Transduction by Integrins: Activation of the Mitogen-Activated Protein Kinase Cascade

Activation of the MAPK cascade is a common event in the responses to many diverse extracellular stimuli (Cobb *et al.*, 1994b; Gotoh and Nishida, 1995; Guan, 1994). These stimuli vary widely in both their chemical nature (e.g., peptide growth factors, phorbol esters, okadaic acid) and in their biological activities [e.g., nerve growth factor (NGF) induces neuronal outgrowth in PC12 cells, whereas EGF induces cell division]. Several groups now have firmly established that integrin-mediated cell adhesion, in the absence of soluble factors, can lead directly to the activation of the MAPK cascade (Chen *et al.*, 1994a; Clark and Hynes, 1996; Miyamoto *et al.*, 1996; Morino *et al.*, 1995; Papkoff *et al.*, 1994; Renshaw *et al.*, 1996b; Schlaepfer *et al.*, 1994; Takahashi and Berk, 1996; Wary *et al.*, 1996; Zhu and Assoian, 1995). Thus, the insoluble proteins of the ECM join the many factors that can activate this cascade. Since the discovery of the MAPKs, an enormous literature has accrued which describes in detail both the mechanism(s) through which soluble factors activate MAPK and the consequences of this activation. Currently, the activation of MAPK by cell adhesion is being similarly dissected. In this section, we will review the biochemical characteristics, possible mechanisms, and significance of integrin-mediated MAPK activation.

A. Experimental Approaches and Cell Types

The general experimental paradigm used to demonstrate direct integrin signaling involves detaching adherent cells from tissue culture plates and replating them onto surfaces coated with integrin ligand, typically a purified ECM protein such as fibronectin, vitronectin, or laminin. The supposition that MAPK activation is caused specifically by adhesion mediated by integrins, rather than by heterologous cell surface receptors or through nonspecific binding, has been confirmed using three experimental approaches. First, surfaces coated with polylysine support firm, nonspecific attachment of cells, but this attachment does not significantly activate MAPK (Chen *et al.*, 1994a; Mainiero *et al.*, 1997; Miyamoto *et al.*, 1995b; Morino *et al.*, 1995; Schlaepfer *et al.*, 1994; Wary *et al.*, 1996; Zhu and Assoian, 1995). Second, several groups have reported activation of MAPK when cells are plated on surfaces or incubated with beads coated with antibodies specific for various integrins but not with antibodies against other cell surface proteins, such as CD44 or MHC class I (Chen *et al.*, 1996b; Lin *et al.*, 1997a; Mainiero *et al.*, 1997; Miyamoto *et al.*, 1995b; Morino *et al.*, 1995; Wary *et al.*, 1996). Third, immobilized recombinant fragments of fibronectin and fibronectin-derived RGD peptides have been used to mediate cell attachment and subsequent MAPK activation (Chen *et al.*, 1994a; Lin *et al.*, 1997a), and soluble RGD peptides

have been used to compete integrin-mediated adhesion to fibronectin and block MAPK activation (Zhu and Assoian, 1995).

Using this paradigm, integrin-mediated MAPK activation has been demonstrated in a wide variety of cell types. The predominant cell type in these studies is the fibroblast, with the source species typically being rodent (NIH or Swiss 3T3, REF52), human (WI38, HDF), and occasionally avian (CEF, primary quail embryo fibroblasts). Other cell types that exhibit integrin-mediated MAPK activation include human epithelial cells (293, HeLa), keratinocytes, and endothelial cells (human umbilical vein endothelial cells). All cell lines listed thus far are derived from solid tissues and are grown as adherent cultures. However, monocytic cells (THP-1) and platelets are nonadherent cells that exhibit integrin-mediated MAPK activation (Clemetson, 1995; Wahl *et al.*, 1996). The activation of MAPK in response to cell adhesion is not an occurrence restricted to mammalian cells, or even to metazoans. The unicellular parasite *Entamoeba histolytica*, which expresses an integrin-like collagen receptor, shows activation of a MAPK homolog upon attachment to collagen (Perez *et al.*, 1996). This suggests that integrin-mediated MAPK activation is likely to be an important biochemical event in many cell types.

B. Time Course of Integrin-Mediated Mitogen-Activated Protein Kinase Activation and Its Consequences

In NIH3T3 cells, MAPK activity peaks 10 to 15 min after plating on fibronectin, whereas lower, but significant, activity persists 40 to 60 min after plating (Chen *et al.*, 1994a; Clark and Hynes, 1996; Lin *et al.*, 1997a; Renshaw *et al.*, 1996b; Zhu and Assoian, 1995). Similar time courses are seen in other rodent (Swiss 3T3, REF52) fibroblast lines (Chen *et al.*, 1994a) as well as in human dermal (Miyamoto *et al.*, 1995b; Morino *et al.*, 1995) and lung (Chen *et al.*, 1994a) fibroblasts. Swiss 3T3 and REF52 cells also show activation of MAPK 10 min after adhesion to laminin-coated surfaces (Chen *et al.*, 1994a). In primary human keratinocytes plated on laminin, peak MAPK activation occurs 30 min after plating (Mainiero *et al.*, 1997), slightly slower than in fibroblasts on fibronectin. Conversely, HUVECs plated on fibronectin show peak levels of MAPK activity within 10 min of plating (Takahashi and Berk, 1996), slightly faster than fibroblasts on the same matrix protein. From its peak, MAPK activity undergoes a gradual decline (Chen *et al.*, 1994a, 1996b; Clark and Hynes, 1996; Lin *et al.*, 1997a; Renshaw *et al.*, 1996a) or a delayed, but relatively rapid decrease (Mainiero *et al.*, 1997; Miyamoto *et al.*, 1996; Morino *et al.*, 1995; Zhu and Assoian, 1995). As a result, integrin-mediated MAPK activity may persist at 60 to 100% of maximum levels for periods ranging from 15 min to 2 h or more. Thus the time course of integrin-mediated MAPK activation is likely to vary based on the type of cell, integrin, and matrix involved.

The durable activation of MAPK by integrins is in stark contrast to that seen in response to many soluble mitogens such as peptide growth factors (e.g., PDGF), which typically elicit a sharp peak of activity within 2 to 5 min (Gotoh and Nishida, 1995; Zhu and Assoian, 1995). It is clear that integrin-mediated MAPK activation alone is insufficient to promote induction of DNA synthesis (Mainiero *et al.*, 1997; Zhu and Assoian, 1995). This is despite the fact that integrin-mediated adhesion, like growth factor stimulation, results in nuclear translocation of MAPK (Chen *et al.*, 1994a; Davis, 1995; Zhu and Assoian, 1995) and activation of MAPK-responsive transcription factors (Davis, 1995; Hipskind *et al.*, 1994; Janknecht *et al.*, 1993; Mainiero *et al.*, 1997; Wary *et al.*, 1996). It has been suggested that the duration of MAPK activity can affect the nature of the cellular response (Marshall, 1995); in PC12 cells, for example, transient or prolonged MAPK activity induces cell division or differentiation, respectively. How the duration of integrin-mediated MAPK activity may affect downstream biological events is not yet understood.

The failure of cells to enter S phase in response to integrin-mediated MAPK activation may be caused by the level of activity induced by adhesion. In most cases, the average induction of MAPK activity by adhesion is three- to four-fold (Chen *et al.*, 1994a; Clark and Hynes, 1996; Lin *et al.*, 1997a; Morino *et al.*, 1995; Renshaw *et al.*, 1996b; Zhu and Assoian, 1995), although some groups have reported more robust responses (from six- to ten-fold) in fibroblasts stimulated with ligand- or antibody-coated beads (Miyamoto *et al.*, 1995b), in HUVECs transiently transfected with a MAPK expression plasmid (Wary *et al.*, 1996), and in primary keratinocytes (Mainiero *et al.*, 1997). Nonetheless, the average level of integrin-induced MAPK activity is significantly less than the 20- to 50-fold activation induced in adherent cells by classical mitogens such as PDGF (Kyriakis *et al.*, 1993; Reuter *et al.*, 1995; Zhu and Assoian, 1995), EGF (Chen *et al.*, 1996b; Zheng *et al.*, 1994a) and phorbol esters (Marquardt *et al.*, 1994; Renshaw *et al.*, 1996b; Ueda *et al.*, 1996). It is likely that a certain threshold of MAPK activity must be reached or exceeded to deliver a cell into S phase, whereas a sub-threshold level of activity, regardless of duration, will lend itself to an altogether different biochemical event(s). The possible nature of this event in the case of integrin-mediated MAPK activation will be discussed in detail below. It should be noted that the robust activation of MAPK by polypeptide growth factors occurs in adherent cells, whereas responses to growth factors are markedly attenuated in cells in suspension. Thus, as discussed in detail in Section IX., both soluble growth factors and integrin-mediated cell anchorage seem to be required for efficient mitogenic signaling and cell cycle traverse.

C. Mechanisms for Integrin-Mediated Mitogen-Activated Protein Kinase Activation

The previous section introduced significant differences that exist between the profiles of growth factor-mediated and adhesion-mediated MAPK activation. Differences between the two pathways also exist at the molecular level. Unlike growth factor receptors, integrins have no intrinsic enzymatic activity, and thus a difference in mechanisms is somewhat expected. In this section, we will first briefly review the canonical pathway leading from growth factor receptors to MAPK; then, we will discuss what is known so far of the mechanism of integrin-mediated MAPK activation.

1. Mechanism of growth factor activation of mitogen-activated protein kinase Upon treatment of cells with peptide mitogens (e.g., PDGF, EGF), the respective receptor tyrosine kinase (RTK) homodimerizes and undergoes autophosphorylation on various tyrosine residues. These modifications create binding sites for proteins containing SH2 domains (Pawson and Gish, 1992). Some of these proteins are enzymes, such as kinases (Src, p85^{PI-3K}, CSK), phosphatases (SHP), GTPase activators (p120Ras^{GAP}), and regulators of phospholipid turnover [phospholipase C (PLC) γ], whereas others are adapter proteins (Shc, Nck, GRB2) that serve to relocate specific target proteins to sites of tyrosine phosphorylation (Schlessinger, 1994). One of these target proteins, SOS, is a Ras guanine nucleotide exchange factor (GNEF) and is recruited to activated RTKs through its interaction with GRB2. Once at the membrane, SOS promotes conversion of Ras to an activated, GTP-bound form (Downward, 1996). Activated Ras binds the amino-terminal domain of Raf-1 (Van Aelst *et al.*, 1993; Vojtek *et al.*, 1993) forming a stable complex (Finney and Herrera, 1995; Hallberg *et al.*, 1994) and thereby localizing Raf to the plasma membrane. Subsequent to this localization, Raf is activated (Leevers *et al.*, 1994; Stokoe *et al.*, 1994) by a mechanism that remains unclear (Cutler and Morrison, 1997), but is likely to involve phosphorylation of tyrosine (Fabian *et al.*, 1993; Marais *et al.*, 1995) or serine (Morrison *et al.*, 1993) residues, and may include interactions with 14-3-3 proteins (Fantl *et al.*, 1994; Fu *et al.*, 1994; Irie *et al.*, 1994; Michaud *et al.*, 1995) or phospholipid (Ghosh *et al.*, 1996; Mott *et al.*, 1996). Additional complexity comes from observations that various isoforms of protein kinase C (PKC) can directly phosphorylate and activate Raf in a Ras-independent manner (Cai *et al.*, 1997; Carroll and May, 1994; Kolch *et al.*, 1993; Ueda *et al.*, 1996). Once activated, Raf phosphorylates and activates MAPK/ERK kinase (MEK), the dual-specificity (threonine/tyrosine) kinase directly responsible for activation of MAPK (Graves *et al.*, 1995).

2. Mechanism of integrin-mediated activation of mitogen-activated protein kinase Tracing the pathway of integrin-mediated MAPK activation backward from MAPK offered no surprises, initially. To date, the only

way to activate MAPK is through direct phosphorylation by MEK (Ahn *et al.*, 1992), and auspiciously, MEK is activated by integrin-mediated adhesion with kinetics similar to those of MAPK activation (Chen *et al.*, 1996b). Moreover, activation of MEK by adhesion is required for the activation of MAPK, as pharmacological inhibition of MEK activation ablates integrin-mediated MAPK activation in fibroblasts (Chen *et al.*, 1996b) as well as human monocytes (McGilvray *et al.*, 1997). It is safe to assume that, as no other mechanisms for activation of MAPK besides phosphorylation by MEK have been discovered, that integrin-mediated MAPK activation will behave no differently in this regard.

The ability to phosphorylate and activate MEK has been ascribed to several proteins including c-Mos (Pham *et al.*, 1995), *Xenopus* Ras-dependent ERK kinase stimulator (REKs) (Kuroda *et al.*, 1995), a 40 to 50 kDa protein from NIH3T3 cells (Reuter *et al.*, 1995), and the upstream regulators of the SAPK/JNK pathway, the MEK kinases (Gardner *et al.*, 1994; Lange-Carter *et al.*, 1993). However, activation of MEK is carried out principally by the Raf family of protein kinases (Daum *et al.*, 1994; Marshall, 1996; Morrison, 1995). In NIH3T3 cells, Raf-1 is activated by adhesion to fibronectin (Chen *et al.*, 1996b), and the time course of activation parallels those for MEK and MAPK, with an activity that peaks at 10 min after plating and then declines slowly over the next 50 min. To date, there is little information on the response of other Raf family members or of the other potential MEK activators to integrin-mediated cell adhesion. However, Raf-1 is the most abundant member of the Raf family in NIH3T3 cells (Reuter *et al.*, 1995) (Lin, Howe, and Juliano, unpublished observations). Furthermore, pharmacological destabilization of Raf-1 by geldanamycin (Schulte *et al.*, 1995; Stancato *et al.*, 1997) inhibits integrin-mediated MEK activation (Howe and Juliano, unpublished observations). Therefore, in the absence of data to the contrary, it is reasonable to assume that, in fibroblasts, integrin-mediated activation of MEK is likely to occur through activation of Raf-1.

a. EVIDENCE FOR RAS- AND FOCAL ADHESION KINASE-DEPENDENT MECHANISMS. Raf activation is a complex matter, and the mechanism of how it occurs after cell adhesion is still unclear. One highly tenable mechanism by which integrins may activate Raf (and therefore MAPK) closely parallels growth factor stimulation. It involves Ras activation using the same signaling intermediates used by RTKs, with FAK acting as a surrogate, integrin-associated, tyrosine kinase domain (Schlaepfer *et al.*, 1994). In this mechanism (fig. 6A), engagement of integrins by fibronectin induces FAK autophosphorylation, which in turn leads to FAK association with and tyrosine phosphorylation by Src, as discussed in Section IV. Src-mediated phosphorylation of FAK promotes the direct binding of the GRB2 adapter protein to Tyr925 of FAK (Schlaepfer *et al.*, 1994, 1996) and also may promote the formation of a complex containing FAK and a

GRB2/Shc complex, which is independent of phosphorylation at Tyr925 (Schlaepfer and Hunter, 1997). Both situations would result in the recruitment of SOS to the juxtamembrane complex (Miyamoto *et al.*, 1995b; Schlaepfer *et al.*, 1994) which subsequently would activate Ras (see fig. 6A). In this mechanism, integrin-mediated MAPK activation depends on Ras (for activation of Raf) and FAK (for activation of Ras). In agreement with these notions, several groups have demonstrated an integrin-mediated activation of Ras (as measured by GTP loading), either by cell adhesion to fibronectin in fibroblasts (Clark and Hynes, 1996; King *et al.*, 1997) or by antibody-mediated engagement of integrins on T cells (Kapron-Bras *et al.*, 1993) and neutrophils (Zheng *et al.*, 1996a). Also, studies using beads coated with integrin ligands or anti-integrin antibodies have demonstrated colocalization of Ras, as well as GRB2 and SOS, with integrins (Miyamoto *et al.*, 1995b). Finally, expression of dominant negative Ras (Ras^{N17}) or Shc (Shc^{F317}) can block integrin-mediated MAPK activation in several systems (Clark and Hynes, 1996; King *et al.*, 1997; Mainiero *et al.*, 1997; Schlaepfer and Hunter, 1997). A role for FAK in integrin-mediated MAPK activation also is supported by experiments in which overexpression of wild-type FAK in transformed human epithelial cells enhances integrin-mediated MAPK activation four-fold, whereas expression of FAK^{F397}, to which Src cannot bind, reduces FN-stimulated MAPK activity (Schlaepfer and Hunter, 1997). Furthermore, fibroblasts isolated from Src $-/-$ mice still show adhesion-induced MAPK activation, but it is ten-fold lower than in Src $-/-$ cells made to re-express c-Src through retroviral infection (Schlaepfer *et al.*, 1997). However, no comparison has been made between the level of MAPK activity in these Src "re-expressers" and normal murine fibroblasts (e.g., NIH3T3), which have a 5-fold lower level of Src expression. These data, in addition to the substantial body of data describing the interaction of FAK with GRB2 (Schlaepfer *et al.*, 1997; Schlaepfer *et al.*, 1994; Schlaepfer and Hunter, 1997; Schlaepfer and Hunter, 1996), support the notion that FAK can contribute to integrin-mediated MAPK activation.

An interesting situation arises in the case of p130^{CAS} and integrin-mediated MAPK activation. It seems feasible, a priori, that p130^{CAS} might contribute to activation of MAPK by adhesion: it binds the adapter proteins CRK and NCK, which bind the GNEF's SOS and C3G (Matsuda and Kurata, 1996; Vuori *et al.*, 1996), it can directly interact with FAK (Harte *et al.*, 1996; Polte and Hanks, 1997; Polte and Hanks, 1995, 1997), and it localizes to focal adhesions (Harte *et al.*, 1996; Petch *et al.*, 1995; Polte and Hanks, 1997). However, both wild-type FAK and the Y397F point mutant are able to bind to p130^{CAS}, but the latter is incapable of mediating integrin-mediated MAPK activation (Schlaepfer and Hunter, 1997). This suggests that p130^{CAS}, despite its a priori characteristics, is not involved in linking integrins and/or FAK

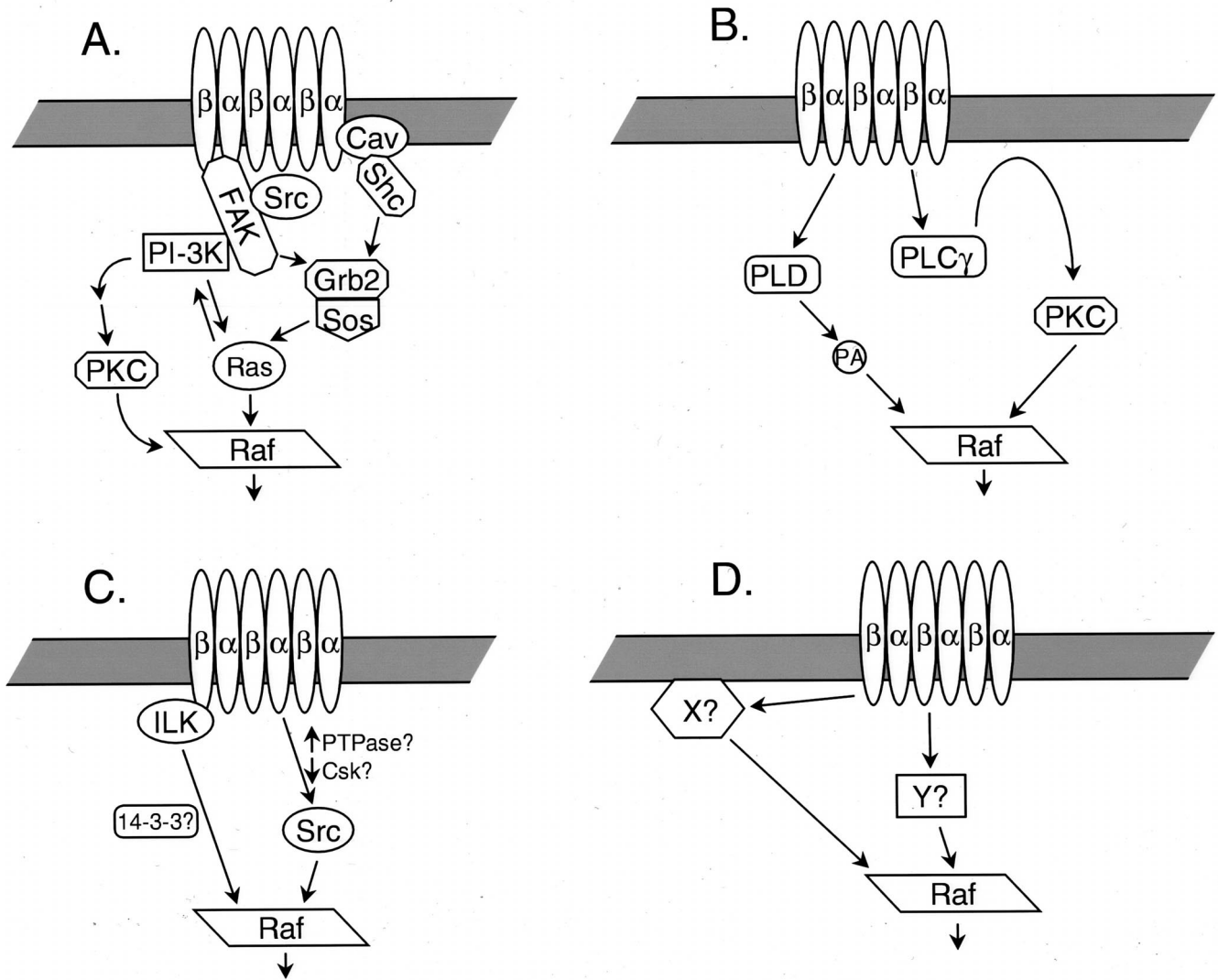


FIG. 6. Possible mechanisms for integrin-mediated activation of the MAPK pathway. Currently, the best characterized mechanisms involve activation through FAK- and Ras-dependent pathways (A), although substantial evidence suggests that other distinct mechanisms exist. Integrin-mediated adhesion regulates several activities that can contribute to MAPK activation, especially through activation of Raf. Among these activities are phospholipases C and D (B), protein kinase C (B), the integrin-linked kinase (ILK) (C), and Src-family tyrosine kinases (C). Activation of Raf coincides with its localization to the plasma membrane and cytoskeleton, raising the possibility that integrin-mediated adhesion may directly recruit Raf to the membrane or may regulate a membrane-associated Raf activator (X in D). Other regulators of various components of the MAPK pathway have been described (D), but their regulation by adhesion has not yet been determined. See text for details.

to MAPK, although it may be important in integrin-mediated signal transduction through other effectors, such as p65^{PAK} (Galisteo *et al.*, 1996) and PKN (Quilliam *et al.*, 1996). Alternatively, p130^{CAS} may only be involved when Src can bind to the Y397 site on FAK and tyrosine phosphorylate sites on p130^{CAS}.

Thus, in some circumstances, there is solid (although not overwhelming) evidence that both FAK and Ras are directly in the signaling pathway leading from integrin-mediated cell adhesion to MAPK activation.

b. EVIDENCE FOR RAS-INDEPENDENT MECHANISMS. In contrast to the discussion above, there is substantial evidence to suggest that other, Ras- and/or FAK-independent mechanisms for integrin-mediated MAPK activation exist. First, our laboratory did not detect a sig-

nificant increase in Ras GTP-loading after NIH3T3 cell adhesion to fibronectin (Chen *et al.*, 1996b). Furthermore, transfection of pZIP-Ras^{N17}, although able to significantly inhibit activation of MEK by constitutively active SOS, did not affect activation of MEK by adhesion to fibronectin (Chen *et al.*, 1996b). Finally, high-level expression of an N-terminal portion of Raf (amino acids 23 to 284), which contains the Ras binding site (Brtva *et al.*, 1995) and completely blocks the activation of the MAPK cascade by oncogenic Ras (Ras^{V12}), presumably by precluding Ras-Raf interaction, also failed to inhibit integrin-mediated activation of the MAPK pathway (Chen *et al.*, 1996b). The reasons for the discrepancy between these results, which suggest that integrin-mediated MAPK activation is Ras-independent, and the

forementioned results, which suggest Ras dependence, are unknown. It has been suggested that the difference may lay in the level of Ras^{N17} expression, as one group mentions a similar lack of effect of pZIP-Ras^{N17} on integrin-mediated MAPK activation, but demonstrates inhibition with a vector in which expression is driven from the powerful cytomegalovirus promoter (Schlaepfer and Hunter, 1997). This would not account for the differences seen in adhesion-mediated Ras GTP loading or for the effect seen with the Raf N-terminus, which was expressed under the cytomegalovirus promoter, however. The existence of Ras-independent mechanisms of integrin-mediated Raf activation has been supported by recent observations using mutant forms of Raf defective in the Ras binding site; nonetheless, these Raf mutants can be activated by integrin-mediated adhesion (Howe and Juliano, unpublished). One possible explanation that might allow for all of these observations is that integrin-mediated MAPK activation may require some degree of Ras function, but not direct stoichiometric Ras-Raf interaction. A candidate Ras effector in this regard may be PI-3K (Rodriguez-Viciano *et al.*, 1996), which may contribute to Raf activation through activation of PKC isoforms (Akimoto *et al.*, 1996; Liscovitch and Cantley, 1995; Toker *et al.*, 1994) and which has been implicated recently in integrin-mediated MAPK activation (King *et al.*, 1997; Mainiero *et al.*, 1997).

c. EVIDENCE FOR FOCAL ADHESION KINASE-INDEPENDENT MECHANISMS. Similarly, reports have suggested that integrin-mediated MAPK activation can occur independently of FAK function. The first report demonstrates that whereas FAK tyrosine phosphorylation requires integrin β subunits, the recruitment and activation (by tyrosine phosphorylation) of the Shc adapter protein apparently is specified by the transmembrane and extracellular juxtamembrane regions of α subunits in a mechanism involving interaction with caveolin (Wary *et al.*, 1996) (fig. 6A). Furthermore, antibody-mediated ligation of the $\alpha 6 \beta 1$ integrin can induce tyrosine phosphorylation of FAK, but not Shc, whereas ligation of an $\alpha 1$ mutant that lacks a cytoplasmic tail can activate Shc, but not FAK. FAK-independent recruitment and activation of Shc also can occur through direct interaction of Shc with the atypical cytoplasmic domain of the $\beta 4$ integrin subunit, which becomes tyrosine phosphorylated after adhesion to laminin (Mainiero *et al.*, 1995, 1997). In a second report, which deals specifically with the matter at hand, several observations indicate that activation of FAK and MAPK by integrins are separable phenomena (Lin *et al.*, 1997a). First, a $\beta 1$ subunit mutant with a deletion that overlaps the putative FAK binding domain can support integrin-mediated MAPK activation but not FAK tyrosine phosphorylation. Second, experiments involving cell adhesion to recombinant fibronectin fragments show that MAPK is activated at a time when there is no detectable tyrosine phosphorylation of FAK, and in time course experiments, activation

of MAPK precedes the low level of FAK tyrosine phosphorylation induced by attachment to the fragments. Third, expression of FRNK (see Section IV.), although able to block integrin-mediated tyrosine phosphorylation of FAK completely, has no effect on integrin-mediated MAPK activation. Taken together, these data strongly suggest that integrin-mediated MAPK activation can occur through a FAK-independent mechanism.

d. THE NATURE OF RAS-INDEPENDENT MECHANISMS OF MITOGEN-ACTIVATED PROTEIN KINASE ACTIVATION BY INTEGRINS. At this point the mechanism(s) underlying Ras- and FAK-independent activation of MAPK by integrins remain largely undefined. Indeed, several mechanisms are theoretically possible (fig. 6B, C). For instance, any one of several lipid mediators may play a role (fig. 6B). Thus integrin-mediated cell adhesion activates PKC (Vuori and Ruoslahti, 1993). Upon activation, several isoforms of PKC are capable of directly phosphorylating and activating Raf in the absence of functional Ras (Arai and Escobedo, 1996; Cacace *et al.*, 1996; Cai *et al.*, 1997; Carroll and May, 1994; Kolch *et al.*, 1993; Sozeri *et al.*, 1992; Ueda *et al.*, 1996; Zou *et al.*, 1996). Ras-independent activation of PKC by cell adhesion could occur through mechanisms that generate lipid co-activators for PKC. For example, PI-4,5-kinase-mediated synthesis of PI(4,5)P₂ and its hydrolysis to diacylglycerol (DAG) and inositol triphosphate (IP₃) by PLC are known to occur in response to integrin-mediated cell adhesion (Chong *et al.*, 1994; Cybulsky *et al.*, 1993; Kanner *et al.*, 1993; Ren *et al.*, 1996; Somogyi *et al.*, 1994); and adhesion-mediated increases in PI-3K lipid products that are potentially able to activate certain PKCs have been reported recently (Khwaja *et al.*, 1996). In addition, integrin-mediated cell adhesion has been shown to activate phospholipase A₂ (PLA₂), leading to production of arachidonic acid products and activation of PKC (Auer and Jacobson, 1995). Several isoforms of PKC, from both conventional (α , β , γ) and novel (δ , ϵ , η , θ) subclasses, are regulated by DAG (Newton, 1997). Also, three novel PKCs (δ , ϵ , η), as well as the atypical isoform PKC λ , can be regulated by phospholipids generated through the activity of PI-3K, namely PI(3,4)P₂ and PI(3,4,5)P₃ (Akimoto *et al.*, 1996; Toker *et al.*, 1994). Like PI4,5K, PI-3K also is activated after integrin-mediated cell adhesion, and this can occur through several mechanisms (see below) (Chen and Guan, 1994a,b; Guinebault *et al.*, 1995; Zhang *et al.*, 1993). Another lipid-based mechanism for Ras-independent activation of Raf by cell adhesion may involve integrin-mediated activation of phospholipase D (PLD) (Serrander *et al.*, 1996) and production of phosphatidic acid, which can contribute to Raf activation through direct interaction with Raf (Ghosh *et al.*, 1996) or through conversion to DAG and activation of PKCs (Singer *et al.*, 1997).

Integrin-mediated activation of Raf also may involve tyrosine phosphorylation (fig. 6C). Although the extent

to which tyrosine phosphorylation contributes to physiological activation of Raf is currently unclear (Morrison, 1995), there is substantial evidence that Raf can be activated by direct tyrosine phosphorylation by Src family members (e.g., Src, Fyn, and Lck) (Cleghon and Morrison, 1994; Cutler and Morrison, 1997; Fabian *et al.*, 1993; Marais *et al.*, 1995; Popik and Pitha, 1996; Thompson *et al.*, 1991). Significantly, Raf activation by tyrosine phosphorylation does not require Ras (Dent *et al.*, 1995; Fabian *et al.*, 1993; Marais *et al.*, 1995; Stokoe and McCormick, 1997), although Ras can augment the process, most likely by recruiting Raf to the membrane (Dent *et al.*, 1995; Jelinek *et al.*, 1994; Leever *et al.*, 1994; Marais *et al.*, 1995; Stokoe *et al.*, 1994). Furthermore, Src is activated after adhesion to fibronectin and localizes to focal adhesions (Fincham *et al.*, 1996; Kaplan *et al.*, 1994, 1995) (see Section IV.). Thus, integrin-mediated activation of Src also may contribute to Ras-independent Raf activation.

Another plausible candidate for Ras-independent activation of Raf and MAPK is the recently described ILK (Hannigan and Dedhar, 1997; Hannigan *et al.*, 1996) (see Section III.). ILK binds to the cytoplasmic tails of $\beta 1$ and $\beta 3$ integrins, and overexpression of ILK can promote anchorage-independent growth. Of importance is the recent observation that ILK can associate with Raf and can induce MAPK activity in a FAK- and Ras-independent manner (Dedhar, 1997), perhaps by recruiting Raf to the membrane (via interaction with β integrin subunits) in a manner analogous to Ras (fig. 6C).

It has been known for some time that activated Raf exists as part of an membrane-associated, NP40-insoluble complex that apparently is associated with the cytoskeleton (Carraway and Carraway, 1995; Stokoe *et al.*, 1994; Wartmann and Davis, 1994). Also, constitutive localization of Raf to the membrane via addition of an isoprenylation sequence results in a constitutively high level of Raf activity, presumably through constant juxtaposition of Raf with an unidentified, membrane-associated activator. Thus, if integrin-mediated adhesion caused recruitment of Raf to juxtamembrane and/or cytoskeletal complexes, this might position Raf for efficient activation independent of Ras-mediated events. The identity of the proteins involved in this putative integrin-dependent Raf recruitment and activation are currently unknown (fig. 6D). In view of the above, it seems possible that any one of several adhesion-triggered mechanisms could help to recruit Raf to the juxtamembrane region and permit its activation. Thus integrin-mediated regulation of cytoskeletal or membrane-associated activities may directly contribute to Raf activation and stimulation of the MAPK cascade.

3. *The role of focal contact hierarchies and cytoskeletal organization in integrin-mediated mitogen-activated protein kinase activation* As might be expected, there is an intricate relationship between integrin-mediated fo-

cal contact assembly and integrin-triggered signal transduction. Recent studies (Miyamoto *et al.*, 1995a,b) have illustrated a hierarchical order in the assembly of cytoskeletal and signaling complexes that depends both on receptor occupancy and on lateral aggregation of integrins in the membrane. Thus, clustering of integrins with antibodies was sufficient to recruit the focal contact proteins FAK and tensin to the cytoplasmic face of the integrin cluster; in the absence of tyrosine kinase inhibitors, FAK was activated and numerous signaling transducing proteins were recruited. Further, integrin clustering by antibodies was sufficient to activate both MAPK and JNK, but these processes were blocked when tyrosine kinase inhibitors were used. However, integrin clustering, without occupancy of the integrin's ligand binding site, did not support additional recruitment of focal contact proteins such as vinculin, α -actinin, and talin. By contrast, these three proteins were recruited when both ligand binding and integrin clustering took place, even in the presence of tyrosine kinase inhibitors. As discussed in more detail below, the use of cytochalasin D disrupted the recruitment of most signaling components. Thus, development of a mature focal contact assembly and its associated signaling complexes requires integrin aggregation, integrin occupancy, tyrosine kinase activity, and actin cytoskeletal integrity in a coordinated and hierarchical manner.

It has been suggested that activation of MAPK is mediated by the overall change in cytoskeletal organization that parallels cell spreading rather than by adhesion alone. In support of this, MAPK can be activated in adherent cells under fluid shear stress, which causes changes in cell shape (Berk *et al.*, 1995; Ishida *et al.*, 1996; Takahashi and Berk, 1996). Also, integrin-mediated MAPK activation is blocked by treatment with cytochalasin D which poisons actin microfilament assembly (Chen *et al.*, 1994a; Miyamoto *et al.*, 1995b; Morino *et al.*, 1995; Takahashi and Berk, 1996; Zhu and Assoian, 1995). However, others have reported significant adhesion-mediated activation of MAPK in the absence of (or at least before) significant cell spreading (Chen *et al.*, 1994a; Lin *et al.*, 1997a). Furthermore, studies using antibody- or ligand-coated beads (Miyamoto *et al.*, 1995a,b; Wary *et al.*, 1996) have demonstrated that these reagents can elicit integrin-mediated MAPK activation, even though it is highly unlikely that beads can promote cytoskeletal reorganization as efficiently as a solid, planar surface (i.e., a fibronectin-coated dish). The weight of the evidence thus far suggests that some degree of focal adhesion assembly and actin filament recruitment is vital for integrin-mediated MAPK activation; however, long-range organization of the cytoskeleton such as occurs in well-spread cells is not essential.

As we will discuss in detail (see Section VI.) the Rho family of small GTPases plays an important role in regulation of the cytoskeleton. Rho appears to be essen-

tial for the activation MAPK by integrin-mediated cell adhesion. Inactivation of Rho by serum starvation of Swiss 3T3 cells (Hotchin and Hall, 1995) or inhibition of Rho function in NIH3T3 cells by a dominant negative mutant (Renshaw *et al.*, 1996b) prevents MAPK activation in response to integrin-mediated cell adhesion. However, cells in which Rho has been inactivated also fail to form proper focal adhesions and stress fibers after cell adhesion. Thus, rather than a direct effect on signaling pathways, the effects of manipulating Rho may reflect that normal focal adhesions, and perhaps some degree of stress fiber organization, are the minimal structural requirements needed for integrin-mediated MAPK activation to occur. This view is consistent with the aforementioned effects of cytochalasin D as well as the concept of hierarchical assembly of adhesion/signaling structures. However, Rho has many functions (see below), and inhibition of Rho activity may have an impact on the cell in several ways, any of which may disrupt MAPK activation.

D. Integrin-Mediated Activation of the Jun N-Terminal Kinase/Stress-Activated Protein Kinase Pathway

Although the picture is presently incomplete, it seems that integrin-mediated cell adhesion can regulate the SAPK or JNK pathway, a kinase cascade which closely resembles the MAPK pathway (Kyriakis *et al.*, 1995; Waskiewicz and Cooper, 1995). Surprisingly, JNK activity has been reported to be both stimulated (Mainiero *et al.*, 1997; Miyamoto *et al.*, 1995a,b; Wary *et al.*, 1996) and suppressed (Cardone *et al.*, 1997; Frisch *et al.*, 1996a) by integrin-mediated adhesion, but the reason for this contradiction is not clear. Integrin-mediated activation of JNK may contribute to adhesion-regulated gene expression through phosphorylation and activation of c-Jun (Minden *et al.*, 1995). Further, integrin-mediated suppression of JNK activity may prevent apoptosis; this topic is discussed at length in Section X.

E. Functions of Integrin-Mediated Mitogen-Activated Protein Kinase Activation

The biological role of integrin-mediated MAPK activation is not known. Adhesion to the ECM is required for several cellular functions. Although integrin-mediated MAPK activation is durable (lasting tens of minutes to hours), its duration is shorter than the time span during which adhesion mediates its effects on cell growth and differentiation. Thus, if integrin-mediated MAPK activation is important for regulating anchorage-dependent events, it must establish some type of "permanent record" to allow adhesion-dependent signaling events to occur properly. This may be through alteration of the cellular architecture, regulation of gene expression, or a complex commixture of both. Alternatively, integrin-mediated MAPK activation may serve a more immediate function in regulating new contacts with the ECM, such as promotion of cell spreading or cell motility. Insight

into the function of integrin-mediated MAPK activation is likely to come from investigation of the known effectors of MAPK and their regulation by adhesion. There is no lack of candidates, because MAPK has diverse targets. However, as discussed earlier, the kinetics of adhesion-mediated MAPK activity differs substantially from the kinetics of mitogen-stimulated MAPK activity. Thus, the subset of potential targets that are affected, as well as the degree to which those targets are affected, may very well differ between adhesion-mediated and mitogenic MAPK activities. An overview of integrin signaling to the MAPK cascade is given in fig. 7.

1. *Transcriptional regulation* One of the hallmark functions for MAPK is the regulation of various transcription factors, including c-Myc, ATF-2, Elk-1, SAP-1, and AP-1 (Davis, 1995; Karin, 1995; Treisman, 1996). As mentioned previously, MAPK translocates to the nucleus after integrin-mediated adhesion (Chen *et al.*, 1994a; Zhu and Assoian, 1995). Integrin-mediated adhesion can activate transcription from the *c-fos* serum response element (SRE) (Mainiero *et al.*, 1997; Wary *et al.*, 1996), a MAPK-responsive promoter element that is regulated, in part, by Elk-1 and SAP-1 (Davis, 1995; Denhardt, 1996; Karin, 1995). Furthermore, it has been known for several years that adhesion to fibronectin rapidly induces the expression of both *c-fos* and *c-jun* (Dike and Farmer, 1988). Whereas any promoter that contains an SRE may potentially be affected by integrin-mediated MAPK activation, it is particularly interesting to note that the vinculin promoter contains an SRE (Moiseyeva *et al.*, 1993) and that expression of vinculin, as well as actin, α -actinin, the β 1 integrin subunit, and fibronectin, is induced with immediate-early kinetics (Ryseck *et al.*, 1989). To date, however, physiological transcriptional targets for integrin-mediated MAPK activity have not been identified. Nonetheless, it is of considerable importance that the role of the MAPK pathway in integrin-induced gene expression be investigated.

2. *Cytoplasmic targets* In addition to transcription factors, potential targets for adhesion-activated MAPK also exist in the cytoplasm. One such target is pp90^{RSK} (a.k.a. MAPKAPK-1) (Denhardt, 1996; Papkoff *et al.*, 1994); MAPK can phosphorylate and activate pp90^{RSK}, which can then move into the nucleus (Chen *et al.*, 1992a) and contribute to the regulation of transcription by AP-1 and cAMP-responsive element binding protein (CREB) (Denhardt, 1996). Another candidate for integrin-mediated MAPK activation is not a single enzyme, but a cytoarchitectural system, the microtubule network. MAPK is associated physically with and has enzymatic activity toward microtubule components (Reszka *et al.*, 1995) (MAPK once stood for microtubule-associated protein-2 kinase). Two recent reports have implicated microtubules and microtubule-associated MAPK activity in the regulation of integrin-mediated signaling events and the formation of focal adhesions and stress fibers (Bershadsky *et al.*, 1996; Reszka *et al.*,

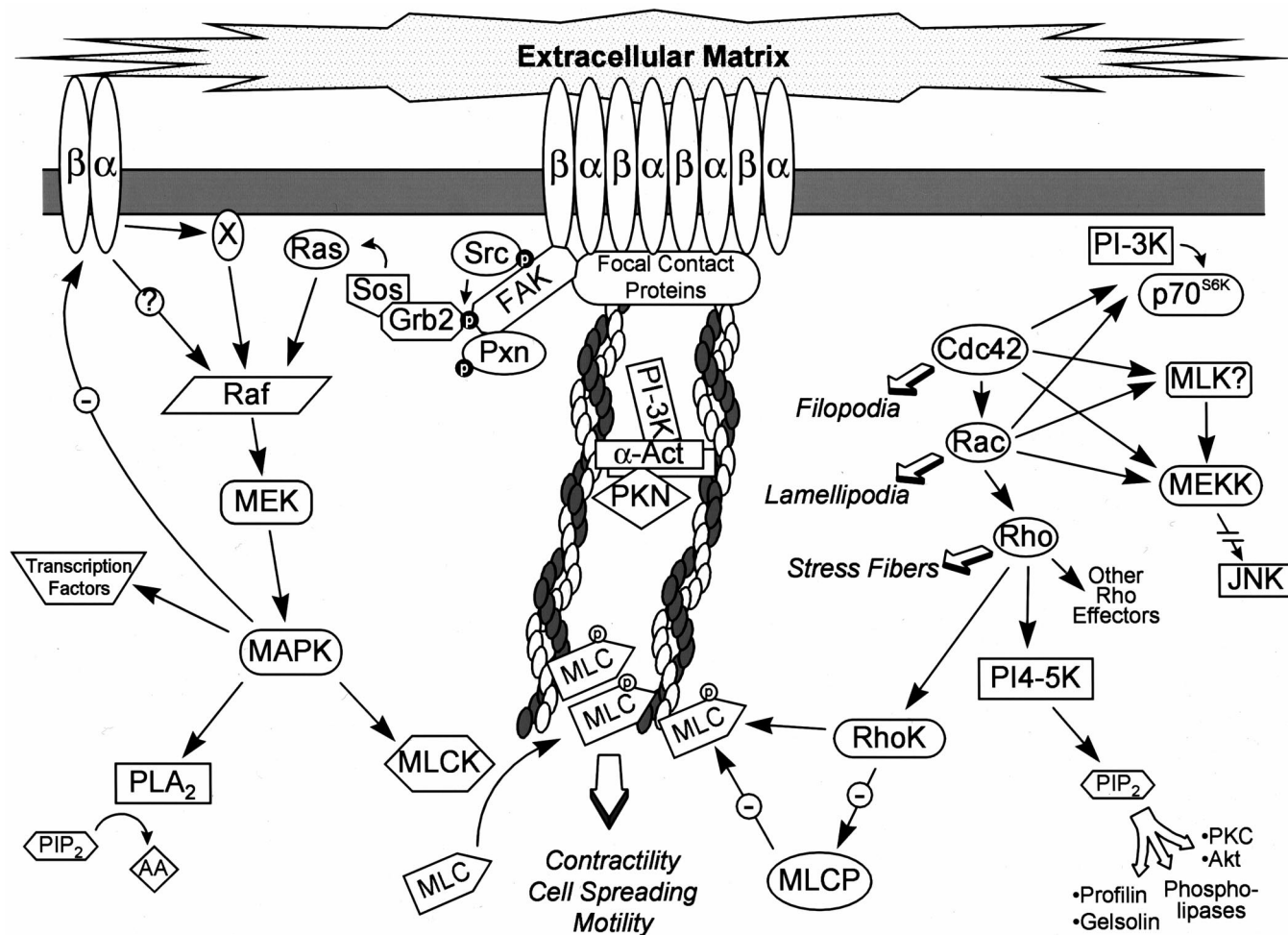


Fig. 7. Hypothetical model for collaboration between integrin-mediated signaling events. The function of integrin-mediated mitogen-activated protein kinase (MAPK) activation is not fully understood but possibilities include activation of transcription factors, PLA_2 , and myosin light chain kinase (MLCK). Rho family GTPases (Cdc42, Rac, Rho) have many effectors, some of which mediate their effects on actin cytoskeletal organization. Among those known for Rho in this regard are PI4-5K, a lipid kinase important for PIP_2 synthesis, and Rho kinase (RhoK). Synthesis of PIP_2 by PI4-5K could provide a substrate for MAPK-activated PLA_2 . Also, RhoK can directly phosphorylate the myosin light chain (MLC) and can inhibit MLCP. These events may synergize with activation of MLCK by MAPK to increase MLC phosphorylation, which can lead to contractility, focal adhesion formation, and other morphological effects. See text for details.

1997). Finally, a very attractive cytosolic target for integrin-mediated MAPK activation is PLA_2 (Clark and Hynes, 1996). PLA_2 is responsible for liberating arachidonic acid (AA) from glycerolphospholipids such as $PI(4,5)P_2$ (Divecha and Irvine, 1995), an activity important for optimal integrin-mediated cell-substrate interactions in some cell types (e.g., HeLa) (Auer and Jacobson, 1995; Chun and Jacobson, 1993) but apparently not in others (e.g., NIH3T3) (Clark and Hynes, 1996).

3. *Cell adhesion and motility* Two recent reports provide a highly satisfying function for integrin-mediated MAPK activation by suggesting that MAPK activated by integrins may "feedback" and contribute to the regulation of cell adhesion and motility. The first demonstrated that constitutively active mutants of MAPK pathway components, namely Ras and Raf, can suppress the activation of integrins (Hughes *et al.*, 1997). Also, activation of Raf inhibits fibronectin matrix assembly

and induces cell rounding. Suppression of integrin activation correlates with activation of MAPK, but is not caused by direct phosphorylation of the integrin and is independent of protein and mRNA synthesis. Inactivation of integrins by MAPK may represent a negative feedback loop for the regulation of integrin function which may contribute to the dynamic modulation of ligand binding affinity. Moreover, the efficacy of this loop in a given cell type may be a determinant of the duration of integrin-mediated MAPK activation in that cell type. The second report demonstrated that expression of a constitutively active MEK mutant stimulates cell migration on collagen and leads to phosphorylation and activation of myosin light chain kinase (MLCK), leading to increased phosphorylation of the myosin light chain (MLC) (Klemke *et al.*, 1997). Inhibition of MAPK activity suppresses both cell migration and phosphorylation of MLCK and MLC, but not cell adhesion or in situ cell spreading, suggesting that integrin-mediated

MAPK activation is not required for these initial events in this system. In vitro experiments showed that MAPK can phosphorylate and activate MLCK directly. Maximal phosphorylation and activation of MLCK by MAPK in vitro required prolonged incubation (approximately 40 min). Hypothetically, a similar requirement in vivo (should it exist) may be met by the extended duration of MAPK activity after cell adhesion. Whereas the evidence linking MAPK and MLCK is compelling, it is clear that other mechanisms may contribute to regulation of MLC phosphorylation and subsequent control of actinomyosin dynamics. Most notable are the phosphorylation of MLC and inactivation of MLC phosphatase by Rho-kinase (Amano *et al.*, 1996b; BurrIDGE and Chrzanowska-Wodnicka, 1996; Kimura *et al.*, 1996), which may govern cell contractility and subsequent signal transduction and cytoskeletal organization (BurrIDGE and Chrzanowska-Wodnicka, 1996; Chrzanowska-Wodnicka and BurrIDGE, 1996; Tapon and Hall, 1997) (see fig. 7 and Section VI.). The relative contributions of MAPK and other regulators of MLCK function still must be determined. Nonetheless, these recent observations have placed MAPK in a central locus for the regulation of cell adhesion, contractility, and locomotion.

F. Summary

The current picture of direct integrin-mediated activation of the MAPK cascade is marked by several controversial aspects. First, as discussed above, there are at least three models of the mechanism of activation, each supported by considerable data. One popular model has integrin signaling recapitulating the peptide growth factor signaling pathway with FAK, rather than the growth factor receptor, serving as the tyrosine kinase. This model posits key roles for FAK and Ras, with signal generation involving activation of FAK via the integrin β subunit cytoplasmic domain. A second model suggests that integrin-mediated focal contact assembly can permit recruitment and activation of Raf, and subsequently of MAPK, without the involvement of Ras or FAK. Finally, a third model emphasizes interactions of integrin α subunit transmembrane and external domains with caveolin and Shc in a Ras-dependent but FAK-independent signaling pathway. Because each of these models enjoys considerable experimental support, perhaps this suggests that integrins can signal to MAPK via several distinct mechanisms, and the dominant mechanism in any particular situation will depend on the cell type and the experimental conditions used.

Perhaps more important than the mechanistic details of the direct integrin-MAPK signaling pathway is consideration of the biological significance of these events. To a substantial degree integrin ligation triggers a set of downstream events (activation of Raf, MEK, MAPK, and transcription factors) similar to that triggered by peptide mitogens. However, integrin ligation itself does not result in mitogenesis. (Note: The key role of integrins in

collaborating with soluble growth factors to permit efficient mitogenic signaling will be considered separately in Section IX.) Further, in a physiological context most cells live with their integrins continually engaged with the surrounding ECM; thus, major global changes in MAPK activity within the cell are unlikely. However, cells do make and break adhesions with the ECM during cell migration and tissue remodeling; thus local changes in MAPK activity within the cytoplasm are quite likely. This hints at the possibility that the key biological role for integrin-triggered MAPK activation may be local regulation of contractility and of movement of cellular processes. However, it also remains possible that a low level of MAPK activity, because of breaking and reforming of integrin-mediated adhesions, may play a permissive role in cell growth.

It is also important to realize that integrin signaling may impinge on effectors other than MAPK. For example, integrin-mediated activation of Raf may have downstream effects that are independent of MAPK. Raf is implicated in the transcriptional activation of NF- κ B (Finco and Baldwin, 1993; Janosch *et al.*, 1996; Li and Sedivy, 1993), the regulation of apoptosis (Troppmair and Rapp, 1997) through interactions with the antiapoptotic proteins BCL-2 (Wang *et al.*, 1994, 1996b) and BAG-1 (a BCL-2 interacting protein) (Wang *et al.*, 1996b), and phosphorylation of the pro-apoptotic protein Bcl-XL/Bcl-2-associated death promotor (BAD) (Wang *et al.*, 1996a). Raf also can phosphorylate the p53 tumor suppressor protein (Jamal and Ziff, 1995) and cdc25, a dual specificity phosphatase that activates cyclin-dependent kinases (Galaktionov *et al.*, 1995). Thus, it is important to remember that the activation of MAPK pathway components is only one of many signaling events triggered by integrin-mediated adhesion (see fig. 7).

VI. Integrins and Rho Family Guanosine 5'-Triphosphatases

A. Introduction

An important consideration in the field of integrin-mediated signaling is what structure is actually eliciting the signals. As discussed in Section V. above, the formation of signaling complexes is coordinated closely with the assembly of focal contact/cytoskeletal structures. Further, it is well documented that focal adhesions and other integrin-associated structures "mature" with increasing time after adhesion (BurrIDGE *et al.*, 1988; Craig and Johnson, 1996; Gilmore and BurrIDGE, 1996a; Jockush *et al.*, 1995). Some integrin-mediated signals simply may promote focal adhesion assembly thus setting the stage for other events, some may require early or intermediate stages of maturation of adhesion complexes, whereas others may be generated only by a fully formed, mature focal adhesion complex and its associated linkages to the actin cytoskeleton.

The Rho family of small GTPases, including various forms of Cdc42, Rac, and Rho, are involved intimately in the regulation of the actin cytoskeleton. Several excellent recent reviews describe practically every aspect of the Rho family (Denhardt, 1996; Lim *et al.*, 1996; Narumiya, 1996; Ridley, 1996; Symons, 1996; Tapon and Hall, 1997), and an exhaustive analysis will not be attempted here, but an introduction is in order. Rho family GTPases couple extracellular signals to the formation and/or organization of higher-order actin structures. Specifically, Rho, Rac, and Cdc42 mediate the formation of stress fibers, lamellipodia, and filopodia, respectively (Nobes and Hall, 1995a; Ridley and Hall, 1992; Ridley *et al.*, 1992). Distinct extracellular signals activate individual members of the Rho family through specific receptors, either RTKs or G-protein-coupled receptors. In this way, LPA, bombesin, and bradykinin stimulate the activity of Rho, Rac, and Cdc42, respectively, to induce formation of the respective actin structures mentioned above. Cdc42 activity can lead to Rac activation, and Rac activity can lead to Rho activation, suggesting a GTPase cascade (Chant and Stowers, 1995). In support of this, LPA induces stress fibers within 2 to 5 min, whereas induction of stress fibers by bombesin and bradykinin (as well as PDGF, EGF, and insulin) occurs during a longer time course of 20 to 30 min (Nobes and Hall, 1995b). However, the regulation of this cascade is not without its intricacies, as the formation of filopodia by Cdc42 correlates with the dissolution of stress fibers (Kozma *et al.*, 1995), and inhibition of Rho can enhance Cdc42 activity (Nobes and Hall, 1995a). An overview of Rho family GTPases in relation to integrin signaling and the cytoskeleton is provided in fig. 7.

B. Regulation of Activity

The pathways between the various receptors and Rho family proteins have not been fully charted, and several candidate intermediaries have been suggested including PI-3K, PKC, and a tyrphostin-sensitive tyrosine kinase (Chrzanowska-Wodnicka and Burridge, 1994; Nobes *et al.*, 1995). However, because Rho family proteins are active when bound to GTP and inactive when bound to guanosine 5'-diphosphate (GDP), the pathways that regulate their function will involve proteins which control the state of the bound nucleotide, namely GAPs and GNEFs (Denhardt, 1996; Lamarche and Hall, 1994; Lim *et al.*, 1996). GAPs enhance the hydrolysis of GTP and thus promote inactivation of Ras-related proteins, whereas GNEFs promote the release of GDP, allowing GTP to bind and activate the proteins. GAPs specific for Rho family members include Ral-BP1 (a.k.a. Cdc42GAP), breakpoint cluster region (BCR) and active, breakpoint cluster region-related (ABR), p190Rho^{GAP}, chimaerins (α , β , and η) (Herrera and Shivers, 1994; Kozma *et al.*, 1996) and an atypical myosin (*myr5*) (Reinhard *et al.*, 1995). Rho family GNEFs include diffuse B-cell lymphoma (DBL), Ost (from osteosarcoma), Lbc,

Vav, Tiam1, the faciogenital dysplasia gene product (FGD1) (Zheng *et al.*, 1996b), and small G-protein GTP dissociation stimulator (smgGDS). Ost acts as an effector for Rac and stimulates nucleotide exchange and activation of Cdc42 and Rho (Horii *et al.*, 1994), and therefore may play a role in mediating a GTPase cascade. Significantly, many Rho family GNEFs contain a PH domain, which can bind to $\beta\gamma$ subunits of heterotrimeric G-proteins, suggesting a possible direct link between G-protein-coupled receptors and Rho family proteins. PH domains can also bind PI(4,5)P₂, which may serve to localize Rho GNEFs to the plasma membrane (Lemmon *et al.*, 1996; Michiels *et al.*, 1997). Another class of GTPase regulators is represented by Rho guanine nucleotide dissociation inhibitor (GDI), which inhibits GDP dissociation (and in some instances GTP hydrolysis), sequesters GDP-bound GTPases in the cytoplasm, prevents the interaction of Rho GTPases with GAPs, and also may be involved in membrane relocation of Rho proteins (Denhardt, 1996).

Although serum components clearly can activate Rho and its relatives, the question of whether cell adhesion also can stimulate their activation remains unanswered. As mentioned previously, formation of focal adhesions and stress fibers is a Rho-dependent process; in Swiss 3T3 cells, this process requires both attachment to the ECM and serum components (Hotchin and Hall, 1995; Burridge and Chrzanowska-Wodnicka, 1996). However, some cell types can form stress fibers and focal adhesions under serum-free conditions and can maintain these structures after the removal of serum (Chrzanowska-Wodnicka and Burridge, 1996); it is not clear whether this is because of persistence of Rho activity after serum withdrawal or persistence of cytoskeletal structures after Rho inactivation. This suggests that in these cells, either Rho (and stress fiber/focal adhesion formation) is not required continually for the maintenance of cell adhesion, or Rho is not fully inactivated by serum withdrawal. If signaling events do depend on the formation of mature focal adhesions, and focal adhesion formation depends on Rho, an elementary step in integrin-mediated signaling must be the activation of Rho; this also implies regulation of Rho GNEFs and Rho GAPs. Thus, an important avenue of future experimentation should be whether (and how) Rho GNEFs and GAPs are regulated by cell adhesion. In support of this, there is certainly emerging evidence that suggests that Rho proteins are mediators of integrin signaling (Barry *et al.*, 1997; Nakahara *et al.*, 1998; Schwartz *et al.*, 1996).

C. Proteins that Interact with Rho Family Guanosine 5'-Triphosphatases

The list of proteins with which Rho family members interact is growing rapidly (table 2) (Ridley, 1996; Tapon and Hall, 1997). With the knowledge that Rho GTPases instigate complex and sometimes diverse cellular events (see below), the notion that these proteins may have

many targets makes sense. Some of these target proteins mediate cytoskeletal organization, whereas others are likely to mediate noncytoskeletal effects, and still others are currently without discernible function. The assignment of effectors to downstream events is still in the early stages, and although substantial progress has been made, it is complicated by several regulatory intricacies. For instance, several effectors are shared by more than one GTPase: the serine/threonine kinase p65^{PAK} is activated by both Cdc42 and Rac, and both Rac and Rho associate with PI4-5K lipid kinase activity (see table 2). Further complexity is added by communication between effectors. A prime example of this is the direct regulation by Cdc42 and Rac of both PI-3K and its effector p70^{S6K}.

D. Rho Guanosine 5'-Triphosphatases, Cell Cycle Progression, and Transcriptional Regulation

In addition to their functions in reorganization of the actin cytoskeleton (see below), Rho, Rac, and Cdc42 are required for the serum-induced progression of fibroblasts into S phase (Olson *et al.*, 1995; Yamamoto *et al.*, 1993), although recent evidence casts doubt on the role of Cdc42 in this regard (Molnar *et al.*, 1997). Furthermore, Rac is required for induction of cell division by the oncogenic tyrosine kinase v-Abl (Renshaw *et al.*, 1996a) and both Rac and Rho are required for efficient trans-

formation by oncogenic Ras (Khosravi-Far *et al.*, 1995; Qiu *et al.*, 1995a,b) and can synergize with a weakly transforming allele of Raf to promote efficient transformation (Khosravi-Far *et al.*, 1995). Further affirmation for the role of Rho family proteins in cell cycle progression comes from the observation that several Rho family GNEFs, namely DBL, Vav, and Lbc have the potential to oncogenically transform fibroblasts (Hunter, 1997; Khosravi-Far *et al.*, 1994; Zheng *et al.*, 1995).

One mechanism through which Rho family proteins could regulate cell cycle progression is by activation of transcription factors. All three Rho family GTPases can activate NF- κ B, and Cdc42 and Rho are required for activation of this factor by TNF α (Perona *et al.*, 1997). Another target for Rho family transcriptional regulation is serum-response factor (SRF). SRF, alone or in a complex with ternary complex factor (TCF) proteins (e.g., Elk-1, SAP-1), regulates transcription from promoters containing an SRE (Treisman, 1995). Activated forms of Cdc42, Rac, and Rho can all induce SRF-dependent expression from the SRE, and Rho is required for activation of the SRE by several stimuli (e.g., LPA, PDGF, phorbol ester) (Hill *et al.*, 1995). Recently it has been demonstrated that activated Rho-kinase (see below) can stimulate transcriptional activity from the *c-fos* SRE (Chihara *et al.*, 1997). The ability of Rac to regulate SRF activity is separable from its cytoskeletal functions

TABLE 2
Proteins that interact with Rho-family guanosine 5'-triphosphatases

GTPase(s)	Effector	Functions/comments	References
Cdc42	p120 ^{ACK} WASP	Tyrosine kinase; phosphorylates NADPH-p47 ^{phox} Wiskott-Aldrich Syndrome protein; binds Nck; may be involved in regulation of hematopoietic cell cytoskeleton	(Aspenstrom <i>et al.</i> , 1996) (Aspenstrom <i>et al.</i> , 1996; Quilliam <i>et al.</i> , 1996; Symons <i>et al.</i> , 1996)
Rac	POR-1	Partner of Rac; contains leucine-zipper; may mediate membrane ruffling	(Van Aelst <i>et al.</i> , 1996)
Cdc42 and Rac	p67 ^{phox} MLK-3	Component of phagocytic NADPH oxidase complex Mixed lineage kinase; may mediate Cdc42- and Rac-dependent activation of JNK/SAPK or p38/RK pathways	(Diekmann <i>et al.</i> , 1994) (Teramoto <i>et al.</i> , 1996a)
	MEKK1	May mediate Cdc42- and Rac-dependent activation of JNK/SAPK pathway	(Fanger <i>et al.</i> , 1997)
	p65 ^{PAK}	Serine/threonine kinase homologous to yeast Ste20p; implicated in activation of p38/RK pathway and in regulation of actin organization	(Manser <i>et al.</i> , 1994; Sells <i>et al.</i> , 1997; Zhang <i>et al.</i> , 1995a)
	p70 ^{S6K}	Ribosomal protein kinase; activated by mitogens and cell adhesion	(Chou and Blenis, 1996; Malik and Parsons, 1996)
	PI-3K	Important lipid kinase (generates 3'-phosphatidylinositols); involved in antiapoptotic signaling (through Akt/PKB); required for activation of p70 ^{S6K} by some stimuli; implicated in PDGF-mediated activation of Rac	(Franke <i>et al.</i> , 1997; Nobes <i>et al.</i> , 1995; Toliás <i>et al.</i> , 1995; Zheng <i>et al.</i> , 1994b)
Rho	PKN	Also called PRK1 or 2; related to PKC; may regulate Rho-mediated gene expression	(Amano <i>et al.</i> , 1996b; Watanabe <i>et al.</i> , 1996b)
	Rhotekin, Rhopilin Rho kinase	Function/activities unknown; Rho-binding domains have homology to that in PKN Also called ROK α ; contains coiled-coil, leucine zipper, PH, and cysteine-rich domains; member of kinase family that includes myotonic dystrophy kinase and p160 ^{ROCK} /ROK β (which also binds Rho); involved in assembly of stress fibers/focal adhesions and in Rho-dependent regulation of MLC phosphorylation	(Ishizaki <i>et al.</i> , 1996; Kimura <i>et al.</i> , 1996; Leung <i>et al.</i> , 1995; Matsui <i>et al.</i> , 1996)
	MLCP	Myosin light chain phosphatase; associates with Rho through myosin binding subunit; activity is inhibited by Rho kinase-mediated phosphorylation	(Kimura <i>et al.</i> , 1996)
Rho and Rac	Citron PI4P-5K	Contains coiled-coil, leucine zipper, PH, and cysteine-rich domains Principal mediator of PI(4,5)P ₂ synthesis; binds GDP/GTP-bound forms of Rac/Rho, but activated only by GTP-bound; different isoforms may mediate Rac- and Rho-associated activity	(Madaule <i>et al.</i> , 1995) (Chong <i>et al.</i> , 1994; Loijens <i>et al.</i> , 1996; Ren <i>et al.</i> , 1996; Toliás <i>et al.</i> , 1995)

(Westwick *et al.*, 1997), and there is evidence to suggest that Rho also can regulate transcription in the absence of actin effects (Thornburn *et al.*, 1997). Expression of a constitutively active form of PI-3K (a putative effector for Cdc42 and Rac) can induce the formation of Rac- and Rho-dependent actin structures (i.e., lamellipodia and stress fibers), but cannot stimulate Rac- and Rho-mediated transcriptional activation (Reif *et al.*, 1996), emphasizing the separation of these functions. The activation of SRF transcriptional activity raises the interesting possibility that Cdc42/Rac/Rho-stimulated SRF may combine with TCF proteins phosphorylated by integrin-mediated MAPK activity to induce transcription of genes containing SREs, such as *c-fos* or vinculin (see Section V.). The induction of *c-fos* expression is particularly interesting in light of the fact that Cdc42 and Rac can also activate JNK (Coso *et al.*, 1995; Minden *et al.*, 1995), which phosphorylates and activates c-Jun. Jun dimerizes with c-Fos to form the AP-1 complex, which is implicated in the response to integrin-mediated cell adhesion in several systems (Dike and Farmer, 1988; Fan *et al.*, 1995; Tremble *et al.*, 1994) (see fig. 6).

Activation of the JNK pathway by Cdc42 and Rac originally was thought to be mediated by p65^{PAK} because of its interaction with the two GTPases and its homology to Ste20p, the upstream activator of a yeast JNK/SAPK homolog (Waskiewicz and Cooper, 1995). However, it seems clear that p21-activated kinase (PAK) is not involved in Cdc42- and Rac-mediated JNK activation (Teramoto *et al.*, 1996a,b; Westwick *et al.*, 1997; Zhang *et al.*, 1995a), although it may mediate the activation of the related p38/RK pathway (Zhang *et al.*, 1995a). Instead, the serine/threonine kinase MLK3, a mixed-lineage kinase that can interact with Cdc42 and Rac and whose overexpression can activate the JNK pathway, seems to be involved in mediating this effect (Teramoto *et al.*, 1996a). Finally, direct interaction between Cdc42 or Rac and MEKK1, a known upstream activator of the JNK pathway, has been described recently (Fanger *et al.*, 1997) and provides another highly plausible mechanism for JNK activation by Rho family GTPases.

E. Rho Guanosine 5'-Triphosphatases and the Cytoskeleton

All three Rho family GTPases have profound effects on cell morphology and microfilament organization (Tapon and Hall, 1997), but the mechanisms by which they exert their effects are not fully understood. All three proteins cycle on and off the plasma membrane (Adamson *et al.*, 1992; Bokoch *et al.*, 1994; Takeichi, 1995), although the role that this plays in the regulation of their function is unclear. Of the known effectors (see above), the best candidate for directly mediating Cdc42-induced actin effects is Wiskott-Aldrich Syndrome protein (WASP). Overexpression of WASP in Jurkat cells induces the formation of large cytoplasmic clusters of

polymerized actin which colocalized with WASP (Symons *et al.*, 1996). However, WASP expression is restricted to hematopoietic cells, and therefore cannot explain the induction of filopodia by Cdc42 in fibroblasts, although the existence of WASP isoforms in other cell types is a possibility. The Rac-interacting protein POR-1 clearly is involved in Rac-mediated lamellipodia formation (truncated POR-1 interferes with Rac-induced membrane ruffling) (Van Aelst *et al.*, 1996), although it has no known catalytic activity and only a leucine-zipper region to suggest a possible function. Finally, the serine/threonine kinase p65^{PAK} has been implicated recently as a possible effector for both Cdc42 and Rac in rearrangement of the actin cytoskeleton (Sells *et al.*, 1997), although this is controversial (Westwick *et al.*, 1997).

1. *Lipid metabolism* PI4P-5K, a kinase regulated by Rac and Rho, is the major enzyme responsible for generating PI(4,5)P₂, a lipid of critical importance in numerous cellular functions. It has been known for several years that the synthesis of this lipid is induced by integrin-mediated cell adhesion (McNamee *et al.*, 1993). By their activation of PI4P-5K, Rac and Rho provide a molecule that can impinge on a wide variety of biochemical pathways. PI(4,5)P₂ is a substrate for phospholipases A, C, and D, which generate AA, DAG plus IP₃, and phosphatidic acid (PA), respectively. Integrin-mediated adhesion induces PLA₂ activity and a concomitant increase in AA which precedes cell spreading (Auer and Jacobson, 1995; Chun and Jacobson, 1992; Cybulsky *et al.*, 1993). It is tempting to speculate that this activity may be attributable to integrin-mediated MAPK activation, but this has not been tested formally. AA release and subsequent generation of leukotrienes may be both necessary and sufficient to activate Rho (through an unknown mechanism) and induce stress fiber formation in a Rho-dependent manner (Peppelenbosch *et al.*, 1995), thus possibly linking stress fiber assembly to the initial cell adhesion event in a positive feedback manner.

Several isoforms of PLC can be found in association with the cytoskeleton (Banno *et al.*, 1996; Plopper and Ingber, 1993), and PLC activity is stimulated by integrin-mediated cell adhesion (Cybulsky *et al.*, 1993; Kanner *et al.*, 1993; McNamee *et al.*, 1993). Furthermore, PLC is required for integrin-mediated hydrolysis of PI(4,5)P₂ to IP₃ (and, presumably, DAG) (Somogyi *et al.*, 1994). DAG and IP₃ are best known for their ability to activate PKC and mobilize intracellular calcium stores, respectively. PKC activity is stimulated by integrin-mediated adhesion (Vuori and Ruoslahti, 1993) and has numerous targets, including Raf-1 (see Section V.). Furthermore, integrin-mediated increases in intracellular calcium occur in many cell types through several distinct integrin-ECM interactions (Schwartz, 1993b; Sjasstad and Nelson, 1997). Mobilization of intracellular calcium by adhesion has the potential to elicit numerous effects, but some of the most relevant are modulation of integrin activity, regulation of protease (e.g., calpain) activity,

activation of CADTK/PYK2/CAK β (see Section IV.), and activation of calmodulin (Gimond and Aumailley, 1992).

PLD can be activated by cell adhesion (Serrander *et al.*, 1996) as well as a host of soluble factors that promote Rho activation (Hess *et al.*, 1997; Malcolm *et al.*, 1996; Martin *et al.*, 1996; Schmidt *et al.*, 1996). PLD-mediated production of PA can enhance PI4P-5K activity (English, 1996), suggesting an amplification loop that results in increased PI(4,5)P₂ synthesis. PA also can regulate stress fiber formation through a Rho-dependent mechanism (Cross *et al.*, 1996), and may contribute to activation of Raf through direct binding (Ghosh *et al.*, 1996). Significantly, PA can be converted into DAG, and thus activation of PLD can partially recapitulate PLC-mediated signaling events (Singer *et al.*, 1997).

Although it is clear that PI(4,5)P₂ is extremely important as a phospholipase substrate, it also is well established that the intact molecule has important functions of its own. PI(4,5)P₂ can bind to several proteins involved in regulating the actin cytoskeleton and associated structures, including gelsolin, profilin, vinculin, α -actinin, and the ERM proteins (Craig and Johnson, 1996; Hirao *et al.*, 1996; Jockush *et al.*, 1995; Kandzari *et al.*, 1996; Mitchison and Cramer, 1996). PI(4,5)P₂ binding to gelsolin or profilin promotes their dissociation from actin, allowing nucleation and polymerization of actin filaments. PI(4,5)P₂ also disrupts the head-tail intramolecular interaction of vinculin and thereby makes available the binding sites for actin and talin (Gilmore and Burridge, 1995, 1996b). The interaction of α -actinin with actin also is enhanced by PI(4,5)P₂ (Burridge and Chrzanowska-Wodnicka, 1996; Fukami *et al.*, 1992; Jockush *et al.*, 1995). Recently, ERM proteins have been implicated as essential cofactors in Rac- and Rho-induced actin reorganization (Mackay *et al.*, 1997), and their regulation by phospholipid may provide a crucial link in this regard.

Recently, some exciting findings have emerged concerning a role for Cdc42, Rac, and PI-3K in cell motility and tumor cell invasiveness. Thus, in mammary carcinoma cells, activation of Cdc42 or Rac disrupts normal epithelial polarization and promotes integrin-dependent motility and invasiveness; these events depend on PI-3K (Keely *et al.*, 1997). In a similar vein, an α 6 β 4-mediated activation of PI-3K has been shown to increase carcinoma cell invasiveness (Shaw *et al.*, 1997). Although these two studies agree on the overall importance of Rho-family GTPases and PI-3K in motility and invasiveness, they disagree as to the placement of Rac and PI-3K in the signal transduction pathway, with the first study suggesting that the GTPases are upstream of PI-3K and the second study suggesting the converse.

Thus, through the activation of lipid kinases and generation of PI(4,5)P₂ and other lipid products, Rac and Rho connect into a vast array of biochemical pathways that regulate the actin cytoskeleton and control several

signal transduction pathways (Downward, 1998). Whereas integrin-mediated cell adhesion has been reported to affect several of the enzymes involved in PI(4,5)P₂ formation and breakdown, the importance of these events in the overall processes of integrin-mediated signaling and integrin influences on cell function has yet to be fully determined.

2. *Rho, mitogen-activated protein kinase, and contractility* In addition to the effects on transcription and on actin filaments mentioned earlier, another intriguing collaboration between signals from the Rho family proteins and integrin-activated MAPK might occur in the regulation of cell contractility (fig. 7). As mentioned above, Rho uses Rho-K as an effector to regulate MLC phosphorylation. Specifically, Rho-K phosphorylates and deactivates the myosin binding subunit of myosin light chain phosphatase (MLCP) (Kimura *et al.*, 1996). Furthermore, Rho-K is able to directly phosphorylate myosin at the same site targeted by MLCK (Amano *et al.*, 1996a). Also, as stated earlier, MAPK activated by integrins can phosphorylate and activate MLCK (Klemke *et al.*, 1997). The result of each of these events is the net increase in MLC phosphorylation, which activates myosin and stimulates contractility (Burridge and Chrzanowska-Wodnicka, 1996). Although highly plausible, synergy between Rho and integrin-mediated MAPK activation in this regard has not yet been demonstrated. It has been proposed that contractility is the driving force behind the Rho-mediated formation of stress fibers and focal adhesions (Burridge and Chrzanowska-Wodnicka, 1996; Chrzanowska-Wodnicka and Burridge, 1996). Several other events mediated by Rho family proteins (discussed above) also may function in this regard. Increased intracellular PI(4,5)P₂ induced by Rac and Rho can stimulate actin polymerization and attachment of filaments to the membrane in primordial focal-adhesion-like structures. MLC phosphorylation is increased through inhibition of MLCP by Rho-K, and possibly by activation of MLCK by MAPK and/or calmodulin. This can stimulate cellular contractility and promote formation of stress fibers and focal adhesions.

F. Summary

There is clearly an intimate as well as intricate relationship between members of the Rho GTPase family and integrins. Rho family members seem to have two basic but separable functions, the first being signaling to nuclear transcription factors, and the second being control of cytoskeletal assembly. Integrins cooperate with Rho proteins in the organization and regulation of the actinomyosin containing cytoskeleton, because in the absence of integrin-mediated anchorage appropriate vectorial assembly of structures such as actin stress fibers cannot occur. As discussed above, Rho family proteins can influence cytoskeletal organization by several different means, including activation of kinases that regulate myosin function, and by the generation of lip-

ids, such as PI(4,5)P₂, that can affect the function of key focal contact proteins. Conversely, Rho-dependent cytoskeletal structures may be essential “scaffolds” for integrin signaling, bringing together molecules that are involved both in direct integrin signaling and in integrin-mitogen collaboration (see Section IX.). Integrin-mediated signaling, for example through the MAPK pathway, then potentially could collaborate with direct Rho-family signaling via the JNK pathway or other pathways to provide a coordinated activation of key transcription factors involved in cell cycle progression. One aspect of our current understanding that is still obscure is whether integrin-mediated adhesion can influence directly the GAPs and GNEFs that regulate Cdc42, Rac, and Rho, although recent work suggests the existence of a link between integrins and Cdc42 (Schwartz, 1997).

VII. Other Aspects of Integrin Signaling

A. Signaling by Specific Integrins

The integrin signaling processes described in Sections IV. and V. above, that is, FAK activation and activation of the MAPK cascade, can be initiated by several different integrins. However, a good deal of the biology of integrins seems to imply specific actions of different integrin subunits. Thus, it seems likely that individual integrins participate in specific signaling events that thus far are delineated very poorly. Along these lines, it is interesting to speculate that several recently discovered proteins that bind selectively to the cytoplasmic tails of individual integrin subunits (described in Section III.) may play an important role in the specificity of integrin-mediated events within cells. Several integrin-specific actions on the cell cycle and on apoptosis are discussed in Section X. Here we will simply tabulate several examples of what are apparently specific biological actions of individual members of the integrin family that cannot be substituted by other members of the family (table 3).

B. Cross-Talk Between Integrins

In this review we have emphasized integrin signaling to downstream processes presumably involved in growth control. However, there is another aspect of integrin signaling that should be mentioned briefly. Several observations indicate that integrins signal to each other;

this usually takes the form of one integrin regulating the ability of another integrin family member to support cell adhesion, motility, or phagocytosis. There are also a few instances of this sort of “cross-talk” between integrins and non-integrin adhesion receptors. Some examples of adhesion receptor cross-talk are given in table 4. In most cases the mechanistic basis of receptor cross-talk is unknown. However, as we proceed with the elucidation of integrin signaling pathways, described in Sections IV. through VI. above, several likely possibilities come to mind, and the issue of adhesion receptor interactions becomes less mysterious. First, recent observations (Hughes *et al.*, 1997) have shown that elements of the MAPK cascade can modulate the ligand binding affinity of integrins. Because integrins can also activate the MAPK cascade (Section V.), this is one obvious way in which one integrin might cross-talk with another. Second, many integrin-mediated activities, such as cell adhesion or cell motility, require engagement with the cytoskeleton. Because of the interplay between integrins, Rho family GTPases, and the cytoskeleton (Section VI.), this suggests another way in which signals generated by one integrin can affect functions mediated by another. Third, it is now clear that several integrins can share interactions with transmembrane or cytoplasmic binding proteins (Section III.), thus hinting at yet another mechanism for cross-talk. With respect to cross-talk between integrins and other adhesion receptors, several interesting examples have emerged recently (table 4); the mechanisms are far from clear, but the biological significance may be substantial.

C. Mechanochemical Aspects of Adhesion Signaling

One interesting hypothesis concerning signaling by cell adhesion receptors is a mechanochemical one; the integrins or other receptors essentially would play the role of a physical link between the ECM and the cytoskeleton, whereas the organization and mechanical tension of the cytoskeleton consequently would influence events in the cytoplasm and nucleus that are involved in cell division or cell differentiation. This “tensegrity” concept has been developed elegantly by D. Ingber and his colleagues (Ingber, 1993, 1997). In this model, overall cell shape and the bulk mechanical properties of cells would be as important or more important than discrete biochemical reactions. Although somewhat unsatisfying

TABLE 3
Specific actions of individual integrins

Specific integrin	Biological effect	Reference
β1c (alternatively spliced β subunit)	Growth inhibition	(Fornaro <i>et al.</i> , 1995)
β6	Growth promotion	(Dixit <i>et al.</i> , 1996)
αvβ3	Control of apoptosis in endothelial cells	(Brooks <i>et al.</i> , 1994)
α6β4	Growth control in epithelial cells	(Mainiero <i>et al.</i> , 1997)
α5β1	Control of apoptosis	(Zhang <i>et al.</i> , 1995c)
α2β1	Growth control in smooth muscle cells	(Koyama <i>et al.</i> , 1996a)
α6β1	Differentiation of skeletal muscle cells	(Sastry <i>et al.</i> , 1996)
αv integrins	Increase cell calcium	(Schwartz and Denninghoff, 1994)

TABLE 4
Cross-talk between integrins or with other adhesion receptors

Adhesion receptors involved	Biological effect	Reference
$\alpha 5\beta 1/\alpha \nu\beta 3$	$\alpha 5\beta 1$ affects motility mediated by $\alpha \nu\beta 3$	(Bauer <i>et al.</i> , 1992)
$\alpha \nu\beta 3/\alpha 5\beta 1$	$\alpha \nu\beta 3$ affects phagocytosis mediated by $\alpha 5\beta 1$	(Blystone <i>et al.</i> , 1995)
$\alpha M\beta 2/\text{FcIII}$	$\alpha M\beta 2$ and the FcIII receptor cooperate in the respiratory burst	(Zhou and Brown, 1994)
$\alpha \nu\beta 5/\text{uPAR}$	A protease receptor modulates $\alpha \nu\beta 5$ -mediated motility	(Yebra <i>et al.</i> , 1996)
L-selectin/ $\beta 2$	L-Selectin activates $\beta 2$ integrins	(Hwang <i>et al.</i> , 1996)
PECAM-1/ $\beta 2$ and $\beta 1$	The Ig-family receptor PECAM-1 activates $\beta 2$ and $\beta 1$ integrins	(Newman, 1997)
N-cadherin/ $\beta 1$ and $\beta 3$	Integrins activate N-cadherin	(Monier-Gavelle and Duband, 1997)
$\alpha \text{IIb}/\beta 3/\text{other } \beta 1$ integrins	Trans-inhibition of one integrin by another	(Diaz-Gonzalez <i>et al.</i> , 1996)

to the biochemically inclined, there is a remarkable amount of support for the idea that cell shape is a key regulator of cell growth and differentiation. For example, by use of powerful engineering techniques similar to those used in microchip manufacturing, very precise microdomains of adhesive material were placed on surfaces. When endothelial cells attached to those surfaces there was an excellent correlation between cell shape and the cell's ability to enter S phase (Chen *et al.*, 1997). Another example of the importance of cell shape in controlling biological processes concerns studies with breast epithelial cells conducted by M. Bissell and colleagues. Here a substantial amount of evidence suggests that appropriate patterns of gene expression and cell differentiation only occur when the cells have both the correct biochemical signals and the correct cell shape (Boudreau *et al.*, 1996; Roskelley *et al.*, 1995). Although at first glance these observations seem at variance with mainstream concepts of biochemically based signaling pathways, this may not be so. As illustrated in this review, the more we learn about signaling pathways the clearer it becomes that the three-dimensional architecture of signaling complexes is a key aspect of efficient signaling. Thus, in the long run, a more detailed analysis of cell signaling may well resolve any conflicts between "tensegrity" models and more conventional biochemical concepts of signaling.

D. Relationships Between Integrin Signaling Pathways

One of the major unresolved issues regarding pathways of integrin signaling concerns the relationships between FAK and MAPK. Under most circumstances there are close parallels in the circumstances and kinetics of FAK activation and MAPK activation. Further, overexpression of FAK has been reported to increase MAPK activation (Schlaepfer and Hunter, 1997). However, the preponderance of evidence suggests that FAK is not required for integrin-mediated MAPK activation (Frisch *et al.*, 1996a; Lin *et al.*, 1997a; Wary *et al.*, 1996). Despite the numerous studies on FAK, the precise biological role of this kinase remains open to question. Many investigators emphasize a key role for FAK in regulating the formation or dissolution of focal adhesion structures and thus in controlling cell motility. Another alternative is that FAK plays a vital role in adhesion regulation of apoptosis (Frisch *et al.*, 1996a); however,

other mechanisms may be equally or more important in this phenomenon (Cardone *et al.*, 1997) (Section X).

Likewise, although many investigations have been concerned with activation of the MAPK pathway by integrin-mediated adhesion, our biological insights into this process remain limited. One important possibility is that integrin signaling to MAPK helps regulate cytoskeletal assembly through PLA₂ and arachidonate metabolites (Auer and Jacobson, 1995; Clark and Hynes, 1996) and contractility of actinomyosin through MLCK (Klimke *et al.*, 1997). However, one should not discount the possibility that durable activation of MAPK via cell adhesion contributes to cell cycle traverse or cell differentiation mediated primarily by soluble growth or differentiation factors. We will return to this theme in Section IX.

In addition to events involving kinases, integrins have been reported to elicit a variety of other signaling events in cells, particularly ones involving mono- and divalent cations. Through regulation of ionic transients, integrin-mediated cell adhesion can affect the intracellular environment which has the potential to affect myriad signaling pathways. Integrin-mediated increases in intracellular calcium play an important role in the regulation of cell adhesion and can also regulate contractility through calmodulin-dependent regulation of MLCK. Furthermore, calcium transients may contribute to the regulation of PKC activity and may negatively regulate FAK-mediated signaling through calpain-mediated proteolysis (Cooray *et al.*, 1996; Schwartz, 1993a; Sjasstad and Nelson, 1997). Integrin-mediated adhesion also elevates intracellular pH (pH_i), largely through activation of the Na⁺/H⁺ antiporter (Ingber *et al.*, 1990; Schwartz *et al.*, 1989, 1991a,b). Regulation of pH_i through antiporter activity plays an important, but not well understood, role in many aspects of cell growth and division (Bianchini and Pouyssegur, 1994; L'Allemain *et al.*, 1984a,b; Pouyssegur *et al.*, 1985; Vairo *et al.*, 1992). One particularly relevant observation is that inhibition of antiporter function (pharmacologically or by genetic deficiency) inhibits Rho-mediated formation of stress fibers (Vexler *et al.*, 1996). MAPK is thought to play a major role in regulation of antiporter function (Bianchini *et al.*, 1997), raising the possibility that integrin-mediated cell adhesion may activate the Na⁺/H⁺

antiporter through a mechanism involving MAPK activation.

A confounding degree of cross-talk exists between various integrin-associated signaling events. One manifestation of this is the convergence of two or more regulators on a single effector. Several of the signaling events discussed previously can be regulated by multiple adhesion-related proteins. A good example is the regulation of PI-3K activity in response to adhesion. PI-3K is implicated as an effector for a host of regulators including Cdc42 and Rac, FAK, and Abl (see Sections IV. and V.), yet the relative contributions of each of these proteins in the adhesion-mediated regulation of PI-3K activity is not fully understood. Finally, the possible regulation of MLCK by integrin-mediated MAPK activation and of MLCP by Rho-kinase illustrates that signaling processes can use separate pathways to achieve the same result, in this case the phosphorylation of the MLC. Another characteristic of the intricacy of integrin signaling is that a given regulator and effector often can be connected through multiple pathways. The multiple potential mechanisms of integrin-mediated MAPK activation exemplify this. Another example is provided by the integrin regulation of intracellular calcium, which can be achieved through IP₃-mediated release of intracellular stores or activation of plasma membrane ion channels (Sjastad and Nelson, 1997).

In summary, integrin-mediated cell adhesion regulates numerous biochemical activities that may impinge on an even greater number of cellular pathways and events. These activities may appear tortuously interwoven and at least a bit redundant. Clearly not all activities will occur in all cells under all adhesive conditions. Indeed, it is most likely a combination of (at least) the species of integrin and the type of matrix that will determine the panel of responses for any given cell type. Furthermore, despite the recent flurry of activity, the field of integrin signaling is still in its infancy. The consequences of integrin-mediated signals, their actual contributions to cell function, have been speculated upon more often than investigated. A simplistic, but functional hypothesis is that signals generated by the interaction of a cell with a physiologically relevant matrix will be permissive for or causal to that cell's function, whereas interaction of a cell with an inappropriate matrix will generate signals that suppress cell function or growth, or perhaps lead to apoptosis. Permissiveness for cell function can include regulation of cell morphology and/or motility, induction of specific acute responses (e.g., in immune or inflammatory cells), or communication of the state of proper attachment to other biochemical pathways to allow appropriate response to other extracellular signals such as differentiation and growth factors. This last aspect is of particular importance and will be discussed in detail in Section IX.

VIII. Signaling by Cadherins, Selectins, and Immunoglobulin-Cell Adhesion Molecules

In this section we will deal with signaling mediated by non-integrin cell adhesion receptors, particularly the cadherins, selectins, and Ig-CAMs. Considerable information is available concerning cadherin signaling, whereas less is known about signaling mediated by the other families of adhesion receptors. As we shall see, there are both similarities and differences in signal transduction pathways mediated by integrins and by non-integrin adhesion receptors.

A. Cadherin Signaling

1. *Cadherins, catenins, adenomatous polyposis coli, and the Wnt pathway* Current understanding of a major aspect of signal transduction involving cadherins comes through a felicitous congruence of research from several different fields. As described above, investigators interested in cadherin-mediated adhesion initially described the catenins as a family of peripheral membrane proteins involved in cadherin function and in the structure of cell-cell adherence junctions. At approximately the same time, developmental biologists were delineating the Wingless signaling pathway in *Drosophila* that is vital for establishing segment polarity in fly embryos and adults. Genetic evidence implicated the Armadillo protein as a key player in Wingless signaling; it turns out that Armadillo is the fly homolog of mammalian β -catenin and has approximately 70% amino acid identity to the mammalian protein. Wingless is a secreted glycoprotein that is a member of the Wnt family of growth factors that control developmental patterning in both vertebrates and insects. Thus, Armadillo/ β -catenin is not only a structural protein of cell adhesion junctions, but also a key player in a vital developmental signal transduction cascade. To make matters even more interesting, tumor biologists identified a tumor suppressor gene called adenomatous polyposis coli (APC) whose loss or inactivation is responsible for a familial predisposition to colon cancer. The protein product of APC can bind to β -catenin and regulate its stability, abundance, and ability to participate in signaling events. At this point the reader can appreciate that cadherins, catenins, APC, and Wnt proteins are all implicated in a complex signaling and growth control network. The past several years have seen a coalescence of our understanding of this network, as we now will discuss.

2. Components of the Wnt-cadherin signaling pathway

a. WNT PROTEINS. *Wnt* genes express a family of glycoproteins of approximately 350 to 380 amino acids in length that play key roles in embryonic development in flies, frogs, and mammals (Nusse and Varmus, 1992; Torres *et al.*, 1996). Wnt proteins have signal sequences and thus seem destined for secretion; however, little soluble Wnt is recovered from cells expressing these proteins because they seem to bind to ECM components,

suggesting that Wnts may act locally in an autocrine or paracrine fashion. In mammals, *Wnt* genes originally were identified as proto-oncogenes and seem to play this role in mammary carcinogenesis. In addition, members of the Wnt family are expressed in a highly localized and regulated manner during mammalian development, and have been implicated in cell fate determination (Nusse and Varmus, 1992). The biological actions of Wnts are complex; for example, members of the Wnt-1 subgroup promote formation of the dorsoanterior axis in *Xenopus*, whereas members of the Wnt-5A subgroup antagonize this process (Torres *et al.*, 1996).

b. FRIZZLED, DISHEVELED, AND GLYCOGEN SYNTHASE KINASE 3. Downstream elements of the Wnt signaling pathway have been delineated largely through genetic screens in *Drosophila*. The receptor for Wingless, a *Drosophila* Wnt protein, is encoded by the *frizzled* gene (Bhanot *et al.*, 1996). This suggests that known vertebrate frizzled homologs may code for other Wnt receptors. The frizzled protein of 694 amino acids has an N-terminal cysteine-rich extracellular domain that is important in Wingless binding, seven putative transmembrane segments, and a cytoplasmic C-terminal region. Although frizzled seems to be a seven-spanner, its homology to well known G-protein-coupled receptors is low. Little is known currently about the nature of the signal immediately generated by frizzled proteins. The *disheveled* gene, which codes for a protein of unknown function, seems to be most immediately downstream of *frizzled*. The next component of the pathway, *zeste white3/shaggy*, has a mammalian homolog in the form of glycogen synthase kinase 3 (GSK3). Wingless signaling leads to inactivation of *zeste white3*/GSK3 kinase activity, and as we shall see subsequently, to the accumulation of cytoplasmic pools of Armadillo/ β -catenin.

c. ARMADILLO/ β -CATENIN. Both Armadillo and β -catenin (781 amino acids) contain 13 consecutive partially conserved 42 amino acid "armadillo" repeats flanked by N-terminal and C-terminal domains (Cowin and Burke, 1996). Interactions of β -catenin with classic cadherins occur through the armadillo repeats as does binding of APC; indeed cadherin and APC compete for binding at overlapping sites (Hulsken *et al.*, 1994). The N-terminal domain of β -catenin binds to α -catenin in the context of adherence junctions (Cowin and Burke, 1996). In addition, the N-most 7 armadillo repeats of cytosolic β -catenin also can bind to transcription factors termed LEF-1/TCF (Behrens *et al.*, 1996; van de Wetering *et al.*, 1997), and the LEF-1/ β -catenin complex can migrate from the cytoplasm to the nucleus. Once there, the DNA binding domain of LEF-1 interacts with specific sequences, whereas the C-terminal domain of β -catenin seems to be involved in transcriptional activation (van de Wetering *et al.*, 1997).

d. ADENOMATOUS POLYPOSIS COLI. The APC gene originally was identified as a key tumor suppressor gene in colorectal carcinogenesis. Individuals with inherited

mutations in APC develop numerous colon polyps and are predisposed to the development of colon cancer. Human APC is a very large protein (2843 amino acids) with several distinct domains. APC interacts with β -catenin, γ -catenin, GSK3, tubulin, as well as several other proteins (Huber *et al.*, 1996; Morin *et al.*, 1997; Su *et al.*, 1993); of most interest here is the ability of APC to bind β -catenin and regulate its activity. The N-terminal region of APC plays a role in oligomerization; this is followed by seven *armadillo* repeats, three 15 amino acid repeats, seven 20 amino acid repeats, and a basic region (Morin *et al.*, 1997; Vleminckx *et al.*, 1997). The 15 amino acid and 20 amino acid repeats are the critical sites for binding of catenins; many of the APC mutations observed in colon cancer result in truncations affecting the 15 amino acid or 20 amino acid repeats, thus preventing APC association with β -catenin.

3. A model for the Wnt-cadherin signaling pathway Here we present a consensus model of the Wnt-Cadherin signal transduction cascade (see fig. 8) (Gumbiner, 1996; Huber *et al.*, 1996; Peifer, 1996). The key feature of this model is the existence of discrete but interchangeable pools of β -catenin/Armadillo which can function both in cell adhesion and in regulation of transcription. One pool exists at the adherence junction where β -catenin can interact with cadherins and, via α -catenin, with the actin cytoskeleton. Another pool of β -catenin exists in the cytoplasm. Molecules from this pool can bind to LEF-1/TCF and the resulting binary complex can migrate into the nucleus forming a ternary complex with DNA (and thus a third pool of β -catenin) that selectively regulates transcription of LEF-1 responsive genes. In the consensus model, the size of the cytoplasmic pool of β -catenin determines the extent of binary complex formation and the degree of transcriptional activation. A fourth pool of β -catenin associates with APC, which targets the β -catenin for degradation by an as yet undetermined mechanism. The Wnt/Wingless signaling pathway regulates the size of the cytoplasmic pool of β -catenin. Thus binding of Wingless to Frizzled (or Wnt-1 to a vertebrate frizzled homolog) sends a signal to Disheveled which in turn negatively regulates the kinase ZW3 (or its vertebrate homolog GSK3). Active ZW3/GSK3 kinase binds the APC/ β -catenin complex, phosphorylates APC, and enhances its association with β -catenin (Rubinfeld *et al.*, 1996). Wingless/Wnt signals inhibit ZW3/GSK3 thus permitting dissociation of the APC/ β -catenin complex and increasing cytoplasmic pools of free β -catenin. The cadherins of the adherence junction compete for β -catenin binding and thus can also influence the pool of free cytoplasmic β -catenin.

Although the general features of this model are widely accepted, several points of controversy remain. One issue concerns the role of cadherins in the Wnt signaling pathway. There is significant evidence that β -catenin plays independent roles in adherence junctions and in signaling (Sanson *et al.*, 1996). For example, mutational

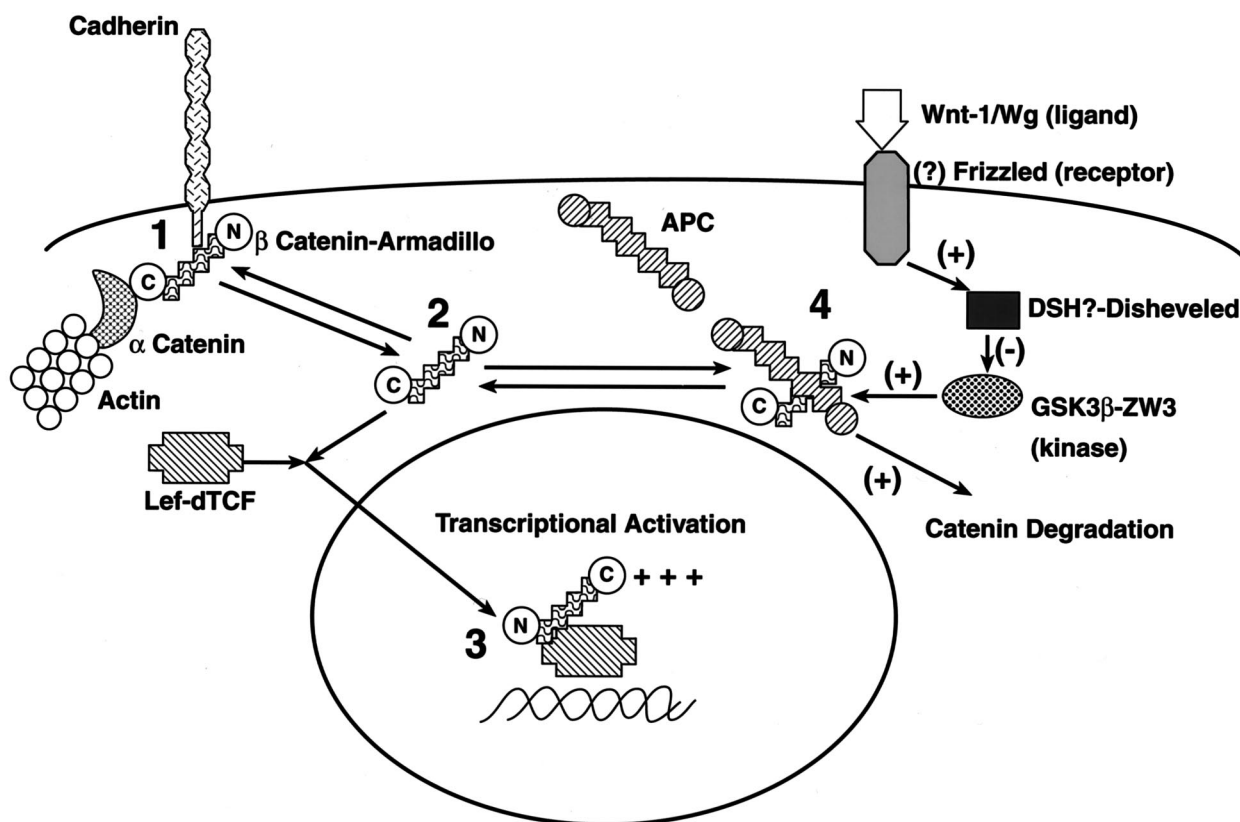


FIG. 8. The cadherin-Wnt signaling pathway. The drawing illustrates the concept that both cadherins and the Wnt/Wingless signaling pathway contribute to the control of cell growth and differentiation by regulating the pool of intracellular β -catenin/Armadillo. Four interchangeable pools of β -catenin/Armadillo are shown: (1) at the cadherin-containing adherence junction; (2) free in the cytoplasm; (3) associated with the Lef-1/TCF DNA binding protein in the nucleus; (4) associated with APC and targeted for degradation. Signaling through the Wnt/Wingless pathway regulates APC- β -catenin association via the GSK3/ZW3 kinase.

studies have been used to identify distinct sites on Armadillo for these two functions (Orsulic and Peifer, 1996). However, there is also considerable evidence that cadherins can influence the Wnt signaling pathway by competing for free β -catenin (Fagotto *et al.*, 1996; Heasman *et al.*, 1994). This controversy seems a matter of emphasis and most investigators would probably agree that the adherence junction pool of β -catenin is not involved directly in signaling, but that recruitment to this pool from the free cytoplasmic pool can negatively modulate the Wnt pathway. Another issue concerns the precise functional relationship between APC and β -catenin. In mammalian cells wild-type APC seems to increase β -catenin turnover/degradation, whereas mutants of APC associated with tumorigenesis fail to do this (Peifer, 1996; Rubinfeld *et al.*, 1996). Recent studies in melanoma cells (Rubinfeld *et al.*, 1997) and in colon carcinoma cells (Morin *et al.*, 1997) have suggested strongly that β -catenin is the key downstream effector of the tumor suppressive effects of APC. However, in *Xenopus* axis formation, APC seems to have a signaling role that is at least partially independent of its role in regulating β -catenin turnover, because overexpression of APC does not alter β -catenin levels perceptibly (Vleminkx *et al.*, 1997).

4. *Other aspects of cadherin signaling* In addition to modulating the Wnt pathway, cadherins have been implicated in other signal transduction events. Cadherin-mediated cell-cell association itself can trigger intracellular tyrosine phosphorylation events (Kinch *et al.*, 1997); the proteins which are tyrosine phosphorylated are quite distinct from those that are phosphorylated in response to integrin-mediated adhesion to the extracellular matrix. Cadherins have been reported to associate with other types of receptors and signaling molecules in the context of the adherence junction. For example, β -catenin seems to mediate an association between the EGF-receptor and cadherins (Hoschuetzky *et al.*, 1994), whereas the cytoplasmic kinase FER is associated with p120cas, another structural component of the adherence junction (Kim and Wong, 1995). Shc, an adapter protein implicated in Ras pathway signal transduction, recently has been reported to bind to the cytoplasmic domain of cadherins via the Shc SH2 domain (Xu *et al.*, 1997). A early report that cadherin/catenin complexes interact with RPTP μ , an Ig-CAM family receptor tyrosine phosphatase (Brady-Kalnay *et al.*, 1995), has been challenged recently (Zondag *et al.*, 1996). However, it seems likely that members of other adhesion receptor families will "talk" to cadherins; for example, an integrin-cad-

herin cross-signaling interaction has been reported recently (Monier-Gavelle and Duband, 1997). Along these lines, an exciting recent study indicates a direct link between integrin signaling and regulation of the cadherin/ β -catenin pathway. Thus, transfection of epithelial cells with ILK (see Section III.A.3.a. above) results in an epithelial to mesenchymal conversion, with a disruption of cadherin-based adherence junctions and the production of a more motile fibroblastic phenotype (Novak *et al.*, 1998). This seems to result from ILK phosphorylation and inhibition of ZW3/GSK3, leading to accumulation of β -catenin and activation of mesenchymal genes responsive to the β -catenin/LEF-1 complex. These results offer important new insights into the complex relationships between cadherin-mediated cell-cell interactions and integrin-mediated cell-matrix interactions.

B. Signaling by Immunoglobulin-Cell Adhesion Molecules

1. *Neural immunoglobulin-cell adhesion molecules* NCAM and L1 are neural Ig-CAM family members that play an important role in neurite outgrowth (Tessier-Lavigne and Goodman, 1996). Several years ago it was established that a signal transduction process involving tyrosine kinases, AA metabolites, and calcium fluxes was responsible for NCAM/L1-mediated growth cone activation and neurite extension (Baldwin *et al.*, 1996). During the past few years two distinct models have been proposed to explain this phenomenon. One model predicated a key role for an fibroblast growth factor (FGF) receptor, suggesting that NCAM (or L1) can interact directly with FGF receptor via a peptide sequence conserved between the two types of molecules, thus activating tyrosine kinase activity and subsequently triggering PLC γ -mediated calcium responses (Saffell *et al.*, 1997). This model is discussed further in Section IX. of this review concerning adhesion receptor-growth factor receptor interactions.

An alternative model implicates Src family tyrosine kinases in NCAM and L1 signaling; thus neurons from *fyn* minus "knockout" mice display inhibition of NCAM-mediated neurite outgrowth, whereas neurons from *src* minus mice display impairment of L1-mediated outgrowth (Beggs *et al.*, 1994; Ignelzi *et al.*, 1994). Recently, biochemical evidence has been developed for a direct association between the 140 kDa isoform of NCAM and Fyn (Beggs *et al.*, 1997). This same group also demonstrated that NCAM binding interactions induce tyrosine phosphorylation of FAK, the cytoplasmic tyrosine kinase closely involved in integrin signaling (see Section IV.). Because activated FAK binds to Src family members, this suggests formation of a macromolecular complex involving NCAM140, FAK, and Fyn that may regulate neurite extension. This represents one of the first times that FAK activation has been observed in response to a non-integrin-mediated adhesion process. The two models of growth cone signaling may not be mutually exclu-

sive, because there are numerous examples in the signaling literature of interplay between RTKs and Src-family kinases.

In addition to activating intracellular kinases, neural Ig-CAMs may also be functionally regulated by phosphorylation. Thus, the p90^{rsk} kinase (a downstream element of the Ras-MAPK pathway) has been shown to phosphorylate L1 on Ser¹¹⁵² and modulate its neurite outgrowth activity. NgCAM, an L1-related protein, has been reported to associate with and be phosphorylated by a casein kinase II (Kunz *et al.*, 1996). Finally, L1 family adhesion molecules in chick retina are tyrosine phosphorylated by Cek5, an Eph family tyrosine kinase which is itself a member of the Ig-CAM superfamily of receptors (Zisch *et al.*, 1997).

Other Ig-CAM family members also have been implicated in neuronal growth cone extension and guidance. Thus DLAR, a *Drosophila* Ig-CAM family receptor tyrosine phosphatase, has been shown to be critical for motor axon pathfinding (Krueger *et al.*, 1996); whether the phosphatase activity of DLAR is essential remains undefined. Also in flies, the *frazzled* gene, which codes for an Ig-CAM related to mammalian DCC (deleted in colorectal cancer), and which is a receptor for netrins, has been shown to play a key role in several aspects of axon guidance (Keino-Masu *et al.*, 1996; Kolodziej *et al.*, 1996).

2. *Other signaling by immunoglobulin-cell adhesion molecules* A vast literature on the role of Ig-CAMs in signal transduction in the immune system is available. Indeed both the T-cell receptor and the B-cell receptor are members of the Ig-CAM family, as are several other key receptors in lymphoid cells. Because of the specialized nature of this extensive literature we cannot pursue it in the context of this article. For further information on Ig-CAM signaling and the immune response the reader is referred to excellent reviews on these topics (Crabtree and Clipstone, 1994; Weiss and Littman, 1994).

There recently has been increased interest in the role of Ig-CAM receptors in nonneural and nonimmune system cells. PECAM-1, a homotypic Ig family receptor found on endothelial cells, platelets, and several types of white blood cells seems to have a particularly interesting and complex biology. It is critically involved in the formation of intercellular junctions between endothelial cells; it is also involved in leukocyte emigration (diapedesis) from the blood through the vascular endothelium and into sites of inflammation (Newman, 1997). PECAM is clearly a signal transducing receptor because antibody-induced dimerization of PECAM on blood cells can result in activation of β 1 and β 2 integrins. Recently, a novel mechanism was elucidated that seems to be responsible for PECAM signaling (Jackson *et al.*, 1997). Upon platelet aggregation the cytoplasmic domain of PECAM becomes tyrosine phosphorylated; this creates a binding site for SHP-2, a cytoplasmic tyrosine phosphatase.

tase that contains two SH2 domains. SHP-2 has been implicated previously in signaling from certain receptor tyrosine kinases to the Ras pathway, thus suggesting a possible mechanism for PECAM signaling. Although these events have been described in platelets, presumably similar activities can occur in other cells expressing PECAM.

Although there has been a great deal of interest in the ICAM family as counter-receptors for $\beta 2$ integrins, ICAMs have been shown only recently to generate signals. Thus antibody cross-linking of ICAM-1 has been shown to trigger an oxidative burst in monocytes, tyrosine phosphorylation in endothelial cells, and induction of the IL-1 β gene in synovial cells (Koyama *et al.*, 1996b).

C. Signaling by Selectins

Relatively little is known currently about signaling by members of the selectin family of adhesion receptors; however, this area is developing very rapidly. One very important aspect of selectin signaling concerns the role of selectins as cosignaling agents in leukocyte activation by vascular endothelial cells (Zimmerman *et al.*, 1996b). Thus, at sites of inflammation, endothelial cells both up-regulate surface expression of P-selectin and also express factors [platelet-activating factor (PAF), C-X-C and C-C family chemokines] act through G-protein-coupled receptors to activate $\beta 2$ integrins on leukocytes. The activating factors such as PAF act in a juxtacrine fashion and require P-selectin-mediated interaction for full effect. This topic is discussed in more detail in Section IX, below, which deals with cooperation between adhesion receptors and other receptors.

Here we will deal with the limited information currently available on direct signaling by selectins or their mucin-type counter receptors. Downey and colleagues provided some of the earliest observation on selectin signaling, showing that engagement of L-selectin contributed to the oxidative burst in neutrophils (Waddell *et al.*, 1994), and later showing that the MAPK pathway in these cells could be triggered through L-selectin (Waddell *et al.*, 1995). In a more detailed analysis, Brenner *et al.* (1996) showed that antibody engagement of L-selectin in wild-type Jurkat T-lymphoid cells led to tyrosine phosphorylation of the selectin, association with GRB2/SOS, GTP-loading of Ras, activation of MAPK, and a Ras/Rac-dependent oxidative burst; however, in Jurkat cells deficient in p56^{lck}, a Src family member, none of these events occurred. Thus, L-selectin signaling requires the Lck kinase as an early upstream signaling pathway component. In contrast to integrin signaling, L-selectin signaling to tyrosine kinases and MAPK does not seem to require extensive cross-linking, because use of a secondary antibody does not enhance the effects of primary anti-L-selectin. In contrast, a pathway leading from L-selectin to activation of $\beta 2$ integrins in T cells seems to require use of a secondary antibody to promote

cross-linking, or use of GlyCAM-1, a physiological ligand for L-selectin that is polyvalent (Hwang *et al.*, 1996). The mucin-like counter receptors for selectins also seem to be able to signal. An early study (Celi *et al.*, 1994) showed that adhesion of monocytes to P-selectin could induce the expression of tissue factor mRNA in these cells; the adhesion presumably is mediated through PSGL-1, the P-selectin counter receptor. In a similar vein, the binding of T cells to P-selectin has been reported to trigger tyrosine phosphorylation of FAK and other cellular proteins (Haller *et al.*, 1997). Thus both selectins and transmembrane counter-receptors for selectins (such as PSGL-1) seem to be able to generate various types of signaling events in leukocytes. This area is still in an early phase of development and much remains to be learned about the biochemistry and biological implications of signaling involving the selectin family.

D. Collagen Binding Receptor Tyrosine Kinases

An important new development in adhesion signaling is the identification of the "orphan" tyrosine kinases DDR1 and DDR2 as collagen binding receptor tyrosine kinases (Shrivastava *et al.*, 1997; Vogel *et al.*, 1997). These molecules have an extracellular domain that is related to discoidin 1, a lectin-like adhesion protein from *Dictyostelium discoideum*, whereas the intracellular domains have typical tyrosine kinase structures. DDR1 and DDR2 bind to native triple helical collagens, whereas collagen-associated carbohydrates also may play a role in binding and stimulation of tyrosine kinase activity. Because integrins (particularly $\alpha 2\beta 1$) also interact with collagens, these novel kinases may well play some sort of cosignaling role with integrins. Activation of DDR1 and 2 proceeds relatively slowly, does not seem to trigger MAPK, but enhances the expression of matrix metalloproteases and thus may play a role in tumor invasion.

E. Summary

The Wnt signaling pathway influenced by cadherins seems to be quite distinct from integrin signaling with its interplay of tyrosine kinases and MAPK cascade components. However, as described above, cadherins also may influence other signaling pathways, including ones involving tyrosine kinases and phosphatases, and thus may have some similarity to integrin signaling. In signaling by Ig-CAMs and selectins, tyrosine phosphorylation is a key event, whereas some studies also are beginning to implicate MAPK cascade components in these pathways. Thus integrins and other cell adhesion receptors may share much of the same basic signal transduction machinery. The biological *raison d'être* for signaling by adhesion receptors remains unclear but presumably is related to the cell's need to process positional information, as well as information about the availability of soluble growth and differentiation factors.

IX. Adhesion Modulation of Signaling by Soluble Mitogens and Differentiation Factors

A. Introduction

It has become increasingly clear that, in addition to generating their own brand of signals, cell adhesion receptors can regulate the cellular response to other extracellular stimuli, such as soluble growth factors and differentiation-inducing agents. Perhaps the best known incarnation of this is anchorage-dependent growth, a phenomenon which has been studied for nearly 30 years (Stoker *et al.*, 1968). Recently many aspects of anchorage control of cell growth have been ascribed to integrin-mediated interactions with ECM proteins, thus placing anchorage dependence in a more biochemical context. At one level, the reason for adhesion-mediated regulation of signal-induced events is simple; not everything a cell can do should happen wherever and whenever a cell happens to be. Thus, the regulation of cellular events, both reversible (e.g., synthesis/secretion of specific proteins) and irreversible (e.g., cell division, differentiation, apoptosis), is through the coordinated effects of positive and negative signals, including ones from soluble factors, as well as ones from the ECM and from adjacent cells. It is this summation of signals conveying both chemical and positional information which tells a cell when the time and place is right to conduct a particular activity. In this section we will deal with cooperation or collaboration between soluble factors and cell adhesion receptors. Once again the emphasis is on integrins, but we will deal with other adhesion receptors as well.

B. Integrin Modulation of Growth Factor Signaling

Integrin regulation of signaling by soluble growth factors has been documented in many types of adherent cells. These include endothelial cells (Short *et al.*, 1998), epithelial cells (Streuli, 1993), fibroblasts (Lin *et al.*, 1997b; Miyamoto *et al.*, 1996), keratinocytes (Mainiero *et al.*, 1997), hepatocytes (Liu *et al.*, 1991), chondrocytes (Arner and Tortorella, 1995), myoblasts (Sastri *et al.*, 1996), astrocytes (Cazaubon *et al.*, 1997), and neurons (Schmidt and Kater, 1995).

There are several ways in which integrins and growth factors may cooperate in mediating cellular events (see fig. 9A). For example, there is compelling evidence that ECM and soluble factors can synergize to regulate the intracellular ionic environment. Specifically, whereas both bFGF and adhesion to fibronectin can independently activate the Na^+/H^+ antiporter and raise pH_i in endothelial cells, growth factor stimulation of adherent cells is more efficient in the process (Ingber *et al.*, 1990). Similarly, in fibroblasts, PKC-dependent activation of the antiporter (and elevation of pH_i) by PDGF requires cell adhesion (Schwartz and Lechene, 1992). Also, PDGF-stimulated Ca^{2+} mobilization, another ion transient required for cell cycle progression in murine fibroblasts, does not occur in cells in suspension but readily

occurs in cells adherent to fibronectin (Tucker *et al.*, 1990). The mechanism underlying this regulation is among the most intellectually satisfying in the field. In adherent cells, PDGF stimulates tyrosine phosphorylation and activation of $\text{PLC}\gamma$, which hydrolyzes $\text{PI}(4,5)\text{P}_2$ into DAG and IP_3 , which in turn activate PKC and increase Ca^{2+}_i . In nonadherent cells, the activation of $\text{PLC}\gamma$ by PDGF still occurs, but there is a dramatic decrease in the level of its substrate, $\text{PI}(4,5)\text{P}_2$, and therefore no appreciable generation of DAG and IP_3 can occur (McNamee *et al.*, 1993). Presumably, the adhesion-dependent synthesis of $\text{PI}(4,5)\text{P}_2$ is largely caused by the activation of PI4-5K activity by Rac and Rho (as discussed in Section VI.).

Perhaps the most direct mechanism whereby integrins can collaborate with soluble factors is the physical interaction of integrins and associated proteins with one or more components of a given growth factor signaling cascade. Assembly of these "signaling scaffolds" would localize, or rather juxtapose normally diffusible elements of the signaling cascade and thereby allow transduction of the signal to occur more efficiently. Receptor-mediated signals are generated at the membrane and propagated through the cytoplasm to their appropriate targets. Within focal adhesions, integrins physically bridge the ECM to the network of cytoplasmic actin microfilaments, a situation that seems particularly well suited for providing an appropriate molecular scaffold for signaling components, as discussed in Section III. above. One of the most direct examples of physical coupling of adhesion and growth factor pathways is the insulin-stimulated association of $\alpha\text{v}\beta 3$ with the IRS-1 (Vuori and Ruoslahti, 1994), and in a recent report, with the insulin receptor itself (Schneller *et al.*, 1997). IRS-1 is heavily tyrosine phosphorylated after insulin stimulation and is required for the transduction of signals initiated at the insulin receptor. Significantly, insulin-stimulated DNA synthesis in $\alpha\text{v}\beta 3$ -expressing cells is enhanced when the cells are plated on vitronectin (an $\alpha\text{v}\beta 3$ ligand) versus other substrates, whereas no enhancement is seen in cells expressing the $\alpha\text{v}\beta 5$ vitronectin receptor (Vuori and Ruoslahti, 1994). There are other examples of scaffolding occurring directly at the level of the growth factor receptor. Thus the EGF receptor directly associates with microfilaments, and its close relative, p185^{neu} , is found in a microfilament- and membrane-associated, high-molecular weight glycoprotein complex that contains elements of several signal transduction cascades, including the Ras-MAPK pathway (Carraway and Carraway, 1995). In epithelial cells, the association of the EGFR with the actin cytoskeleton appears to be regulated by cell density, being far less stable in subconfluent versus confluent cells, and this affects the efficiency of EGF-induced signal transduction (Bedrin *et al.*, 1997). Recently, it has been reported that a highly tyrosine-phosphorylated subfraction of PDGF receptor from stimulated cells coimmunoprecipitates

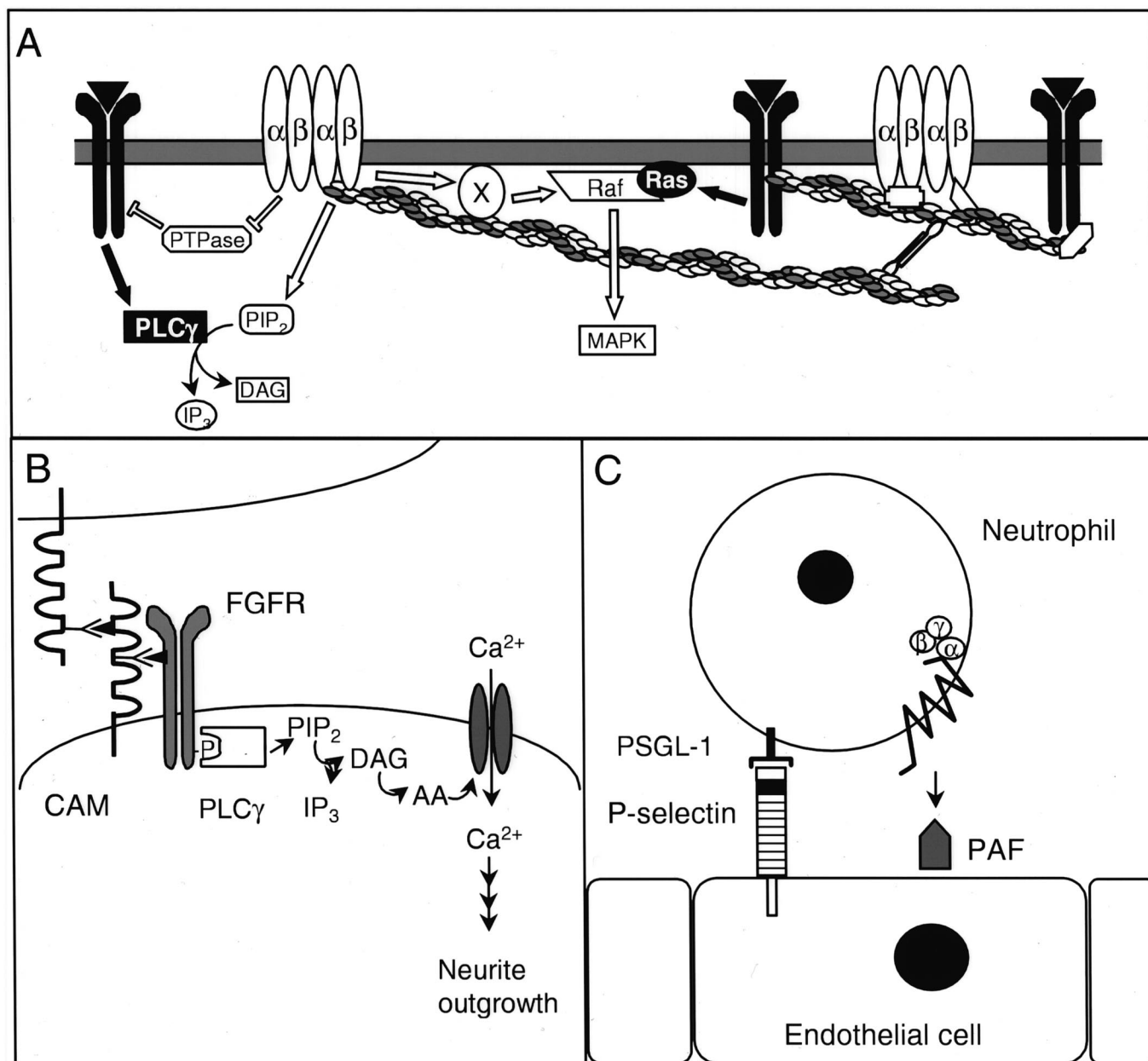


FIG. 9. Collaboration between cell adhesion molecules and soluble factors. (A) Mechanisms for collaboration between integrins and soluble factors. Integrin-mediated adhesion and signaling may down-regulate a factor that inhibits receptor signaling [e.g., a protein tyrosine phosphatase (PTPase)] or may provide a necessary substrate (e.g., PIP $_2$) or cofactor for a growth factor-mediated activity (e.g., PLC). Also, integrin-mediated adhesion may establish "signaling scaffolds" that juxtapose cytoskeleton-associated coactivators (X) with growth factor-regulated activities (e.g., Ras/Raf), or which physically interact with growth factor receptors themselves. (B) Signal transduction events stimulated by N-cadherin, L1, and NCAM leading to neurite outgrowth. Homophilically-bound N-cadherin, L1, or NCAM (CAM) molecules are postulated to recruit the FGF receptor (FGFR) via binding motifs to the CAM homology domain (filled triangle). FGFR recruitment causes autophosphorylation of the FGFR at Tyr766 in the cytoplasmic domain creating a binding site for the SH2 domain of phospholipase C gamma (PLC γ). Activation of PLC γ triggers a cascade through the utilization of phosphatidylinositol 4,5-bisphosphate (PIP $_2$) to yield diacylglycerol (DAG) and inositol trisphosphate (IP $_3$), conversion of DAG to arachidonic acid (AA) culminates in the AA-dependent influx of calcium ions through N- and L-type calcium channels. Diagram adapted from Doherty and Walsh, 1996. (C) Collaboration between P-selectin and platelet-activating factor (PAF) in the activation of leukocytes. Upon activation of endothelial cells P-selectin and PAF are expressed rapidly on the endothelial cell surface. P-selectin is capable of binding carbohydrate moieties such as PSGL-1 on the surface of leukocytes, such as neutrophils. Tethering of neutrophils to the vascular vessel wall promotes binding of PAF with its receptor on the neutrophil membrane. Resultant activation of neutrophils leads to increases in intracellular calcium, cell polarization and activation of β_2 integrins by an inside-out signaling mechanism. Diagram adapted from Zimmerman *et al.* (1996).

with the $\alpha\beta_3$ integrin, and significantly, engagement of $\alpha\beta_3$ by adhesion to vitronectin can enhance PDGF-induced mitogenesis (Schneller *et al.*, 1997).

In another report of integrin-mediated scaffolding, cells incubated with microbeads coated with integrin

ligands (fibronectin or RGD peptides) form focal contact-like structures at the cell-bead interface, and these structures are enriched for several signaling molecules, including Src, FAK, PLC γ , PI-3K, the Na $^+$ /H $^+$ antiporter, and the high-affinity FGF receptor flg (Plopper

and Ingber, 1993; Plopper *et al.*, 1995). Significantly, the bead-associated complexes, after isolation, retained tyrosine kinase and inositol lipid-metabolizing activities. As discussed in Section V. above, other experiments, using ligand- or anti-integrin antibody-coated beads, have identified more than 20 signaling molecules associated with focal contacts (Miyamoto *et al.*, 1995b, 1996), including several Src family kinases, the PTP-1D tyrosine phosphatase, PLC γ , and PI-3K, as well as Rho family GTPases Rac and Rho, several components of the MAPK and JNK cascades, and most recently, receptors for bFGF, PDGF, and EGF. The association of RTKs with integrin/focal adhesion complexes is transient (lasting approximately 60 min after bead addition) and independent of growth factor binding, but it seems to require both integrin aggregation and occupancy (Miyamoto *et al.*, 1996). Integrin engagement apparently is sufficient to induce ligand-independent RTK tyrosine phosphorylation in some systems (Sundberg and Rubin, 1996), but not in others (Lin *et al.*, 1997a; Miyamoto *et al.*, 1996).

Recent findings hint at several different mechanisms whereby integrins may regulate growth factor signal transduction. Several reports have described cooperation between adhesion and growth factors in the activation of MAPK (Cybulsky and McTavish, 1997; Inoue *et al.*, 1996; Lin *et al.*, 1997b; Miyamoto *et al.*, 1996; Renshaw *et al.*, 1997). In most instances, growth factor stimulation of nonadherent cells or cells attached nonspecifically to polylysine or plastic has little or no effect on MAPK activity [but not so in some systems (Hotchin and Hall, 1995)], and in all cases, engagement of integrins through adhesion to ligand-coated substrata or beads significantly enhances growth factor activation of MAPK. The adhesion dependence of growth factor activation of MAPK is ablated in cells transformed by viral oncogenes, including *v-ras*, *v-src*, and *v-mos* (Inoue *et al.*, 1996). Dissection of the pathway between growth factor receptors and MAPK suggests that in nonadherent fibroblasts, the early stages of growth factor signaling, including RTK autophosphorylation (Chen *et al.*, 1996b) and Ras GTP-loading, are intact, whereas the activation of downstream kinases (i.e., Raf, MEK, and MAPK) is attenuated significantly, with Raf activation being particularly affected (Lin *et al.*, 1997b). Observations in a recent report partially reiterate these results, but suggest that attenuation of mitogenic MAPK activation in suspended cells may occur at the level of MEK (Renshaw *et al.*, 1997). However, effects of adherence also have been observed on receptor autophosphorylation in fibroblasts and epithelial cells (Cybulsky and McTavish, 1997; Miyamoto *et al.*, 1996). In both cases, integrin engagement through either adhesion to ligand-coated plates (Cybulsky and McTavish, 1997) or incubation with antibody- or ligand-coated beads (Miyamoto *et al.*, 1996) was shown to synergize with growth factors to allow efficient tyrosine phosphorylation of the growth

factor receptor. Thus, there is convincing evidence in several cell types that integrin-mediated cell adhesion can increase the efficiency of growth factor signaling to the MAPK pathway. Whether the locus of adhesion modulation of growth factor signaling is at the level of the receptor tyrosine kinase or at a downstream step may vary among cell types or be influenced by experimental conditions.

C. Signal Modulation by Other Cell Adhesion Molecules

1. Neuronal cell adhesion molecules Neuronal axon guidance and outgrowth involve the integration of signals emanating from growth factors and guidance molecules, and from interactions of cell adhesion receptors with ligands on surrounding cells and the ECM. As mentioned in Section VIII., there are currently two theories concerning the mechanism of signaling by neuronal adhesion molecules involved in neurite outgrowth. One version suggests direct intracytoplasmic interactions between the cytoplasmic domains of adhesion receptors such as NCAM and Src-family kinases. Another theory, now also supported by compelling data, is that in the neuronal growth cone, homotypic cell adhesion receptors such as N-cadherin, NCAM and L1 promote axon extension through their ability to modulate the fibroblast growth factor receptor (FGFR) (reviewed in Doherty and Walsh, 1996).

To study neurite outgrowth mediated by different neuronal cell adhesion molecules, Doherty and colleagues extensively used a coculture system whereby rat cerebellar neurons or PC12 pheochromocytoma cells are plated onto a transfected 3T3 cell monolayer. Expression of physiological levels of N-cadherin, NCAM or L1 in the 3T3 cells enhances neurite extension from cerebellar neurons or PC12 cells relative to that exhibited by cells plated onto untransfected 3T3 cells (Doherty *et al.*, 1991). In this experimental paradigm, a battery of pharmacological agents have been used to define the downstream components of the pathway. The initial step depends on an erbstatin-sensitive tyrosine kinase and subsequent events activate PLC γ and in turn stimulate the liberation of DAG from PI(4,5)P₂ and the production of AA by a DAG lipase (Williams *et al.*, 1994b,c,d). AA production can lead to the influx of calcium via N- and L-type channels. Hence agents that block Ca²⁺ influx also inhibit the neurite outgrowth supported by neuronal CAMs and conversely, agonist-induced activation of calcium channels enhances neurite extension in the absence of intercellular neuronal CAM interactions (see fig. 9B) (Doherty *et al.*, 1991; Saffell *et al.*, 1992; Williams *et al.*, 1994b). A similar cascade of events is triggered by FGF binding to its receptor, as indicated by the finding that FGF treatment of cerebellar neurons or soluble L1 treatment evokes the same pattern of tyrosine phosphorylation (Williams *et al.*, 1994a).

The FGFR family of receptor tyrosine kinases is expressed widely in the developing nervous system and activation of the receptor results in neurite outgrowth (Green *et al.*, 1996; Jaye *et al.*, 1992; Wanaka *et al.*, 1990; Williams *et al.*, 1994b). A conserved 20 amino acid region of the FGFRs contains three sequences that are individually homologous to short extracellular sequences in N-cadherin, L1, and NCAM and this region in FGFRs has been termed the CAM homology domain (CHD) (Williams *et al.*, 1994a). Several lines of experimental evidence suggest that regions of this FGFR-CHD bind via intracellular *cis*-interactions with regions in N-cadherin, NCAM, and L1 (Doherty and Walsh, 1996; Saffell *et al.*, 1997; Williams *et al.*, 1994b). The same group recently has shown that treatment with soluble forms of L1 and NCAM leads to FGFR tyrosine phosphorylation (Saffell *et al.*, 1997) and that a peptide that inhibits FGF binding to its receptor does not perturb N-cadherin, L1, and NCAM-induced neurite outgrowth (Williams *et al.*, 1994a). Thus, NCAM modulation of the RTK apparently occurs in the absence of FGF ligand. These effects are seemingly specific to N-cadherin, NCAM, and L1 versus integrin-mediated outgrowth (Williams *et al.*, 1994a), and neurite outgrowth induced by NGF, potassium depolarization, or cholera toxin is unaffected in cells expressing a dominant-negative FGFR (Saffell *et al.*, 1997). In summary, these studies indicate that neurite outgrowth supported by N-cadherin, NCAM, and L1 is mediated through the activation of the FGFR, with the CHD of the FGFR being critical to this response. Despite these data, no direct evidence of N-cadherin, NCAM, or L1 binding to FGFR has been presented to date. An additional complexity is the finding that the cytoplasmic domain of NCAM is required for its ability to act as a neuronal receptor and promote neurite outgrowth (Saffell *et al.*, 1995). A likely explanation here is that the cytoplasmic domain is necessary for the localization of NCAM to sites of cell-cell contact.

Thus, in the neuronal growth cone, homophilic binding of N-cadherin, L1, and NCAM may lead to their recruitment to sites of cell-cell contact. Neuronal CAM aggregation, directly or indirectly, leads to coclustering of FGFR family members into a complex, resulting in FGFR activation and initiation of a cascade that leads to calcium influx and neurite outgrowth (fig. 9B). In this scenario, activation of FGFR via heterophilic binding of two neuronal CAMs, for example different cadherin family members, is not ruled out. This model, which relies on a similar cellular distribution of the relevant cell adhesion molecule and FGFR, and a common downstream mechanism of action for all three adhesion molecules, seemingly is opposed to studies indicating divergent pathways whereby L1 signals via Src and NCAM via Fyn and FAK (Beggs *et al.*, 1994, 1997; Ignelzi *et al.*, 1994).

2. Cadherin-catenin complexes In a manner analogous to the neuronal CAM-FGFR model, cadherin-catenin

complexes have been proposed to interact with membrane-bound signaling molecules. As described above, cadherins in complex with cytoplasmic catenins mediate cell-cell interactions in a variety of cell types. Hoschuetzky and colleagues have shown that in epithelial cells, the autophosphorylated EGF receptor coimmunoprecipitates with cadherin-catenin complexes (Hoschuetzky *et al.*, 1994). Binding may be mediated through β -catenin, as under *in vitro* assay conditions the central core of β -catenin can bind directly to the EGF receptor (Hoschuetzky *et al.*, 1994). β -Catenin does not contain regions with obvious homology to SH2 domains, and so in this case, the binding is not through SH2-phosphotyrosine interactions. Another putative physical association of this class of cell adhesion molecules is with the receptor-type protein tyrosine phosphatase, PTP μ ; coimmunoprecipitation and immunofluorescence studies demonstrated an interaction in mink lung cells and rat tissue lysates (Brady-Kalnay *et al.*, 1995). However, whether this is true, nonartifactual interaction recently has been questioned, because Zondag and colleagues failed to detect PTP μ -cadherin-catenin complexes in reciprocal immunoprecipitation experiments in the same cell line (Zondag *et al.*, 1996). The physiological relevance of these putative interactions, as yet, remains unclear. Tyrosine phosphorylation is critical to the function of cadherin-catenin complexes and the integrity of cell junctions (Behrens and Birchmeier, 1994; Behrens *et al.*, 1993; Birchmeier, 1995); hence, their association with RTKs or PTPs may influence adhesion by altering the phosphorylation state of cadherins and catenins. In accord with this theory are the findings that β - and γ -catenins are tyrosine phosphorylated in epidermoid carcinoma cells after EGF stimulation (Hoschuetzky *et al.*, 1994) or after treatment with a broad specificity phosphatase inhibitor (Brady-Kalnay *et al.*, 1995). The influence of catenin binding on the respective pathways downstream of the EGF receptor and PTP μ is open to conjecture.

3. Selectins Cellular interactions between leukocytes and endothelial cells are critical during inflammation because circulating blood cells adhere to and subsequently migrate through the endothelial layer of the blood vessel. Initial binding of leukocytes to and rolling along the endothelium is mediated by low-affinity interactions with the selectin family of cell adhesion receptors (Lasky, 1992; McEver, 1994). Studies in this field have provided a model of collaboration whereby a cell adhesion receptor, P-selectin, and signaling molecule, PAF, act in combination on the endothelial cell surface to elicit a signaling pathway in an attached leukocyte. Endothelial stimulation by agents, such as thrombin or histamine, results in both the translocation of P-selectin to the plasma membrane and cell surface expression of PAF. P-selectin relocation enables leukocytes to adhere loosely and then roll along the endothelial cell surface through binding of ligands, e.g., PSGL-1. This

initial stage results in signaling to leukocytes leading to an elevation of intracellular calcium levels, functional activation of $\beta 2$ integrins, cell polarization, and priming for enhanced degranulation (Ben-Baruch *et al.*, 1995; Lorant *et al.*, 1991, 1993). However, none of these activation steps are direct responses to P-selectin binding alone (Lorant *et al.*, 1993). Enhanced granular secretion from neutrophils bound to activated endothelial cell monolayers or CHO cells expressing transfected P-selectin on their surface is inhibited by an adhesion-blocking anti-P-selectin antibody or by competitive agonists for the PAF receptor (Lorant *et al.*, 1993). Similarly, immobilized P-selectin moderately enhances $\beta 2$ integrin function only when bound neutrophils are costimulated with PAF (Lorant *et al.*, 1993). These data indicate that P-selectin and PAF act together to bind and activate neutrophils. P-selectin acts to capture neutrophils on the endothelial cell surface enabling activation of neutrophils by the juxtacrine signaling molecule, PAF (see fig. 9C). The resultant increase in $\beta 2$ integrin affinity tethers the leukocytes to the surface of an inflamed vessel and is essential for transmigration to extravascular sites. This model depicts endothelial cell-mediated activation of leukocytes occurring in a localized fashion, thereby targeting the response and reducing widespread reaction. These concepts were discussed in more detail in a recent review (Zimmerman *et al.*, 1996a).

D. Summary

The ability to influence the downstream signaling of receptors that are activated by soluble growth and differentiation factors may be one of the most vital biological functions of cell adhesion receptors. This is a relatively new and rapidly growing area of investigation and much remains to be learned; however, several alternative models for collaboration between adhesion receptors and other receptors have emerged already, as discussed in detail above. Perhaps the simplest situation is where interaction with an adhesion receptor directly activates a growth factor receptor without the need for that receptor's soluble ligand. This seems to be true when NCAM interacts with FGFR during neurite outgrowth, or when $\beta 1$ integrins directly activate PDGF β receptor. A second model entails the ligand-mediated activation of growth factor receptors that have been recruited to integrin-dependent adhesion sites. The increased concentration of receptor tyrosine kinases (and possibly direct interactions with integrins) increases the efficiency of receptor activation; this type of event has been reported for EGF receptor, PDGF receptor, and insulin receptor. A third model suggests that integrin-dependent adhesion structures act as molecular "scaffolds" for the downstream components of signaling cascades, thus allowing more efficient propagation of the signal. This type of event has been reported in fibroblasts for activation of the MAPK cascade. Finally, cell adhesion receptors may simply bring two cells into juxtaposition, thus allowing efficient

transmission of paracrine signals, as has been described in leukocyte-endothelial cell interactions.

X. Cell Adhesion Receptors and the Control of Cell Cycle and Programmed Cell Death

A. Cell Adhesion and Cell Cycle Control

1. Overview of the cell cycle machinery The importance of adhesion in the control of cell growth and division has been known for many years. One of the key differences observed between transformed cells and their normal counterparts is that transformed cells can respond to soluble growth factors and proceed through the cell cycle while in suspension, whereas nontransformed cells require adherence to a substratum to respond (Clarke *et al.*, 1970). In recent years some of the molecular and biochemical events underlying anchorage dependence of cell growth and division have begun to come to light. Before discussing the details of recent findings, however, it is worthwhile to briefly review some of the basic features of the cell cycle.

The molecular machinery that dictates movement of a cell through the phases of the cell cycle comprises a series of cyclins and CDKs. Levels of expression of various cyclin proteins change during different phases of the cycle, typically with gradual increases followed by precipitous drops as the cell passes the phase where a particular cyclin-CDK complex is needed. By contrast, the expression levels of the kinases do not vary so dramatically throughout the cycle. To a first approximation, cyclin A/B-CDC2 complexes are required for entry into M phase, cyclin A-CDK2 complexes for transit of S phase, whereas cyclin D-CDK4/CDK6 complexes are associated with G1, and cyclin E-CDK2 with the G1/S transition (Sherr, 1993, 1996) (see fig. 10).

The G1 phase of the cell cycle is regulated by external factors such as peptide mitogens, whereas later events in the cycle are more autonomous and reflect the inexorable turning of the cell cycle machine. Several years ago, Pardee (1989) defined a point late in G1 termed "R" the "restriction point" (equivalent to "START" in yeast) that is the demarcation between mitogen-dependent and mitogen-independent cell cycle progression. Recently, it has become clear that anchorage dependence of cell growth also is confined to G1, and that once cells pass a point essentially identical with R, they no longer require adhesion to a substratum to complete the cycle (Bohmer *et al.*, 1996). Thus, mitogens and anchorage need to play coordinate roles in transit through a key point of the cell cycle. Movement through the R point is associated with activation of cyclin D-CDK4,6 complexes and (possibly to a lesser degree) with activation of cyclin E-CDK2 complexes (Assoian, 1997; Sherr, 1994). A key function for these cyclin-CDK complexes is to phosphorylate Rb protein and its family member p107. These so-called "pocket proteins," in their hypophosphorylated forms, tightly bind members of the E2F transcription factor family;

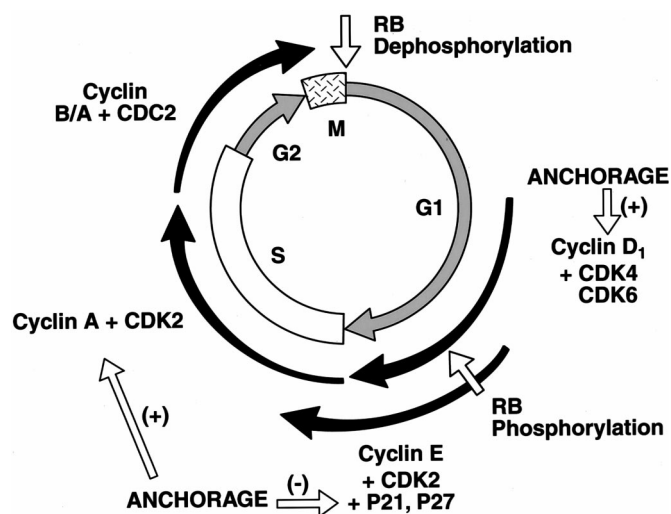


FIG. 10. Anchorage regulation of the cell cycle. Key regulators of the cell cycle including cyclins, cyclin-dependent kinases (CDKs), the p21 and p27 inhibitor proteins, and the Rb protein are shown at the approximate times of their major periods of activity. Positive (+) or negative (-) influences of integrin-mediated cell anchorage on these cell cycle regulators are shown. Adapted from Sherr (1993).

upon phosphorylation of Rb by activated cyclin-CDK complexes, E2F is released and then can transcriptionally activate genes that are required for further progress through the cell cycle (fig. 10).

The kinase activity of cyclin-CDK complexes is regulated by the interplay of several factors. First, CDKs require association with their cognate cyclin to be active. In addition, an upstream CDK-activating kinase phosphorylates both CDK2 and CDK4, and this event is also required for activation. Cyclin-CDK complexes are negatively regulated by the of binding cyclin-dependent kinase inhibitor proteins (CDIs), with the ratio of CDI to CDK in a complex regulating its level of activity. There are two families of CDIs: the cip/kip group (p21^{cip1/waf1}, p27^{kip1}, p57^{kip2}) that are essentially universal negative regulators of cyclin-CDK complexes, and the INK4 group (p15, p16, p18, p19) that specifically act on cyclin D-CDK4,6 complexes (Sherr, 1994). Thus, there is intricate regulation of the G1 cyclin-CDK complexes, affording both mitogens and cell anchorage many opportunities to affect progression through this phase of the cell cycle.

2. Links between signaling pathways and the cell cycle
Initiation of the G1 phase of the cell cycle is under the control of external signals such as polypeptide growth factors. However, the mechanistic connections between growth factor-triggered signaling cascades and the events of the cell cycle are just beginning to be worked out. The immediate-early gene *c-myc* seems to be a key link between growth factor-induced signals and cell cycle components. Expression of *c-Myc* is low in quiescent cells and is induced shortly after exposure to growth factors by signals projected through the Ras/MAPK cascade. As discussed above, regulation of cyclin D-depend

ent kinases is a key aspect of early events of cell cycle traverse and seems a convergence point for mitogenic signals (Lukas *et al.*, 1996). Expression of cyclin D1 message and protein is increased by ectopic expression or activation of *c-Myc* (Daksis *et al.*, 1994; Russell *et al.*, 1995). In addition, *c-Myc* seems to act to cause the sequestration of the p27 CDI in an inactive form, thus potentiating the activation of G1 CDKs (Vlach *et al.*, 1996). There is also direct evidence that MAPKs can increase expression of cyclin D1. Promoter activity for cyclin D1 is stimulated by overexpression of MAPK and inhibited by a dominant negative form of this kinase (Albanese *et al.*, 1995). Cells transfected by SV40 small t-antigen display increased cyclin D1 promoter activity via a mechanism that involves t-antigen inhibition of phosphatases that regulate MAPK and JNK pathways (Watanabe *et al.*, 1996a). Finally, recent work has shown that cyclin D1 expression in fibroblasts is positively regulated by p42/p44 MAPK and negatively regulated by the related p38 kinase pathway (Lavoie *et al.*, 1996). By this means early signaling events in the MAPK pathway and related pathways can have an impact on the G1 cell cycle transition.

3. Cell cycle activities regulated by cell adhesion
Most of the work done thus far on anchorage regulation of the cell cycle has been done in various fibroblast lines, although there have been some studies in other cell types. Whereas different studies disagree about some of the details, there seems to be a general consensus emerging on the overall mechanism. The key event of hyperphosphorylation of the Rb protein requires both soluble mitogens and cell anchorage; this is seen in both human and rodent fibroblasts (Kang and Krauss, 1996; Schulze *et al.*, 1996; Zhu and Toews, 1994). The identity of the cyclin-CDK complexes most important for regulating Rb phosphorylation in response to adhesion is somewhat uncertain at this point. Two groups have found that the expression of cyclin D1 mRNA and protein is strongly adhesion-dependent (Assoian, 1997; Resnitzky, 1997; Zhu *et al.*, 1996), thus placing emphasis on cyclin D1-CDK4,6 complexes. This emphasis is supported by the observation that ectopic expression of cyclin D1 can relieve anchorage dependence of cell cycle traverse in rat fibroblasts (Resnitzky, 1997). However, another group found no difference in levels of cyclin D1, D3, or CDK4 or 6 proteins, and no difference in cyclin D-CDK6 kinase activity in anchored versus suspended cells (Fang *et al.*, 1996). Several investigators have observed an anchorage dependence of the activity of cyclin E-CDK2 complexes because of changes in the levels of associated CDIs. In suspended cells, the expression of p21 is increased and the turnover of p27 is decreased (Schulze *et al.*, 1996; Zhu *et al.*, 1996); this may lead to an increase in the amount of p21 and p27 associated with cyclin E-CDK2 complexes and thus a reduction in activity (Fang *et al.*, 1996). In situations where there is reduced

expression of cyclin D-CDK4,6 complexes, this also can lead to a redistribution of p21 and p27 to cyclin E-CDK2 complexes, thus furthering the inhibitory process (Assoian, 1997). Cyclin A expression and the S phase transition are also affected by adherence in NRK and NIH3T3 fibroblasts (Guadagno *et al.*, 1993; Schulze *et al.*, 1996; Zhu *et al.*, 1996). In NIH3T3 cells, the expression of cyclin A is regulated transcriptionally through an E2F site in the cyclin A promoter (Schulze *et al.*, 1996). The effects of loss of anchorage in these cells could be reversed by overexpression of cyclin D1, whereas overexpression of p27 could repress cyclin A promoter activity in adherent cells. The underlying mechanisms here presumably relate to the abundance and state of activation of cyclin D- and cyclin E-dependent kinase complexes and their ability to phosphorylate Rb and p107 and thus release E2F family transcription factors. A generally similar picture, relating cyclin A induction to anchorage regulation of cyclin E-CDK2 complex activity, also was observed in NRK cells (Carstens *et al.*, 1996). Although, the mechanisms involved in cell anchorage regulation of cell cycle activities are still obscure, one interesting possibility is that the anchorage regulation of MAPK described at length above can influence activation of the cyclin D1 promoter in the same manner as growth factor regulation of MAPK pathways.

In addition to anchorage dependence of cell growth, many normal (untransformed) fibroblasts also display contact inhibition of cell growth; that is, as cells reach high density cell cycle progression ceases (this is clearly because of cell contact, not nutrient depletion). Contact inhibition is presumably a consequence of cell-cell adhesion between fibroblasts, although the cell surface molecules responsible for this process have not been identified. In contact-inhibited rat fibroblasts, there was a reduction in cyclin D-CDK4 kinase activity (Kato *et al.*, 1997). This was not caused by reduced levels of the cyclin or kinase proteins, but rather by maintenance of high levels of the p27 CDI; thus the picture in contact-inhibited cells resembles that seen in some types of fibroblasts where accumulation of CDIs blocks G1 CDK activity in nonanchored cells (Fang *et al.*, 1996).

One of the hallmarks of oncogene-transformed cells is their loss of the anchorage requirement for cell cycle progression. Recently there have been a few direct studies of the interplay between oncogenes and anchorage in cell cycle regulation. For example, Kang and Krauss (1996) used a mutant fibroblast cells line (ER-1-2), that upon *ras* oncogene transformation, retains anchorage dependence of cell growth, although displaying other aspects of the transformed phenotype. In wild-type fibroblasts cyclin A expression was highly anchorage dependent; as expected, *ras* transformation of these cells abrogated anchorage dependence of cyclin A expression. By contrast, in the ER-1-2 line both untransformed and *ras* transformed cells displayed anchorage-dependent cyclin A expression. Interestingly, *ras* transformation of

both wild-type and ER-1-2 fibroblasts led to anchorage-independent activation of cyclin E-dependent kinases and Rb hyperphosphorylation. Further, overexpression of cyclin A in *ras*-transformed ER-1-2 cells, but not in untransformed ER-1-2, supported anchorage-independent growth, suggesting that cyclin A is the key limiting factor in the *ras*-transformed cells, but that other anchorage-dependent factors are vital in the untransformed cells. Thus in the ER-1-2 *ras* system there seems to be an uncoupling between G1 CDK activation and Rb phosphorylation, and the subsequent induction of cyclin A, in contrast to the observations of Schulze *et al.* (1996) in 3T3 cells. Interestingly, the defect in anchorage control in ER-1-2 *ras* cells is rescued by extracellular ATP, presumably acting through a P₂ purinergic receptor (Yang and Krauss, 1997). The studies of Krauss and colleagues thus have underlined the multifactorial nature and complexity of anchorage regulation of the cell cycle, especially in the context of oncogene effects.

4. Anchorage regulation of the cell cycle: a role for specific integrins? The investigations described thus far compared anchored and nonanchored cells, but did not consider in detail the underlying mechanism of cell anchorage. Thus, an issue arises as to whether anchorage regulation of the cell cycle is an integrin-mediated phenomenon, or whether any form of cell attachment to a substratum would do (for example, tethering cells to a positively charged polymer or via an adsorbed antibody to a cell surface protein not known to be involved in cell-ECM adhesion). Several recent publications suggest that, at least in some circumstances, anchorage control of cell cycle activities is a specific integrin-mediated process. For example, vascular smooth muscle cells can attach to both polymerized type I collagen fibrils and to denatured "monomer" collagen; however, cells on polymerized collagen arrest in G1, whereas cells on monomer collagen can proliferate (Koyama *et al.*, 1996a). The biochemical basis for cell cycle arrest on polymerized collagen seems to be an inhibition of cyclin E-CDK2 kinase activity because of increased levels of the p21 and p27 CDIs and is thus similar to the effect of loss of anchorage. Cells attach to both polymerized and monomer collagen using the $\alpha2\beta1$ integrin; however, on polymerized collagen fewer focal contacts are formed and the cells are less spread. This suggests that integrin-mediated focal contact formation supports cell cycle traverse and that disruption or reduction of focal contacts promotes arrest. This notion is supported by the observation that an anti- $\alpha2$ integrin antibody Fab fragment inhibited cell cycle traverse of cells attaching on monomer collagen. It should be noted that the cells remain "anchored" under all these circumstances but that the extent of the integrin-mediated connections between an ECM protein (collagen) and the cytoskeleton is different in the two cases.

In another situation, in vascular endothelial cells, engagement of the integrin $\alpha v\beta3$, but not other integrins,

led to effects on both apoptotic pathways (see below) and on components involved in the cell cycle, particularly p21^{cip1} (Stromblad *et al.*, 1996). In a study that indirectly connects integrin-specific events to cell cycle control, Radeva *et al.* (1997) showed that overexpression of the ILK induced anchorage-independent, but not serum-independent, growth of rat intestinal epithelial cells. The associated biochemical events included activation of CDK4 and cyclin E-associated kinases and phosphorylation of Rb protein. Because ILK binds to $\beta 1$ and $\beta 3$ integrin subunits and is regulated by integrin-mediated cell adhesion, this suggests that the anchorage regulation of cell cycle in this system is integrin dependent.

There are also several other studies where expression of specific integrin subunits has had a profound impact on cell growth control. In most of these cases investigation of changes in the cell cycle machinery was pursued to only a limited degree, but it seems clear that such effects will underlie the alterations in growth control. A very interesting set of observations concerns the $\beta 1c$ subunit; this alternatively spliced version of the $\beta 1$ subunit contains a unique 48 amino acid C-terminal sequence. Potentially $\beta 1c$ can complex with several integrin α subunits. Expression of $\beta 1c$ in mouse fibroblasts or CHO cells reduced expression of key cell cycle components and inhibited DNA synthesis (Meredith *et al.*, 1995). The $\beta 1c$ subunit is expressed in normal prostate epithelium, but not in prostate carcinoma (Fornaro *et al.*, 1996). These observations suggest that $\beta 1c$ is a negative regulator of growth in normal cells, and its loss in tumor cells may contribute to their uncontrolled growth. Another case of specific integrin effects on cell growth control concerns the $\beta 6$ subunit. This subunit complexes with the αv subunit to form $\alpha v\beta 6$ integrins that bind the matrix proteins fibronectin and tenascin. Expression of $\beta 6$ in a tumor line that lacks this subunit results in enhanced proliferation; this function has been mapped to a unique 11 amino acid region of the $\beta 6$ cytoplasmic domain that is not required for cell adhesion or focal contact formation (Yokosaki *et al.*, 1996). The $\alpha 6\beta 4$ integrin is a receptor for laminins and usually is found in hemidesmosomes rather than focal contacts. The extremely long cytoplasmic tail of $\beta 4$ interacts with cytoskeletal elements of hemidesmosomes and may have signaling functions as well. Ligation of $\alpha 6\beta 4$ leads to phosphorylation of the $\beta 4$ subunit, recruitment of the Shc adapter protein, and activation of the Ras-MAPK pathway in keratinocytes (Mainiero *et al.*, 1997). However, in colorectal carcinoma cells (Clarke *et al.*, 1995) and in bladder carcinoma (Kim *et al.*, 1997), overexpression of the $\beta 4$ subunit arrests cell growth, likely because of increased expression of the p21 CDI (Clarke *et al.*, 1995). Thus the $\beta 4$ subunit seems to have a unique and complex role in regulating cell cycle events in epithelial cells. Other recent work (Wary *et al.*, 1996) has suggested that specific α subunits may be involved in integrin signaling and cell cycle control.

In summary, integrin-mediated cell anchorage is a key regulator of cell cycle traverse. Nonanchored fibroblasts display impairment of cyclin D-, cyclin E-, and cyclin A-dependent kinase activity, with the most impacted kinase complex being a function of the cell type and experimental conditions. In addition to effects caused by the dramatic change between anchored and nonanchored states, the expression or ligation of specific integrins also can modulate cell cycle events.

B. Cell Adhesion and Apoptosis

1. Overview of apoptosis Cell adhesion is an important regulator of apoptosis. The induction of a programmed cell death pathway through abrogation of anchorage to the substratum occurs most prominently in epithelial and endothelial cells, and has been termed "anoikis." In addition, more subtle changes in engagement of specific integrins also can affect programmed cell death pathways. Before discussing cell adhesion effects on cell death, it is worthwhile to review briefly present concepts concerning the mechanisms of apoptosis.

Programmed cell death (apoptosis) plays an important physiological role in shaping the tissues of the developing organism and a pathophysiological role in many diseases, including neurodegenerative disorders, as well as in cellular responses to toxic insults such as ionizing radiation and DNA-damaging drugs (Steller, 1995). In contrast to death by necrosis, which involves generalized intracellular damage, a loss of membrane integrity, and release of cell contents, death by apoptosis involves a very specific set of intracellular changes that culminates in cell death without loss of plasma membrane integrity. Some of the hallmark changes in apoptosis include internucleosomal cleavage of DNA leading to the formation of DNA "ladders" readily visualized by gel electrophoresis, the "blebbing" of plasma membrane and shedding of membrane vesicles, nuclear condensation and fragmentation, and eventual engulfment of membrane-bound cellular fragments by phagocytes (Martin *et al.*, 1994).

The genes involved in the programmed cell death pathway have been most clearly defined in *Caenorhabditis elegans* where *ced-3* and *ced-4* were identified as "killer" genes, whereas *ced-9* antagonizes the death process (Vaux, 1997). The *ced-3* gene was shown to encode a member of the caspase family of cysteinyl proteases, formerly termed "ICE" proteases in mammalian systems. The *ced-9* gene product finds its mammalian homolog in BCL-2, a member of a family of proteins that operate as homo- or heterodimers to either promote or antagonize apoptosis. A mammalian homolog of the *ced4* gene product has been identified only recently; this protein, termed Apaf-1, has a caspase recruitment domain and can bind to and activate pro-caspase-3. In *C. elegans*, the apoptotic pathway is rather simple; CED-4 activates CED-3 and this process is inhibited by CED-9,

presumably by direct interactions among these proteins (Golstein, 1997; Vaux, 1997).

As one would expect, regulation of apoptosis is more complex in mammalian cells. There are at least 10 caspases in human cells. In most cases these CED-3 homolog proteins are present as proenzymes that are cleaved and activated by other members of the caspase family, indicating the existence of a proteolytic cascade that ultimately leads to cell death (Porter *et al.*, 1997). Caspases are relatively promiscuous enzymes and thus there are numerous caspase substrates in cells, including key proteins such as Rb, nuclear lamins, poly(ADP-ribose) polymerase, DNA-dependent protein kinase and many others. It is not clear whether any one substrate is the key to cell death; more likely there are multiple parallel pathways contributing to the death process. However, it is clear that activation/overexpression of a single caspase is sufficient to trigger programmed cell death (Porter *et al.*, 1997).

The BCL-2 family of proteins (CED-9 homologs) plays a key role in the regulation of apoptosis (White, 1996). There are multiple representatives of this family in mammals, both as individual gene products and as proteins derived by alternative splicing. In this family, suppressors of apoptosis include BCL-2 itself, BCL-XL, and BCL-W, whereas BAX, BAK, and BAD promote or accelerate apoptosis. These molecules function as homo- and heterodimers with the ratio of death promoters to death suppressors presumably determining whether or not a cell enters the apoptotic pathway. It seems clear that BCL-2-related proteins operate upstream of caspases and regulate their proteolytic activity (White, 1996); however, the precise mechanism involved still is not settled completely. Some powerful recent evidence suggests that CED-4 can interact simultaneously with a BCL-2 family member (BCL-XL) and with mammalian caspases 1 and 8, indicating that BCL-2 regulates the mammalian caspase cascade through CED-4, a situation essentially parallel to that in *C. elegans* (Chinnaiyan *et al.*, 1997). However, the story may be more complicated than that. BCL-2 and BCL-XL are membrane-bound proteins; in particular they are located on the mitochondrial outer membrane. It has been proposed that BCL-2 can function as a pore or channel (Reed, 1997), perhaps regulating the loss of mitochondrial membrane potential known to occur in apoptotic cells (Golstein, 1997). BCL-2 and BCL-XL can prevent the release of cytochrome C from mitochondria, whereas release of cytochrome C into the cytoplasm can activate the caspase cascade (Kluck *et al.*, 1997; Vander Heiden *et al.*, 1997; Yang *et al.*, 1997). Thus BCL-2 may regulate apoptosis by controlling the mitochondrial membrane permeability barrier and the consequent release of cytochrome C and other proapoptotic factors (Li *et al.*, 1997b).

A cell's decision whether or not to enter the apoptotic pathway will depend on signaling processes that monitor extracellular and intracellular events. The clearest

example of intracellular regulation concerns the *p53* tumor suppressor gene, sometimes called the "guardian of the genome" because it is up-regulated in response to DNA damage and, in turn, can slow cell cycle progression and/or promote apoptosis (Ko and Prives, 1996). An important mechanism whereby *p53* promotes apoptosis is through transcriptional activation of the *bax* gene, thus changing the BAX/BCL-2 ratio to favor cell death (White, 1996). The clearest example of extracellular regulation involves "death factors" such as TNF and Fas-ligand (Nagata, 1997). These trimeric proteins bind to their cognate cell surface receptors, thus triggering formation of multimeric receptor complexes. The receptors contain so-called "death domains" that tend to self-aggregate; in addition, the receptor death domains bind to similar domains in cytoplasmic adapter proteins (FADD/MORT1 for Fas, TRADD for TNF-R). The adapter proteins can bind to downstream effectors; among these are caspase 8 which can interact with FADD/MORT1 (bound to Fas, or via TRADD to TNF-R); the caspase and FADD/MORT both possess a "death effector domain" that sustains protein-protein interaction and self-activation of the caspase. Thus cognate ligand binding to Fas or to TNF-R can directly trigger a caspase cascade leading to cell death.

Other extracellular factors provide survival rather than death signals. It has become clear that several peptides initially identified as "growth factors" are probably better described as survival factors; this would include the cytokines IL-2, 3, 4, 7, as well as granulocyte-macrophage colony-stimulating factor, NGF, and insulin-like growth factor-1 (Gajewski and Thompson, 1996). Recently, evidence has emerged that Raf-1, a key kinase in the signaling cascade activated by growth factors, may exert a powerful influence on apoptosis. Thus BCL-2 can recruit Raf-1 to the mitochondrial membrane; once there, activated Raf can (directly or indirectly) phosphorylate and inactivate BAD, relieving its inhibition of BCL-2 and providing an antiapoptotic effect (Wang *et al.*, 1996b). Thus, some of the linkages between growth regulatory signaling pathways and the control of apoptosis are beginning to emerge. As we will see below, signals from the ECM also play an important role in regulating apoptosis.

2. Anchorage regulation of apoptosis A key set of early observations showed that when untransformed epithelial (Frisch and Francis, 1994) or endothelial (Meredith *et al.*, 1993; Re *et al.*, 1994) cells are deprived of anchorage to an ECM substratum, the cells die by an apoptotic mechanism. By contrast, fibroblasts respond to loss of anchorage by G1 arrest. A new term "anoikis" was used to describe programmed cell death initiated by loss of anchorage (Frisch and Francis, 1994). In epithelial cells overexpression of BCL-2 countered anoikis, as did transformation with oncogenic forms of ras or src (Frisch and Francis, 1994). The role of anoikis in normal physiological function is unclear at this point; one possibility is

that it serves a tumor-suppressive function, because cells that lose contact with their normal supporting extracellular matrix would die rather than survive to invade or metastasize elsewhere.

The mechanistic basis of anoikis is the subject of very active investigation. Because anchorage to ECM is mediated largely by integrins, one would presume that integrins provide the initial signals governing anoikis; however, this has not been studied in detail. It seems clear that the FAK tyrosine kinase plays an important role in antagonizing this process. Expression of an activated, membrane-anchored form of FAK rescued epithelial cells from anoikis; also, the cells became anchorage independent for growth and tumorigenic in nude mice (Frisch *et al.*, 1996a). Cell transformation seemed to result primarily from resistance to apoptosis rather than activation of cell growth pathways, because the activated FAK-expressing cells showed no change in MAPK activity. Further study of the anoikis pathway showed that activation of JNKs and of ICE/LAP3 (caspase 7) occurred in response to loss of cell anchorage (Frisch *et al.*, 1996a). As expected, both caspase activity and anoikis were blocked by the protease inhibitor crmA and by overexpression of BCL-2. Activation of the JNK pathway also may be essential to anoikis because expression of a dominant negative JNK-kinase blocked the process. Surprisingly, crmA and BCL-2 blocked the activation of JNK, suggesting that a caspase might be involved in activation of the JNK pathway. This novel concept was supported in a recent report which demonstrated that loss of matrix anchorage activates a caspase that cleaves and activates MEK kinase-1 (MEKK-1), an upstream kinase in the JNK pathway (Cardone *et al.*, 1997). Cleavage of MEKK-1 occurred in cytoplasmic extracts of suspended epithelial cells and this could be blocked by peptide inhibitors of Asp-Glu-Val-Asp (DEVD) specific caspases. When overexpressed in cells, the MEKK-1 cleavage product triggered apoptosis, whereas full-length MEKK-1 sensitized cells to anoikis. These investigations also suggested the existence of a positive feedback loop between MEKK-1 activation and caspase activation. In summary, the investigations of Frisch and colleagues (Cardone *et al.*, 1997; Frisch *et al.*, 1996a,b) suggested a pathway that links integrins, BCL-2 family proteins, MEKK-1/JNK signaling kinases, and caspases as regulators of anchorage-dependent apoptosis (see fig. 11).

There are still issues regarding this scheme that must be resolved. The role of the JNK pathway in apoptosis seems to be quite variable in different model systems. For example, JNK has been implicated in apoptosis in response to growth factor deprivation (Xia, 1995) but has been ruled out in TNF-mediated apoptotic responses (Liu *et al.*, 1996). Thus it is unclear whether the pathway described above, which is based largely on observations in MDCK cells, will be conserved elsewhere. Indeed, recent observations (Khwaja and Downward,

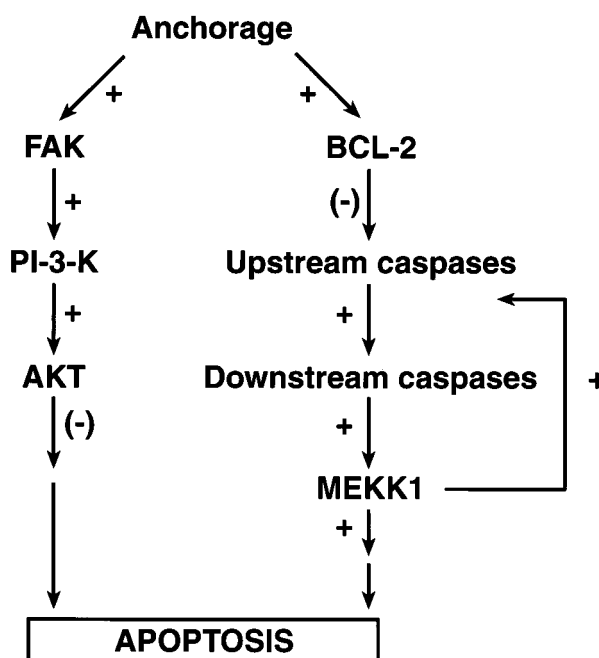


FIG. 11. Anchorage regulation of apoptosis. Two major pathways of anchorage regulation of apoptosis have been described. One involves FAK, PI-3K, and the Akt kinase. The other apparently parallel pathway involves Bcl-2, Caspases, and MEKK1/JNK. Positive (+) or negative (-) regulation of each factor on the next factor in the pathway is indicated. The two pathways may be operant to different degrees in different cell types. Adapted from Frisch and Rouslahti (1997).

1997) have questioned the role of JNK in the suppression of apoptosis by cell anchorage. Another loose end concerns the role of FAK, which seems to play no obvious part in the caspase/MEKK-1 connection. One interesting possibility is that FAK lies on a different pathway of apoptosis regulation, one that also involves PI-3K. As described in Section IV. above, FAK can bind to PI-3K and may be involved in its regulation. Recently, it became clear that PI-3K and its downstream effector the Akt/PKB serine-threonine kinase play a key role in regulation of apoptosis caused either by anchorage loss or by radiation-induced DNA damage (Khwaja *et al.*, 1997). Thus overexpression of activated forms of Ras or PI-3K (but not of other effectors downstream of Ras) conferred protection against anoikis. Cell anchorage was shown to result in elevation of 3'-phosphorylated phosphoinositides and stimulation of the Akt kinase; further, overexpression of Akt provided protection against anoikis. These studies provide a convincing demonstration of the role of PI-3K in regulation of programmed cell death. At this point, however, it is still not entirely clear how cell anchorage might regulate PI-3K activity, thus linking the two sets of events.

Although fibroblasts ordinarily respond to loss of integrin-mediated cell anchorage by arresting in G0/G1 rather than entering an apoptotic pathway, recent studies have shown that this choice can be modulated by oncogenes. Thus Myc/Ras- or E1A/Ras-transformed cells display anoikis when placed in suspension or when their

integrins were blocked with inhibitory peptides (McGill *et al.*, 1997). When cells were transformed with Src rather than Ras, the cells were resistant to anoikis. In a study with rather different outcomes, Ras-transformed fibroblasts either were allowed to attach to a substratum or not, and then treated with farnesyl transferase inhibitors (Lebowitz *et al.*, 1997). Exposure to farnesyl transferase inhibitors provoked massive apoptosis in cells; this was p53 independent and could be blocked by BCL-XL. Apoptosis also was attenuated by transfection of a farnesyl-independent form of Rho B, arguing that the Ras and farnesyl transferase effects were mediated through a Rho protein. Although the mechanistic foundations for these effects are unclear, they illustrate that specific signals from different oncogenes can cooperate with anchorage signals to regulate the choice between cell cycle arrest and apoptosis.

Anoikis involves a radical disruption of the cell's association with its extracellular matrix substratum. However, matrix effects on apoptosis also have been observed when more limited perturbations of cell-matrix interaction have occurred, and evidence points to the involvement of specific integrins. For example, as part of a series of studies concerning integrins in angiogenesis, Cheresh and colleagues examined the role of the $\alpha\beta3$ integrin in regulating apoptosis in endothelial cells (Brooks *et al.*, 1994; Stromblad *et al.*, 1996). The $\alpha\beta3$ integrin is highly expressed in angiogenic but not in resting endothelial cells. When $\alpha\beta3$ is blocked in an in vivo tumor angiogenesis model using inhibitory peptides or antibodies, proliferative endothelial cells in capillary sprouts undergo apoptosis, leaving cells in quiescent vessels unaffected. This very interesting observation eventually may form the basis of a novel approach to cancer therapy. It should be noted that, in contrast to anoikis, the endothelial cells in these studies remain attached to ECM via adhesion receptors other than $\alpha\beta3$ integrin; it is the specific disruption of the linkage between $\alpha\beta3$ and matrix that initiates the apoptotic response.

In a somewhat similar fashion, Bissell and colleagues studied the effects of ECM on breast epithelial cell growth and differentiation (Boudreau *et al.*, 1995a). They found that engagement with complex ECM, but not with purified collagen or fibronectin, could suppress mammary cell apoptosis (Boudreau *et al.*, 1995b). Apoptosis also could be induced by anti- $\beta1$ integrin antibodies or by expression of a metalloprotease which degrades matrix. The apoptotic process was blocked by inhibitors of ICE proteases, as might be expected. It should be noted that the cells always remained attached to the matrix in these cases, and thus the situation was unlike the simple loss of anchorage. In further work this group suggested that a three-dimensional ECM is important in allowing full epithelial cell differentiation and in suppressing both inappropriate secretion of growth stimulatory autocrine factors and consequent apoptosis (Bou-

dreau *et al.*, 1996). Although some of the underlying mechanisms remain obscure, these studies illustrate that very subtle changes in the manner in which cells engage the ECM can strongly influence the cell's commitment to growth, differentiation, or apoptosis.

Another example of a specific integrin effect in apoptosis concerns $\alpha5\beta1$, the "classic" fibronectin-binding integrin. Two groups have shown that overexpression of $\alpha5\beta1$ can confer resistance to apoptosis because of mitogen deprivation in both colon carcinoma cells (O'Brien *et al.*, 1996) and in CHO cells (Zhang *et al.*, 1995c). The effect was quite specific in that expression of the integrin $\alpha\beta1$, also a fibronectin receptor, failed to prevent apoptosis; the effect of $\alpha5\beta1$ probably was mediated through increased expression of BCL-2 (Zhang *et al.*, 1995c).

In summary, it is now clear that integrin-mediated anchorage plays a key role in the regulation of programmed cell death. Complete deprivation of anchorage leads to anoikis in epithelial and endothelial cells via a pathway that involves caspase-dependent cleavage and activation of MEKK1, a regulator of the JNK pathway. Another, possibly completely independent, pathway seems to involve FAK, PI-3K, and the Akt kinase (fig. 11). More subtle changes in integrin engagement with the ECM also can modulate apoptotic pathways, and some examples of integrin subunit-specific effects on apoptosis are beginning to emerge.

XI. Cell Adhesion Receptors and the Regulation of Gene Expression

Several lines of investigation have converged to show that cell-matrix and cell-cell adhesion have important consequences in terms of the regulation of gene expression during development and in adult tissues. Many important insights into the role of adhesion molecules in cell differentiation and tissue development have been provided through study of targeted mutation of cell adhesion genes. What is often striking is the exquisite specificity of some of the phenotypes observed; for example, an $\alpha8$ integrin subunit knockout results primarily in a profound disruption of epithelial-mesenchymal interactions in the kidney (Muller *et al.*, 1997). The approach of targeted mutations recently has been reviewed thoroughly (Huber *et al.*, 1996; Hynes, 1996) and will not be discussed further here. The general topic of adhesion molecules regulating gene expression also has been reviewed extensively by us previously (Juliano and Haskill, 1993; Rosales *et al.*, 1995). Here we will briefly recapitulate selected aspects of the earlier work, and add to that a presentation of some more recent findings. Most of the work to be discussed relates to integrins. There is much less information available concerning gene regulation by adhesion receptors other than integrins. Early studies have been discussed in a previous review (Rosales *et al.*, 1995), whereas additional examples are listed in table 5.

Three experimental systems have supported a significant fraction of the investigations thus far on adhesion molecules and gene expression, namely fibroblasts, monocytic cells, and breast epithelial cells, although some work has been done in other cell types. In fibroblastic cells, a particularly interesting example of gene regulation by adhesion receptors is the control of metalloprotease expression by $\alpha 5\beta 1$ and $\alpha 4\beta 1$ integrins. Attachment of cells to a fibronectin fragment containing the RGD site, which binds $\alpha 5\beta 1$, or attachment to anti- $\alpha 5\beta 1$ antibodies induces the expression of collagenase and stromelysin, although expression was not induced by adhesion to full-length fibronectin (Werb *et al.*, 1989). The full-length protein also contains a sequence recognized by $\alpha 4\beta 1$, and this interaction generates a signal that suppresses metalloprotease expression (Huhtala *et al.*, 1995). A similar situation is seen in cells plated on a mixed matrix containing fibronectin and tenascin, which induces metalloprotease expression, whereas fibronectin alone does not (Tremble *et al.*, 1994). There are many other examples of regulation of protease expression and function by adhesive interactions with the ECM; this topic has been reviewed recently (Werb, 1997).

A robust example of direct integrin-regulated gene expression occurs in monocytes (Eierman *et al.*, 1989; Haskill *et al.*, 1991; Yurochko *et al.*, 1992). This system has allowed new insights into the relationships between matrix proteins, integrins, protein kinases, transcription factors, oncogenes, and gene regulation (Juliano and Haskill, 1993). In physiological terms, monocytes need to engage with the ECM as they migrate from the bloodstream into sites of inflammation. Experimentally, when peripheral blood monocytes are plated onto substrata coated with ECM ligands such as fibronectin, collagen, or laminin, there is a rapid and profound induction of several immediate-early genes including transcription factors such as c-fos, c-jun, I κ B, as well as cytokines such as IL-1 β , IL-8, and TNF α . The blood coagulation component, tissue factor, also is induced in monocytic cells by integrin engagement (Fan *et al.*, 1995; McGilvray *et al.*, 1997). Cross-linking of $\beta 1$, but not $\beta 2$,

integrins with antibodies is sufficient to trigger the induction of multiple immediate-early genes, indicating a critical role for $\beta 1$ integrins in this response (Yurochko *et al.*, 1992). Many of the genes induced by integrin ligation have NF- κ B motifs in their upstream regulatory regions (Juliano and Haskill, 1993). Use of electrophoretic mobility shift assays and NF- κ B reporter constructs have provided direct evidence that the NF- κ B transcription factor is activated after integrin ligation (Lin *et al.*, 1995). It seems likely that integrin-mediated tyrosine phosphorylation is critically involved in immediate-early gene induction in monocytes (Lin *et al.*, 1994), and that the key integrin-responsive tyrosine kinase in monocytic cells may be SYK (Lin *et al.*, 1995). Ligation of $\beta 1$ integrins in monocytic cells also causes activation of MAPK (McGilvray *et al.*, 1997), and inhibition of the MAPK pathway with a specific inhibitor of MEK-1 was sufficient to block integrin-mediated activation of NF- κ B and expression of tissue factor. By contrast, intentional overexpression of activated elements of the Ras/MAPK cascade can block integrin-mediated activation of NF- κ B (Rosales and Juliano, 1996); however, this may be caused by Ras/MAPK effects on the activation status of integrins (Hughes *et al.*, 1997). In addition to activation at the transcriptional level, integrin engagement also can regulate message stability in monocytic cells (Lofquist *et al.*, 1995; Sirenko *et al.*, 1997). In summary, some of the connections between integrin ligation and monocyte gene induction are beginning to be worked out; however, the picture is complex and much remains to be done.

In addition to examples of direct integrin effects on gene expression, there are several interesting situations where integrin engagement modulates cellular responses to growth and differentiation factors. A good example of this is myoblast differentiation as monitored by expression of muscle-specific genes and formation of myotubes (Sastri *et al.*, 1996). Thus, ectopic expression of the $\alpha 5$ subunit in primary quail myoblasts can synergize with bFGF and insulin to allow cell survival and induce proliferation in serum-free conditions. By con-

TABLE 5
Regulation of gene expression by cell adhesion receptors

Receptor Involved	Gene Regulated	Cell Type	Reference
$\beta 1$ integrins	IE genes (cytokines TFs)	Human monocytes	(Yurochko <i>et al.</i> , 1992)
$\beta 1$ integrins	Tissue factor	Human monocytes	(Fan <i>et al.</i> , 1995)
$\beta 1$ integrins (other integrins?)	Milk genes	Mouse breast cells	(Streuli <i>et al.</i> , 1991)
$\beta 1$ integrins	Muscle-specific genes	Quail myoblasts	(Sastri <i>et al.</i> , 1996)
$\beta 1$ integrins	Metalloproteases	Synovial fibroblasts	(Werb <i>et al.</i> , 1989)
$\beta 1$ integrins	Cytokines	Chondrocytes	(Yonezawa <i>et al.</i> , 1996)
$\beta 2$ integrins	E-selectin	Endothelial cells	(Noble <i>et al.</i> , 1996)
$\alpha v\beta 3$	Metalloproteases	Melanoma	(Seftor <i>et al.</i> , 1992)
$\alpha v\beta 3$	p53	Endothelial	(Stromblad <i>et al.</i> , 1996)
$\alpha 5\beta 1$	IE genes	Colon carcinoma cells	(Varner <i>et al.</i> , 1995)
$\alpha 5\beta 1$	Bcl-2	CHO cells	(Zhang <i>et al.</i> , 1995c)
Integrins	IL-2	T cells	(Udagwa <i>et al.</i> , 1996)
Matrix (integrins?)	Integrins	Breast epithelia	(Delcommenne and Streuli, 1995)
Matrix (integrins?)	Liver specific	Hepatocytes	(DiPersio <i>et al.</i> , 1991; Liu <i>et al.</i> , 1991)
CEA (an Ig-CAM)	NCAM, MUC18	Melanoma	(Grimm and Johnson, 1995)
E-cadherin	Syndecan (a proteoglycan)	Mammary tumor cells	(Leppa <i>et al.</i> , 1996)

trast, myoblasts expressing human $\alpha 6$ exhibit decreased proliferation and a high propensity to differentiate into myotubes, whereas myoblasts expressing $\alpha 5$ proliferate to a high cell density and resist differentiation even at confluence. Another interesting example that may be mediated at the level of gene expression concerns the regulation of smooth muscle cell responsiveness to the neuropeptide substance P (Dahm and Bowers, 1996). Thus, an integrin-dependent interaction with the matrix protein thrombospondin results in a suppression of substance P responsiveness in these cells.

Another system in which differentiation is regulated by both integrins and soluble factors is mammary epithelia. When cultured in the presence of a reconstituted basement membrane matrix (Matrigel) and the lactogenic hormone, prolactin, mouse mammary epithelial cells form multicellular, alveoli-like structures and secrete milk proteins (e.g., β -casein, lactoferrin) unidirectionally into the lumen of the structure (Schmidhauser *et al.*, 1990). In the absence of cell-cell contact (i.e., no alveolus formation), single cells cultured with Matrigel and prolactin also express milk proteins but fail to do so when on collagen, despite the presence of hormone (Streuli *et al.*, 1991). Milk production in collagen cultures can be restored by allowing cell-cell contact (Streuli *et al.*, 1991). Addition of anti- $\beta 1$ integrin antibodies to the cultures can block casein production, suggesting that the signal from the matrix is transduced through integrins. Furthermore, the contribution of the complex basement membrane matrix could be singularly provided by laminin (Streuli *et al.*, 1995a). The identification of a matrix-responsive promoter element upstream of the β -casein gene (Schmidhauser *et al.*, 1992), and the observation that interaction with the ECM regulates the DNA binding activity of the prolactin-responsive transcription factor STAT-5 (Streuli *et al.*, 1995b) demonstrate that matrix- or integrin-mediated signals collaborate with lactogenic hormones at the level of transcription. Recently, it has become clear that matrix engagement prevents the activation of a tyrosine phosphatase that negatively regulates the activation of STAT-5 caused by lactogenic hormones (Edwards *et al.*, 1997). The establishment of cell polarity by integrin-mediated matrix interaction is not required for prolactin-dependent expression of milk proteins, as rounded, single cells actively produce β -casein (Streuli *et al.*, 1991). However, in the multicellular alveolar structures, unidirectional secretion of β -casein indeed does occur, implying cellular polarity. Although it is clear that matrix interaction is necessary and sufficient to allow β -casein expression, it is likely that signals from matrix interactions cooperate with signals from cell-cell contact to establish cell polarity and allow proper, unidirectional secretion (Gumbiner, 1996).

Another interesting example of integrin-related gene expression concerns ILK, the integrin-linked kinase described in Section III. Very recent work (Dedhar S.,

personal communication) has suggested that ILK may play a key role in epithelial-mesenchymal transitions, as often occur in development. Thus, ILK phosphorylates and inhibits GSK3 β , a key element of the Wnt signal pathway (see Section VIII.), eventually leading to increased transcription of mesenchymal genes regulated by the LEF-1/ β -catenin complex. This exciting result introduces an important new link between the integrin and cadherin signaling pathways at the level of gene regulation.

XII. Conclusions

The concept that adhesion receptors such as integrins or cadherins can serve as signal transducers is only approximately 8 years old. Before that time adhesion molecules were regarded as being vital structural components that contributed to the architecture of cells and tissues, but were not thought to play a role in the dynamic aspects of cell regulation. Now the field of adhesion receptor signaling is burgeoning, as indicated by the numerous very recent citations in this review. Adhesion receptors are known to be critically involved in fundamental cellular processes including cell differentiation, cell cycle control, and programmed cell death, whereas cooperation or coordination between cell adhesion receptors and receptors for soluble growth and differentiation factors may be the norm rather than the exception.

The teleological basis for this receptor-receptor collaboration seems quite clear. In making decisions to commit to growth, differentiation, or death, cells need information about the availability of nutrients and soluble growth/differentiation factors. However, cells also need positional information about whether they are in an appropriate relationship to the ECM and surrounding cells; this information is provided through cell adhesion receptors. Perhaps the most surprising thing about this process is the mechanism for coordinating biochemical and positional signals. It would be conceivable a priori to have quite distinct signaling pathways for soluble factors and for positional cues; however, what seems to have evolved is a situation whereby adhesion receptors contribute to or modulate the signaling cascades used by receptors for soluble factors. Perhaps the best described example of this is the role of integrin-mediated adhesion in modulating signaling through the peptide growth factor-MAPK pathway, but other examples also exist, as we have discussed in Section IX. above. The primary mechanism for the coordination of soluble and positional signals is also becoming clear. It involves the adhesion-dependent formation of "scaffolds" that allow the efficient assembly and interaction of signaling molecules such as GTPases and kinases. Much remains to be learned concerning the molecular details of "who talks to whom," but the concept of adhesion receptors and cytoskeletal components forming supramolecular struc-

tures that allow the recruitment and assembly of signaling cascades seems to have broad validity.

Thus, in a few short years our concepts of the biological role of cell adhesion receptors have changed completely, leading to the opening of an exciting and dynamic new field of investigation. It is hoped that studies in this area will result not only in important cell biological insights, but also in novel approaches to pharmacological intervention in a variety of disease processes.

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