0031-6997/98/5001-0001$03.00/0
PHARMACOLOGICAL REVIEWS
Vol. 50, No. 1
Printed in U.S.A.

Signal Transduction Pathways Regulating Hematopoietic Differentiation

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I. Introduction and Scope

Hematopoiesis, the process by which pluripotent stem cells give rise to functionally diverse mature blood cells, involves a complex array of hematopoietic growth factors. These factors regulate the survival and proliferation of early progenitors, influence differentiation commitment, and modulate the functional activities of the end-stage cells. The purpose of this review is to survey recent developments in some of the major signal transduction pathways that influence hematopoietic growth and differentiation, with a particular emphasis on myeloid cells. Related topics covered by other recent re-

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views will not be considered in detail here (e.g., cytokines and cytokine receptors, Jaks and Stats, and hematopoietic stem cell biology).

II. Biology of Hematopoiesis: Brief Overview

A. The Hematopoietic Stem Cell

Blood cells of all lineages are derived from a small population of stem cells localized to the bone marrow in adults. Hematopoietic stem cells exhibit two essential properties. First, they are able to give rise to all types of mature blood cells, including those of both the lymphoid (B- and T-cells) and myeloid (monocytes, granulocytes, erythrocytes, megakaryocytes) lineages. Second, they are capable of self-renewal, the ability to divide into two daughter cells with developmental potential identical with that of the parent cell. Estimates have placed the number of hematopoietic stem cells at approximately 0.05% of nucleated cells in the adult bone marrow, making their isolation and characterization difficult. Characterization of the hematopoietic stem cell and the mechanisms that control the decision for self-renewal versus differentiation are areas of intense investigation with major clinical relevance, especially for bone marrow transplantation. For a comprehensive review of hematopoietic stem cell biology, see Morrison et al. (1995).

B. Control of Myeloid Differentiation by Colony-Stimulating Factors and Other Cytokines

The proliferation, differentiation, and functional activities of hematopoietic cells are regulated by a diverse group of protein factors that will be referred to collectively as cytokines. Although a discussion of the biological activities of individual cytokines is beyond the scope of this review, some important general comments are relevant to a more in-depth discussion of the signaling pathways they regulate. First, cytokines produce multiple biological effects that are often dependent upon the stage of differentiation of the target cell. The pleiotropic actions of cytokines can be explained by the array of signaling components and transcription factors present in a given cell at the time of challenge with the factor. In addition, a given cytokine receptor can activate a variety of downstream signaling pathways. Second, many cytokines produce overlapping and, in some cases, nearly identical effects in the same target cell. The molecular basis for this observation can be explained, in part, by the finding that the receptors for many cytokines share common subunits that are important for signal transduction.

Despite the pleiotropic and redundant actions of cytokines, certain cytokines appear to be more restricted in their actions than others, both in terms of the lineage and stage of maturation of the target cells on which they act. For example, some cytokines promote the growth and survival of progenitors and support the development of more than one lineage of cells. Examples of cytokines in this category include stem cell factor (SCF)[b], interleukin (IL)-3, and granulocyte-macrophage colony-stimulating factor (GM-CSF). In contrast, other cytokines act primarily on a particular lineage of cells. Cytokines in this group include granulocyte colony-stimulating factor (G-CSF), colony-stimulating factor-1 (CSF-1), and erythropoietin (EPO), which promote the expansion and differentiation of granulocytes, macrophages, and erythrocytes, respectively. Cytokines also can act synergistically to promote maximal outgrowth of various lineages of cells. For example, the combination of IL-3 or GM-CSF with G-CSF greatly enhances the production of mature neutrophils. In this case, the earlier-acting factors help promote expansion of progenitors that in turn lead to greater numbers of terminally differentiated cells in response to G-CSF. For a more detailed discussion of these concepts, see Lowry (1995).

A final point regards whether cytokines are actually the driving force behind terminal differentiation or if they play, instead, a permissive role to allow a preordained differentiation program to proceed. Discovery of transcription factors unique to hematopoietic cells and subsequent gene knock-out experiments argue strongly for a predetermined differentiation program that is modulated by cytokines. For example, knock-out of the hematopoietic transcription factor GATA-1 leads to a disruption of erythropoiesis (Pevny et al., 1991). Nevertheless, the potent ability of cytokines to initiate hematopoiesis both in vitro and in vivo strongly suggests that the transcriptional regulators ultimately controlling differentiation are influenced by hematopoietic cytokines. The cell-surface receptors and signal transduction machinery that are required for cytokine action are a major focus of this review, with a particular emphasis on the differentiation of myeloid cells. A discussion of the role of hematopoietic transcription factors in the control of hematopoiesis is the subject of several recent reviews (Orkin, 1995a,b, 1996).

III. Overview of Cytokine Receptor Structure

Receptors for hematopoietic growth factors can be broadly divided into two groups: those that have ligand-binding and tyrosine kinase domains within the same polypeptide chain, and those that lack intrinsic tyrosine

[b] Abbreviations: CNTF, ciliary neurotrophic factor; CSF-1, colony-stimulating factor-1; CT-1, cardiotrophin-1; EGF, epidermal growth factor; EPO, erythropoietin; G-CSF, granulocyte colony-stimulating factor; GAPs, GTPase activating proteins; GDP, guanosine diphosphate; GH, growth hormone; GM-CSF, granulocyte-macrophage colony-stimulating factor; gp, glycoprotein; GTP, guanosine triphosphate; GTPase, guanosine triphosphatase; HCP, hematopoietic cell phosphatase; IL, interleukin; JNK, c-Jun N-terminal kinase; LIF, leukemia-inhibitory factor; M-CSF, macrophage colony-stimulating factor; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated protein kinase kinase; MEPs, Myb-Ets-transformed progenitors; OSM, oncostatin M; PH, pleckstrin homology; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PTB, phosphotyrosine-binding domain; SCF, stem-cell factor; SH, Src homology; Stat, signal transducers and activators of transcription; TH, Tec homology; TPO, thrombopoietin.

kinase activity but associate with and activate multiple families of nonreceptor protein-tyrosine kinases. This section is intended only as a brief primer on the elements of receptor structure and activation important for the following sections on downstream signaling. Reference is made to some of the many excellent reviews available on this topic.

A. Hematopoietic Growth Factor Receptors With Intrinsic Tyrosine Kinase Activity

This group includes the receptors for the hematopoietic growth factors CSF-1, SCF, and an SCF-related factor known as the Flk-2/Flt-3 ligand (reviewed by Paulson and Bernstein, 1996). CSF-1, alternatively known as macrophage colony-stimulating factor (M-CSF), is essential for the proliferation and differentiation of macrophage progenitors as well as osteoclasts, the specialized cells essential for bone remodeling. The requirement for CSF-1 and its receptor in these developmental events is illustrated by a naturally occurring mouse mutation known as osteopetrosis, which maps to the CSF-1 coding sequence (Yoshida et al., 1990). Mice bearing this mutation exhibit a specific deficit in macrophages and osteoclasts. The receptor for CSF-1 is identical with the product of the c-*fms* proto-oncogene and is very similar in structure to its transforming retroviral homolog, v-*fms* (Sherr et al., 1985). This landmark finding, together with related studies of the v-*erb*B/epidermal growth factor (EGF) receptor system (Downward et al., 1984), were among the first to demonstrate that growth factor receptor tyrosine kinases can be captured as retroviral oncogenes. Two other naturally occurring mutations in mice led to the identification of the Kit receptor tyrosine kinase and its ligand, referred to as SCF. These mutations, known as *W* and *S1*, both result in mice with a severe hematopoietic stem cell defect. Injection of mice with antibodies to Kit leads to suppression of all myeloid progenitors, indicating the requirement for this tyrosine kinase signal in both the self-renewal and lineage commitment of primitive hematopoietic cells (Ogawa et al., 1991). A final member of the receptor tyrosine kinase family specific to hematopoietic cells is Flk-2/Flt-3. This receptor and its cognate ligand appear to function in actively cycling hematopoietic stem cells (Lyman et al., 1994; Matthews et al., 1991).

The mechanism of activation of hematopoietic growth factor receptors is essentially the same as that described for other members of the receptor tyrosine kinase family (Lemmon and Schlessinger, 1994; Fantl et al., 1993; Heldin, 1995). Engagement of the extracellular ligand-binding domain of the receptor by the factor induces receptor dimerization. The catalytic domains of each half of the receptor dimer are brought into close proximity, resulting in autophosphorylation in trans. Autophosphorylation serves two essential functions. First, autophosphorylation of tyrosines within the receptor kinase domain activation loop is often essential for full catalytic activity toward substrate proteins (Johnson et al., 1996). Second, tyrosine autophosphorylation creates binding sites for effector proteins with Src homology (SH) 2 domains, which are essential mediators of the activated receptor's signal.

B. Cytokine Receptors Share Subunits and Lack Intrinsic Tyrosine Kinase Activity

Most hematopoietic cytokines bind to members of the cytokine receptor superfamily, a large group of transmembrane proteins with some shared structural features (reviewed by Wells and de Vos, 1996; Ihle et al., 1995). In the extracellular domain, most cytokine receptors are characterized by a 200 amino acid region with 4 positionally conserved Cys residues and a signature WSXWS motif (where W = Trp, S = Ser, and X = any amino acid). The cytoplasmic domains of receptor subunits that are involved in signal transduction also exhibit limited homology in the membrane proximal region. This region is often referred to as the box-1/box-2 motif and is essential for mitogenic signaling.

Although protein-tyrosine phosphorylation is an essential early event in cytokine signaling, cytokine receptors lack intrinsic tyrosine kinase activity. Instead, cytokine receptors associate with multiple members of various cytoplasmic tyrosine kinase families, which are described in detail in Section IV. A second feature of some cytokine receptors is the use of a common subunit for signal transduction in combination with a unique subunit to confer binding specificity for a particular ligand. The following sections (see Sections III.B.1. to III.B.4.) will describe briefly three groups of cytokines that share these basic properties. Also described are several examples of single-chain receptors that do not require additional subunits to generate high-affinity ligand binding sites. Representatives of each of the cytokine receptor subfamilies are illustrated in figure 1.

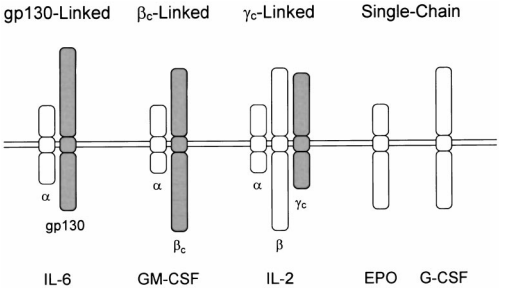

FIG. 1. Representatives of the major subfamilies of cytokine receptors. Some cytokine receptors can be classified on the basis of shared subunits (*shaded*). These include the gp130-linked receptors, for which the IL-6 receptor is a prototype. The receptors for LIF, OSM, CNTF, IL-11, and CT-1 also use gp130. β_c-linked receptors include those for GM-CSF (*shown*), IL-3, and IL-5. Receptors sharing a common γ subunit (γ_c) include those for IL-2 (*shown*), IL-4, IL-7, IL-9, and IL-15. Also shown are examples of receptors that consist of a single type of subunit (EPO, G-CSF). Although none of these receptors contain tyrosine kinase domains, all activate cytoplasmic protein-tyrosine kinases in response to ligand binding.

1. Receptors for interleukin-3, granulocyte-macrophage colony-stimulating factor, and interleukin-5 share a common β subunit (β_c). The overall structures of these cytokines are closely related, and they induce similar responses in common target cells. Receptors for these cytokines in humans consist of a unique α subunit and a common β subunit, referred to as β_c (reviewed by Sato and Miyajima, 1994; Taga and Kishimoto, 1995). The α-subunits confer specificity in ligand binding and are able to bind by themselves to their respective ligands with low affinity. Recent work with the GM-CSF receptor α-chain demonstrates an essential role for the short cytoplasmic segment of this subunit in GM-CSF-induced growth and differentiation (Matsuguchi et al., 1997). The β_c subunit, although unable to bind cytokine on its own, is required for high-affinity binding by the receptor complex and plays a major role in downstream signaling. Distinct regions of the β_c protein are responsible for activation of downstream signaling pathways, including signal transducers and activators of transcription (Stats) and the Ras-mitogen-activated protein kinase (MAPK) cascade (Sato et al., 1993; Quelle et al., 1994). Phosphorylation by associated nonreceptor tyrosine kinases links β_c to downstream signaling by promoting interaction with the SH2 domains of effector proteins.

2. Interleukin-6 receptor subfamily and the glycoprotein 130 signal transducer. IL-6 and the related pleiotropic cytokines leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), oncostatin-M (OSM), IL-11, and cardiotrophin-1 (CT-1) also share structural features and induce similar biological responses in common target cells (reviewed by Hibi et al., 1996). The functional redundancy among this group of cytokines is explained in part by their use of a shared signal transducer known as glycoprotein (gp)130. As for β_c-mediated signal transduction, gp130 also couples to multiple signaling pathways via a tyrosine kinase-dependent mechanism. Distinct regions of gp130 transmit independent signals for growth and differentiation (see Section V.C.2.).

3. The interleukin-2 receptor subfamily shares a common γ chain (γ_c). The overall structures of IL-2, IL-4, IL-7, and IL-9 are related, and each of these molecules is involved in various stages of T-cell growth and development (reviewed by Taniguchi and Minami, 1993; Taga and Kishimoto, 1995). The IL-2 receptor consists of three subunits, α, β, and γ. The α-subunit binds IL-2 alone with low affinity, whereas the other subunits do not exhibit IL-2 binding by themselves. The combination of β and γ binds IL-2 with intermediate affinity and transduces signals in the absence of the α-subunit. However, all three subunits are required for the highest IL-2 binding affinity. The γ_c subunit of the IL-2 receptor is also part of the receptor complexes for IL-4, IL-7, IL-9, and IL-15. The shared function of γ_c is illustrated dramatically by the phenotype associated with X-linked severe combined immunodeficiency. The biological function of this entire cytokine subfamily is affected as a result of a mutation in the γ_c subunit gene, leading to a profound T-cell deficiency (reviewed by Sugamura et al., 1996).

4. Single chain receptors. Although many cytokines use receptor systems with shared components, several important exceptions exist, including the receptors for EPO, thrombopoietin (TPO), and G-CSF (reviewed by Wells and de Vos, 1996; Ihle, 1995). Also related to this group are the receptors for prolactin and growth hormone. Receptors for these factors consist of a single polypeptide chain with an extracellular portion for high-affinity binding of the factor and a cytoplasmic portion essential for the transmission of signals for proliferation and differentiation. As described in Section III.C., distinct regions of these and other receptor cytoplasmic domains appear to control distinct functional responses by interacting with discrete downstream effector proteins.

C. Distinct Receptor Domains Transmit Proliferation and Differentiation Signals

Individual cytokines can control proliferation, differentiation commitment, and functional activities of a given target cell. Isolation of molecular clones of cytokine receptors has allowed for a detailed analysis of structural regions that are responsible for individual effects. The receptor for G-CSF serves as an illustrative example, although parallels can be found in other systems including the EPO receptor as well as gp130.

G-CSF stimulates the proliferation and differentiation of myeloid progenitor cells to neutrophilic granulocytes. In addition, it enhances the survival and functional activities of mature neutrophils (see Avalos, 1996 for a recent review). Although the receptor for G-CSF consists of a single polypeptide chain, it shares several features with other members of the cytokine receptor superfamily outlined above. These include an Ig-like domain, conserved cysteines, and a WSXWS motif, as well as fibronectin type III domains in the extracellular portion. The membrane-proximal region of the cytoplasmic domain contains the box-1/box-2 motif, whereas the more distal portion of the cytoplasmic domain contains several tyrosine phosphorylation sites.

Introduction of G-CSF receptor mutants into myeloid cell lines lacking endogenous receptors has allowed for a clean analysis of the contribution of various receptor regions to downstream signaling events. For example, the membrane-proximal 53 amino acids of the receptor containing the box-1/box-2 region are sufficient for transmission of mitogenic signals (Avalos, 1996). The box-1/box-2 motif has been implicated in the interaction with the Jak family of tyrosine kinases, which are the likely initiators of mitogenic signaling in this and many other cytokine receptor systems (see Section IV.A.). However, tyrosine phosphorylation of the receptor itself is not required for the initiation of proliferative signals.

The more distal portion of the G-CSF receptor appears to be critical for transmission of signals related to neutrophilic maturation. Truncated G-CSF receptors capable of inducing a mitogenic response are unable to promote differentiation (Fukunaga et al., 1993). Unlike the proliferative response, tyrosine phosphorylation of the receptor may play an essential role in the transmission of signals for differentiation. The C-terminal region of the receptor contains four tyrosine residues, all of which are rapidly phosphorylated in response to G-CSF treatment (Yoshikawa et al., 1995). Tyrosine to phenylalanine substitutions of two of these residues resulted in receptors that were unable to promote growth arrest and myeloperoxidase gene expression. These results suggest that signaling molecules with SH2 domains are likely to be recruited to the activated receptor-kinase complex via these tyrosine phosphorylation sites and mediate the observed effects on differentiation. This concept, as well as several candidates for differentiation-related SH2 effector molecules, are described in more detail in Section V.

IV. Cytokine Receptors Activate Multiple Cytoplasmic Tyrosine Kinase Families

The essential initial event that occurs in response to cytokine-receptor interaction is tyrosine kinase activation. For the majority of cytokines, this involves one or more members of the Jak, Src, Fps/Fes, Tec/Btk, and Syk/ZAP70 families of nonreceptor tyrosine kinases (fig. 2). Detailed in sections A through E below are the major features of each kinase family, with a particular emphasis on kinases that have been linked to myeloid growth regulation.

A. Jak Family

This group of kinases currently includes four members (Jak1, Jak2, Jak3, and Tyk2) and has been the subject of intense research activity since it was directly linked to cytokine signaling several years ago (reviewed by Leaman et al., 1996; Ihle, 1995; Schindler and Darnell, 1995). Jak1, Jak2, and Tyk2 are expressed in many cell types and are activated by a variety of cytokines and growth factors. In contrast, expression of Jak3 is restricted primarily to hematopoietic cells of myeloid and lymphoid lineages (Gurniak and Berg, 1996). Induction of terminal differentiation of a myeloid leukemia cell line with G-CSF correlates with a strong induction of Jak3 mRNA, suggesting that this kinase may have an important function in mature myeloid cells (Rane and Reddy, 1994). More recent studies show that Jak3 associates with the γ_c subunit shared by the IL-2 receptor subfamily. A necessary role for Jak3 in B and T-cell development is clearly evident from the phenotype of Jak3 knockout mice, which closely resembles that of severe combined immunodeficiency (Thomis et al., 1995; Nosaka et al., 1995).

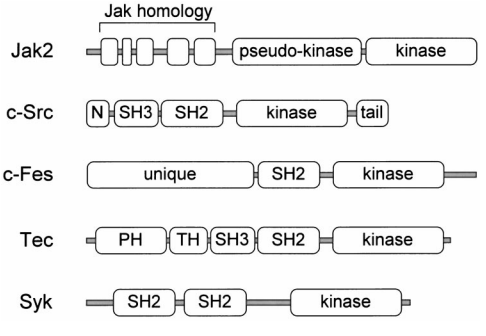

FIG. 2. Domain structures of protein-tyrosine kinases linked to cytokine receptors. Illustrated are representatives of the five major tyrosine kinase families activated in response to various cytokines. Major structural elements shared within and across the various families are shown (not to scale).

Jak-2 represents a prototype of the Jak kinase family, and its structure is shown in figure 2. Jak-2 is a 130 kDa protein distinguished by the presence of two kinase homology domains, the more C-terminal of which is catalytically active. The second kinase domain lacks several residues that are essential for kinase activity, and the precise function of this additional "pseudokinase" domain is unknown. Jak kinases also lack SH2 and SH3 domains, which are conserved features associated with most other nonreceptor tyrosine kinases. However, sequence alignment of the Jak kinases reveals several blocks of homology that may serve important protein-protein interaction functions, such as association with cytokine receptors (Zhao et al., 1995).

The membrane-proximal box-1/box-2 region of cytokine receptors is necessary and sufficient to induce a mitogenic response. Mutations or deletions within this region abolish mitogenesis and activation of Jaks, suggesting that it is essential for association with Jak kinases. For example, association of Jak2 with the EPO receptor both in vitro and in coimmunoprecipitation experiments requires the box-1/box-2 region of the receptor (Miura et al., 1994; Witthuhn et al., 1993).

Activation of Jaks in response to cytokine binding is likely to occur as a result of receptor oligomerization. The ligand-induced oligomerization event brings the receptor-associated Jaks into close proximity, allowing for autophosphorylation in trans and activation via a mechanism reminiscent of growth-factor receptor tyrosine kinases. Strong evidence for the oligomerization model of Jak activation comes from studies of chimeric receptors. For example, fusion of the extracellular ligand-binding domain of the EGF receptor with the cytoplasmic domain of the EPO receptor resulted in a chimeric molecule that responds to EGF with oligomerization, Jak2 activation, and mitogenesis (Maruyama et al., 1994). This mechanism may also contribute to the activation of other tyrosine kinases associated with cytokine receptors.

B. Src Family

Src is the prototype of large group of nonreceptor tyrosine kinases that share similar structural features and regulation (for an excellent comprehensive review,

see Brown and Cooper, 1996). The family consists of several members with widespread tissue distribution (Src, Yes) as well as another group with expression that is restricted primarily to hematopoietic cells (including Blk, Fgr, Fyn, Lck, Hck, and Lyn). Important structural features of Src kinases include N-terminal sequences for lipid attachment (myristylation and, in some cases, palmitylation), a unique domain, SH3, SH2, and kinase domains, followed by a C-terminal negative regulatory tail (fig. 2). Myristylation of Src allows for its membrane localization that is essential for biological activity (Resh, 1994). The SH3 domain may play a role in binding substrate molecules (Briggs et al., 1995; Weng et al., 1994), and, together with the SH2 domain, it is critical for the regulation of kinase activity (Briggs et al., 1997; Okada et al., 1993; Murphy et al., 1993; Superti-Furga et al., 1993). SH3 domains bind with high affinity and specificity to target sequences rich in proline and other hydrophobic amino acids. These sequences form a polyproline type II helix that associates with a hydrophobic pocket on the surface of the SH3 domain (for reviews of SH2 and SH3 structure and function, see Pawson, 1995; Cohen, et al., 1995). SH2 domains also function in protein-protein interaction by virtue of their affinity for phosphotyrosine-containing sequences (see Section V.A.). In the context of Src, the SH2 domain contributes to the negative regulation of kinase activity. Phosphorylation of a highly conserved tyrosine residue in the tail region induces intramolecular interaction with the SH2 domain, folding the kinase into an inactive conformation. This closed form of the kinase is stabilized by an additional intramolecular interaction of the SH3 domain with a polyproline type II helix formed by a loop connecting the SH2 and kinase domains. The latter interaction was discovered only recently with the solution of high-resolution X-ray structures of the inactive, tail-phosphorylated forms of c-Src and Hck (Sicheri et al., 1997; Xu et al., 1997). The tail residue is phosphorylated by a distinct regulatory kinase known as Csk (for C-terminal Src kinase; Nada et al., 1991). Gene-knockout experiments strongly suggest that Csk is the master regulator of all Src family kinases (Imamoto and Soriano, 1993; Nada et al., 1993). Without Csk, embryonic lethality is observed with a concomitant elevation in overall Src family kinase activity. By contrast, knockouts of individual Src kinases often show more subtle phenotypes, suggesting functional compensation by other family members.

Src-related kinases have been implicated in signal transduction by both cytokines and growth factors. In the case of CSF-1 and other growth factors, activation of Src kinases is required for cell-cycle progression in response to factor treatment (reviewed by Parsons and Parsons, 1997). These results are somewhat surprising, given that the receptors for CSF-1 and other growth factors possess intrinsic tyrosine kinase activity and are capable of coupling directly to downstream effector molecules. The mechanism of Src kinase activation by growth factor receptor tyrosine kinases may involve recruitment of Src to the activated, autophosphorylated form of the receptor via the SH2 domain of Src. Binding of the Src SH2 domain to the receptor may activate the kinase domain by displacing the negative regulatory tail, by direct phosphorylation by the receptor tyrosine kinase, or both (Parsons and Parsons, 1997; Brown and Cooper, 1996). The mechanism of Src kinase activation by cytokine receptors is less clear but may involve recruitment of Src to the activated, oligomeric form of the receptor (Taniguchi, 1995).

Although Src kinases are activated by a variety of cytokines, their contribution to cytokine signal transduction and their role in differentiation commitment is less clear. Experiments with mice in which individual and multiple members of the Src kinase family have been inactivated via homologous recombination have begun to shed some light on this important issue. For example, mice with a homozygous deletion of Hck do not exhibit defects in hematopoiesis, although phagocytosis is affected (Lowell et al., 1994). The activity of the related Lyn kinase was increased in macrophages from the Hck knockout animals, suggesting a possible compensatory mechanism. Additional evidence for the functional overlap of Hck with other Src family members comes from analysis of double-mutant animals. For example, Hck/Fgr double-knockout mice develop macrophages that are unable to respond to infection with *Listeria monocytogenes* (Lowell et al., 1994). These double-knockout animals also demonstrated defective integrin-mediated responses in neutrophils, whereas animals with individual knockouts showed essentially wild-type responses (Lowell et al., 1996). These results suggest that Hck and Fgr have evolved to serve very specific functions that allow for highly specialized signaling events in macrophages.

In addition to its roles in phagocyte function, Hck also has been linked to signal transduction pathways for multiple hematopoietic cytokines. Hck is activated in response to IL-3, GM-CSF, and LIF (Anderson and Jorgensen, 1995; Ernst et al., 1994a; Linnekin et al., 1994). Overexpression of Hck causes a substantial increase in cellular protein-tyrosine phosphorylation after IL-3 treatment (Anderson and Jorgensen, 1995), suggesting a direct relationship to the receptor. Overexpressed Src did not couple to the IL-3 receptor in these studies, arguing in favor of specificity in the response. More recent studies have demonstrated direct association of Hck with the β subunit of the IL-3 receptor (Burton et al., 1997). This interaction was reported to involve the SH2 and SH3 domains of Hck and the distal portion of β_c. Constitutive activation of Hck by gene targeting dramatically reduced the LIF requirement for the maintenance of totipotency in embryonic stem cells (Ernst et al., 1994a). This study also demonstrated direct interaction of Hck with gp130, the signal-transducing component of the LIF receptor that is shared with the recep-

tors for IL-6 and other cytokines (see Section III.B.2.). In addition to Hck, other members of the Src kinase family have been implicated in hematopoietic cytokine receptor signal transduction, including Fyn, Lyn, and Lck (Corey et al., 1993, 1994; Anderson and Jorgensen, 1995; Hatakeyama et al., 1991; Torigoe et al., 1992). Lck and Fyn are also critical for T-cell development and antigen responsiveness (reviewed by Qian and Weiss, 1997).

Direct evidence implicating Src family kinases in differentiation signaling downstream of cytokine receptors is provided by recent experiments with a differentiation-defective clone of the erythroid precursor cell line, J2E (Tilbrook et al., 1997). Treatment of J2E cells with EPO normally induces proliferation followed by erythroid differentiation, including hemoglobin synthesis and enucleation. In these studies, a defect in EPO-induced J2E differentiation was observed to correlate with a dramatic reduction in cellular expression of the Src family kinase, Lyn. Introduction of Lyn with a recombinant retrovirus restored the ability of the cells to undergo differentiation. Consistent with these results, suppression of Lyn activity in EPO-responsive parental J2E cells with antisense oligonucleotides or a dominant-negative Lyn mutant suppressed EPO-induced differentiation. However, the effects of EPO on viability in this cell line were not affected, indicating a specific role for Lyn in the differentiation but not the survival pathway. Using coimmunoprecipitation and yeast two-hybrid approaches, a direct association of Lyn with the EPO receptor was demonstrated.

C. Fps/Fes Family

The human c-*fes* gene is the normal cellular counterpart of several viral oncogenes including v-*fps*, the transforming oncogene associated with Fujinami sarcoma virus (Hanafusa et al., 1980; Shibuya and Hanafusa, 1982), as well as the v-*fes* oncogenes associated with several feline sarcoma viruses (Hampe et al., 1982). The c-*fes* locus (Roebroek et al., 1985) encodes a non-receptor protein-tyrosine kinase that, together with the related *fer* gene (Feldman et al., 1986; Pawson et al., 1989), defines a structurally distinct kinase family (fig. 2). Unlike its transforming counterparts, c-Fes tyrosine kinase activity is very tightly regulated in cells, even under conditions of strong overexpression (Greer et al., 1988). As a consequence, c-Fes has been reported to be either nontransforming (Greer et al., 1988) or to exhibit weak transforming activity in fibroblasts (Feldman et al., 1989).

Human c-Fes is a 93 kDa protein-tyrosine kinase consisting of a long N-terminal unique domain, a central SH2 domain, and a C-terminal kinase domain (fig. 2). Fes lacks an SH3 domain and negative regulatory tail, setting it apart from the Src kinase family. Also in contrast to c-Src, the SH2 domain of Fes may function as a positive regulator of kinase activity, as the deletion of this domain has a negative impact on kinase activity in vitro (Hjermstad et al., 1993) and on the ability of Fes to induce terminal differentiation of myeloid leukemia cells (Rogers, J. and Smithgall, T., unpublished data). Recent studies have also shown that the Fes SH2 domain may interact with specific substrate proteins in macrophages (Jucker et al., 1997).

Work from our laboratory suggests that the Fes unique N-terminal domain may also serve regulatory and substrate recruitment functions. Together with the SH2 domain, the Fes unique N-terminal region mediates interaction with the breakpoint cluster region (Bcr) protein (Maru et al., 1995). Bcr is a multifunctional signaling protein with an N-terminal serine/threonine kinase domain as well as regulatory activities for small guanosine triphosphatases (GTPases) of the Rho family (Chuang et al., 1995; Diekmann et al., 1991; Maru and Witte, 1991). Tyrosine phosphorylation of Bcr by Fes leads to the formation of Bcr-Grb2-SOS complexes (Li and Smithgall, 1996; Maru et al., 1995). As described in more detail below (see Section V.B.2.), the Grb2-SOS guanine nucleotide exchange complex serves to link many tyrosine kinases to the activation of Ras. Thus, Bcr may provide a unique signaling intermediate between the Fps/Fes family of tyrosine kinases and the activation of Ras and other small G-proteins. In addition to substrate binding, the unique N-terminal region of Fes may also help to regulate kinase activity. Computer analysis of both Fes and the closely related tyrosine kinase Fer revealed strong consensus sequences for coiled-coil oligomerization domains (Read et al., 1997; Kim and Wong, 1995). Recently it was observed that the active form of Fes is a large oligomeric complex, suggesting that Fes activation may result from oligomerization and transphosphorylation (Read et al., 1997). The presence of coiled-coil motifs within Fes and Fer is unique among the known nonreceptor tyrosine kinase families.

Initial studies of the tissue distribution of c-Fes suggested that it was expressed primarily in hematopoietic cells of myeloid origin, particularly in differentiated granulocytes and monocytes (MacDonald et al., 1985; Feldman et al., 1985; Smithgall et al., 1988). Analysis of Fes expression in a panel of leukemia cell lines showed a strong correlation with responsiveness to a variety of myeloid differentiation agents, suggesting that Fes may play an active role in the maturation process (Glazer et al., 1986; Chapekar et al., 1986; Smithgall et al., 1988). Direct support for this hypothesis comes from work with K-562 cells, a chronic myelogenous leukemia-derived cell line that does not express c-Fes. Transfection of these cells with a c-*fes* expression vector resulted in growth suppression and terminal differentiation (Yu et al., 1989), despite the fact that the cells carry the Philadelphia chromosome and express oncogenic Bcr/Abl. These data are consistent with earlier work showing that infection of chicken bone marrow progenitors with the Fujinami sarcoma virus, which carries a transforming avian homolog of c-*fes*, promoted differentiation to

macrophages without the addition of CSF-1 (Carmier and Samarut, 1986). These results suggest that Fps/Fes tyrosine kinases can generate signals sufficient for myeloid differentiation when expressed in an appropriate hematopoietic context.

The finding that Fes can suppress the growth and promote the differentiation of a chronic myelogenous leukemia-derived cell line indicates that Fes is able to overcome the differentiation block initiated by Bcr/Abl in these cells (Yu et al., 1989). Recent work from our laboratory suggests that Fes may interact directly with Bcr/Abl and influence its transforming activity. We observed that the normal Bcr protein associates with and is phosphorylated by Fes, providing a possible biochemical link between Fes and small G-proteins of the Ras and Rho families (Li and Smithgall, 1996; Maru et al., 1995). We have also observed that Fes can transphosphorylate Bcr/Abl (Lionberger and Smithgall, unpublished data), consistent with a previous report showing that Bcr/Abl can phosphorylate c-Fes (Ernst et al., 1994b). Current studies in our laboratory are focused on the effect of Fes-Bcr/Abl interaction on transformation by Bcr/Abl.

Although c-Fes may be sufficient to induce terminal myeloid differentiation, whether c-Fes is required for differentiation has not been firmly established. Evidence supporting a requirement for c-Fes in myeloid differentiation comes from antisense experiments. Suppression of c-Fes expression in HL-60 promyelocytic leukemia cells using antisense oligonucleotides blocked the induction of granulocytic differentiation by dimethylsulfoxide and retinoic acid. Interestingly, the cells were not only rendered differentiation-resistant by c-Fes antisense oligonucleotides, but they responded to treatment with differentiation inducers by undergoing apoptosis (Manfredini et al., 1993). These results indicate that expression of c-Fes may be required not only for differentiation but for the suppression of apoptosis as well. In a similar study, antisense inhibition of c-*fes* expression blocked macrophage differentiation of HL-60 cells in response to phorbol ester, although no apoptotic effect was reported (Manfredini et al., 1997). Although these results strongly implicate c-Fes or closely-related tyrosine kinases in the differentiation response, definitive evidence for a specific requirement for this gene in myeloid maturation will require a knock-out experiment. Given that recent studies have demonstrated Fes expression in nonhematopoietic sites such as vascular endothelium and neurons (Haigh et al., 1996; Greer et al., 1994), the knockout may produce a surprising result.

The finding that c-Fes can influence myeloid differentiation suggests that it may be coupled to receptors for CSFs and other cytokines. Supporting this view are reports that Fes is activated by GM-CSF, IL-3, IL-4, IL-6, and EPO (Matsuda et al., 1995a; Izuhara et al., 1994; Hanazono et al., 1993a,b). However, other laboratories have not been able to establish a connection between c-Fes and some of these cytokines (Anderson and Jorgensen, 1995; Witthuhn et al., 1993). Other evidence suggesting an association between Fes and cytokine receptor signaling includes the finding that c-Fes and its transforming homolog v-Fps can activate the myeloid differentiation-related transcription factor, Stat3 (Nelson et al., 1997; Garcia et al., 1997). As discussed in Section V.C.2., activation of Stat3 is required for myeloid differentiation of the leukemia cell line M1 in response to IL-6, one of the cytokines known to activate Fes (Matsuda et al., 1995a).

D. Tec/Btk Family

The Tec kinase was originally identified in hepatocytes (Mano et al., 1990) and was subsequently shown to exhibit expression in a wide range of hematopoietic cells (Mano et al., 1993). Tec is closely related to Bruton's tyrosine kinase (Btk), mutations which are associated with X-linked agammaglobulinemia (reviewed by Rawlings and Witte, 1995). Together with several other structurally-related kinases exhibiting primarily hematopoietic expression, they define the Tec/Btk kinase family. Structurally, Tec/Btk kinases exhibit SH3, SH2, and kinase domains in an arrangement most closely resembling the Src kinase family (fig. 2). However, the presence of an N-terminal pleckstrin homology (PH) domain, a novel Tec-homology (TH) domain containing a proline-rich region as well as the absence of an N-terminal myristylation signal, or a C-terminal negative regulatory tail set this family apart from Src and other nonreceptor tyrosine kinases.

The presence of a PH domain is unique to the Tec/Btk tyrosine kinase family, where it may serve functions related to kinase regulation and localization. PH domains are often associated with proteins involved in signal transduction and cytoskeletal interactions (for a review, see Musacchio et al., 1993; Gibson et al., 1994). A recent study has reported that a point mutation in the Btk PH domain results in increased membrane targeting as well as enhanced tyrosine kinase and transforming activities (Li et al., 1996). These findings suggest that the PH domain may play a unique role in regulation of Tec/Btk kinase activity and that membrane targeting may be an important step in the activation mechanism. In addition to the PH domain, the proline-rich region may also contribute to kinase regulation by binding intramolecularly to the SH3 domain (Andreotti et al., 1997). This region may mediate interactions with Src kinase family members as well.

Tec is activated downstream of several cytokine receptors, including those for IL-3 (Mano et al., 1995), IL-6 (Matsuda et al., 1995b), SCF (Tang et al., 1994), EPO (Machide et al., 1995), and G-CSF (Miyazato et al., 1996). In addition to Tec, Btk has also been shown to constitutively associate with gp130, the signal transducer for the IL-6 family of cytokines (Matsuda et al., 1995b). Both Tec and Btk were activated in response to

IL-6 in this study. In myeloid cells, IL-3 and EPO have been shown to induce the association of Tec with Vav (Miyazato et al., 1996; Machide et al., 1995), a hematopoietic signaling molecule believed to be involved in regulation of small GTPases of the Rho family (see Section V.C.1.). Cytokine treatment also led to tyrosine phosphorylation of Vav. Although these studies did not conclusively establish that Vav is tyrosine-phosphorylated by Tec, they suggest that Tec may serve to connect cytokine receptors to Vav and Rho signaling downstream.

Both Tec and Btk have also been shown to associate with members of the Src kinase family (Cheng et al., 1994; Mano et al., 1996; Afar et al., 1996). One interaction mechanism may involve the proline-rich domain of Tec/Btk and the SH3 domain of the Src family kinase. In the case of Tec and the Src-related kinase Lyn, Tec can be transphosphorylated by Lyn but not vice-versa, suggesting that Tec may be a downstream effector for Lyn under some circumstances (Mano et al., 1996). Whether Tec is activated directly by cytokine receptors or requires a Src family member or other intermediate tyrosine kinase will require further analysis. However, this interaction raises interesting general questions regarding direct interactions between nonreceptor tyrosine kinase families.

E. Syk/ZAP70 Family

This group of tyrosine kinases is most prominently associated with signal transduction by B-cell and T-cell antigen receptors as well as Fc receptors (reviewed by Bolen, 1995). The Syk/ZAP70 kinases are structurally distinct, with tandem N-terminal SH2 domains and a C-terminal kinase domain (fig. 2). The SH2 domains promote recruitment of Syk/ZAP70 to the activated antigen receptor after phosphorylation by members of the Src kinase family. The SH2 domains of Src family kinases may mediate direct interactions between Src kinases and Syk as well (Aoki et al., 1995), providing another example of crosstalk between tyrosine kinase families. Homozygous inactivation of Syk by gene targeting resulted in mice that suffered from severe hemorrhage in utero and died shortly after birth, indicating a critical role for the kinase in maintenance of vascular integrity or wound healing (Turner et al., 1995; Cheng et al., 1995). Studies of lymphoid or fetal liver cells from the Syk-deficient animals revealed a critical role for this kinase in B-cell development and pre-B-cell receptor signal transduction. Syk can also be activated in response to hematopoietic cytokines such as G-CSF (Corey et al., 1994). However, fetal liver progenitors from Syk-deficient mice retained responsiveness to G-CSF and other cytokines, suggesting that Syk may not be required for signal transduction through the G-CSF receptor (Turner et al., 1995).

V. Hematopoietic Cytokines Activate Multiple Downstream Signaling Pathways

A. Tyrosine Kinase Effectors Share Src Homology 2 Domains

Hematopoietic growth factors and cytokines induce oligomerization and autophosphorylation of their receptors. The resulting autophosphorylated tyrosine residues, together with the surrounding amino acids, form docking sites for the SH2 domains of downstream signaling proteins (reviewed by Pawson, 1995; Cohen et al., 1995). SH2 domains were first described in the context of nonreceptor tyrosine kinases such as Src and Fps, where they play critical roles in the regulation of kinase activity and interaction with signaling partners. Since that time, SH2 domains have been observed in a diverse array of signaling proteins otherwise structurally unrelated to tyrosine kinases. The essential function of the SH2 domain is to bind with high affinity and specificity to tyrosine-phosphorylated sequences within target proteins. By contrast, they display no affinity for the unphosphorylated sequence. In this way, SH2 domains mediate protein-protein interactions in response to tyrosine kinase activation at the plasma membrane. Thus, SH2-mediated interaction often results in relocalization of signaling proteins from the cytoplasm to the membrane. Relocalization may result in effector phosphorylation and activation by the receptor-associated tyrosine kinase (e.g., Stat transcription factors) or bring the effector in proximity to its substrate or the next component of the signal transduction cascade (e.g., phospholipase-Cγ or the Ras guanine nucleotide exchange complex, Grb2-SOS). The sections below (V.B. and V.C.) will highlight SH2 effector proteins linking cytokine receptors to some of the major signaling pathways that affect the proliferation and differentiation of hematopoietic cells, as well as the pathways themselves.

B. Ras/Raf/Mitogen-Activated Protein Kinase Pathway

The *ras* proto-oncogenes (N-*ras*, K-*ras*, H-*ras*) encode low molecular weight GTPases that play a central role in growth regulatory signal transduction (referred to collectively as Ras). Ras is active in the GTP-bound form, and returns to the inactive, GDP-bound state via its intrinsic GTPase activity. Both the activation (GDP-GTP exchange) and the inactivation (hydrolysis of GTP) processes are modified by protein factors that often couple Ras to tyrosine kinases and other signaling proteins. Association of Ras with GTP induces a major conformational change in the protein, causing it to expose a region that is responsible for direct interactions with downstream effector molecules, such as Raf. Identification of biologically relevant effectors for Ras is an area of intense investigation and is the subject of several recent reviews (McCormick and Wittinghofer, 1996; Katz and McCormick, 1997).

Ras also requires lipid modification of its C-terminus to be biologically active (Lowy and Willumsen, 1993). These modifications, farnesylation and geranylgeranylation, serve to localize Ras to the cytoplasmic face of the plasma membrane. Addition of the 15 carbon farnesyl group to Ras is required for its ability to transform cells. For this reason, the farnesyl-protein transferase responsible for this modification has emerged as an exciting therapeutic target (Gibbs et al., 1994).

1. Activation of Ras occurs downstream of tyrosine kinases. Ras is an essential downstream component of most tyrosine kinase signal transduction pathways. Stimulation of cells with growth factors or transformation with tyrosine kinase oncogenes induces rapid conversion of Ras to the active, GTP-bound state (reviewed by Satoh et al., 1992a). The requirement for Ras activation downstream of tyrosine kinases was established more than 10 years ago. In these experiments, microinjection of neutralizing Ras antibodies was shown to block cellular proliferation in response to growth factors and tyrosine kinase oncogenes such as v-Src (Smith et al., 1986; Mulcahy et al., 1985). Subsequent development of a dominant-inhibitory mutant of Ras provided another powerful tool to demonstrate the requirement for Ras activation in tyrosine kinase signaling (Cai et al., 1990). These and other strategies have been used to demonstrate the requirement for Ras in many other tyrosine kinase-dependent signal transduction pathways, including those initiated by cytokines.

2. Ras activation is mediated via guanine nucleotide exchange. Studies by many laboratories in recent years have led to a clearer picture of the biochemical events that lead from tyrosine kinase activation to Ras. Ras activity is positively controlled by protein factors that promote GDP-GTP exchange (guanine nucleotide exchange factors or GEFs). Conversely, other factors accelerate the intrinsic GTPase activity of Ras, promoting the termination of the Ras signal (GTPase-activating proteins or GAPs). Both types of Ras regulatory proteins provide a biochemical link between Ras activation and tyrosine kinases and are linked to them via SH2 domains. For example, activated, autophosphorylated growth factor receptors often interact directly with the guanine-nucleotide exchange complex, Grb2/SOS. This complex consists of the guanine-nucleotide exchange activity (SOS) coupled to an adaptor protein (Grb2), which consists of a single SH2 domain flanked by two SH3 domains. The SH3 domains of Grb2 bind to proline-rich sequences within SOS, whereas the Grb2 SH2 domain serves to bind the Grb2/SOS complex to specific autophosphorylated tyrosine residues on the activated growth factor receptor. The net effect of this interaction is to redistribute the exchange activity from the cytoplasm to the plasma membrane, thus placing it in proximity to Ras and promoting the guanine-nucleotide exchange reaction. This basic paradigm for Ras activation has been described for many tyrosine kinase-mediated signaling events (for a review, see Schlessinger, 1993).

3. Shc: regulator of Ras and independent effector. Although many tyrosine kinases are able to activate Ras by directly binding Grb2/SOS via the Grb2 SH2 domain, an important variation on this theme has emerged in cytokine receptor signal transduction and other systems. This pathway to Ras involves an additional adaptor protein known as Shc, which contains an SH2 domain as well as another phosphotyrosine-binding (PTB) domain (reviewed by Bonfini, et al., 1996). Although the PTB domain serves the same function as the SH2 domain, it is structurally distinct, bearing instead a striking similarity in terms of structure to the PH domain (Zhou et al., 1995). Shc is recruited to the tyrosine-phosphorylated receptor, becomes phosphorylated on tyrosine, and then interacts with Grb2/SOS via the Grb2 SH2 domain. Shc may serve to provide an additional level of control over Grb2/SOS interaction, as one study has shown that Shc may regulate the binding of Grb2 to SOS (Ravichandran et al., 1995).

Several studies have shown that Shc is phosphorylated on tyrosine in response to cytokine treatment, suggesting that Shc phosphorylation is an important early event in the signaling response (Matsuguchi et al., 1994; Cutler et al., 1993). As described in the preceding paragraph for growth-factor receptors, tyrosine phosphorylation of Shc can lead to interaction with Grb2/SOS as part of the link between the cytokine receptor and Ras activation. In addition to Grb2, cytokine-induced tyrosine phosphorylation of Shc also leads to its association with a 140 to 150 kDa protein (Liu et al., 1994; Damen et al., 1993) that has been subsequently identified as an inositol 5-phosphatase (Ware et al., 1996). Interaction of Shc with both of these proteins may regulate proliferation, as overexpression of Shc potentiates the proliferative response to GM-CSF, and this enhanced response correlates with increased recruitment of Grb2/SOS and Grb2/p145 complexes to the β_c subunit of the receptor (Lanfrancone et al., 1995).

Shc has also been linked to the ability of IL-3 to promote survival of the pro-B-cell line Ba/F3 (Gotoh et al., 1996). Overexpression of the Shc SH2 domain acted in a dominant-inhibitory fashion to block endogenous Shc phosphorylation and induced apoptosis. Cells overexpressing a Shc mutant lacking the phosphorylation site essential for Grb2 binding (Tyr 317) were unable to activate Ras but retained resistance to apoptosis, suggesting that Shc is linked to the apoptotic pathway in a Ras-independent manner in this cell line. Two additional sites of IL-3-dependent Shc tyrosine phosphorylation were identified in this study: tyrosines 239 and 240. Cells expressing Shc mutants lacking these sites retained sensitivity to apoptosis, despite the ability to activate the Ras pathway. These results provide strong evidence that Shc may represent an important branch point in the signaling pathway from the IL-3 receptor,

with separate downstream effectors driving proliferation and suppressing apoptosis.

Other work suggests that Shc may contribute to differentiation signaling in hematopoietic cells in a Ras-independent manner as well. In a recent study (Hill et al., 1996), the human TPO receptor was introduced into the murine IL-3-dependent cell line, 32Dcl3. The resulting cell population responded to TPO by differentiation to a granulocytic phenotype, and the differentiation response was dominant to the proliferative effect of IL-3. Treatment of the cells with TPO and subsequent differentiation correlated with potent tyrosine phosphorylation of Shc but did not lead to activation of MAPK, which is usually activated downstream of Ras (see Section V.B.5.). Interestingly, a mutant of Shc lacking the phosphotyrosine-binding PTB domain blocked the TPO-mediated differentiation response without affecting IL-3-mediated proliferation in this system. These results suggest that Shc may serve to transmit signals for differentiation in myeloid cells that are independent of the Ras/Raf/MAPK pathway. It will be important to determine whether this effect is shared by other cytokine receptor systems, many of which are linked to the tyrosine phosphorylation of Shc.

4. Ras transmits signals for proliferation in some contexts and differentiation in others. Activation of Ras occurs downstream of many growth factor receptors and tyrosine kinase oncogenes. The same is true for most hematopoietic growth factors and cytokines and is often linked to a proliferative response. For example, earlier work showed that the proliferative response of a murine cytokine-dependent cell line to IL-3 or GM-CSF directly correlates with the accumulation of Ras in its active, GTP-bound state (Satoh et al., 1991). Further work showed that cytokine-dependent Ras activation and the proliferative response were both blocked by the tyrosine kinase inhibitor herbimycin A, demonstrating the requirement for a tyrosine kinase upstream of Ras activation (Satoh et al., 1992b).

Although Ras activation may be essential for proliferative signaling in response to cytokines, the differentiation response may require additional or separate signals under certain conditions. For example, expression of a dominant-negative allele of Ras (N17Ras) in the IL-3-dependent cell line 32Dcl3 completely blocked proliferation in response to IL-3 by inducing cell cycle arrest (Okuda et al., 1994). The cells remained viable, however, despite their inability to grow. However, the N17Ras-expressing cells were still able to differentiate in response to G-CSF either in the presence or absence of IL-3, suggesting that Ras activation does not contribute to the differentiation response in this context.

In contrast to the results of Okuda et al. (1994), other studies suggest that activation of Ras may be necessary and sufficient to induce myeloid differentiation. For example, Katagiri, et al. (1994) have shown that the same dominant-negative mutant of Ras (N17Ras) can block monocytic differentiation of HL-60 leukemia cells in response to phorbol ester. Consistent with this finding is the work of Maher et al. (1996), showing that a constitutively active mutant of Ras (D12Ras) induced marked growth suppression and monocytic differentiation of the human monoblast cell line, U937. However, when the same investigators introduced the activated allele of Ras into the human IL-3-dependent cell line TF-1, a growth factor-independent phenotype was obtained. The resulting TF-1/Ras cells exhibited enhanced growth responsiveness to GM-CSF or erythropoietin, but no evidence of differentiation was seen. Taken together, these results suggest that the effects of Ras activation on proliferation versus differentiation are dependent upon the cell lineage and/or the state of maturation of the cells under study. It is interesting to note that both of the studies cited above (Okuda et al., 1994; Maher et al., 1996) in which activated Ras contributed to proliferation used progenitor cell lines that retained cytokine dependence (TF-1 and 32Dcl3). Similar results have been observed in normal bone marrow cultures after infection with a retrovirus carrying an activated allele of Ras (Maher et al., 1994). In contrast, the leukemia cell lines in which Ras activation contributed to the differentiated phenotype were both cytokine-independent and blocked at a later stage of differentiation (HL-60 and U-937).

5. Ras stimulates the Raf/MEK/mitogen-activated protein kinase pathway downstream. The GTP-bound, active form of Ras adopts a conformation that allows it to bind several downstream target proteins. A growing number of molecules have been identified as potential Ras effectors, including phosphatidylinositol 3′-kinase, p120 Ras GAP, and the Ser/Thr kinase, Raf (for recent reviews of Raf and other Ras effectors, see Morrison and Cutler, 1997; Katz and McCormick, 1997; Marshall, 1996). Raf is currently the best characterized effector for Ras. Raf binds to the GTP-bound, active form of Ras and is translocated from the cytoplasm to the plasma membrane as a result of this interaction.

Several studies have shown that full activation of Raf-1 requires tyrosine phosphorylation in addition to the presence of Ras-GTP (Marais et al., 1995; Jelinek et al., 1996; Fabian et al., 1993). In the case of cytokine receptors, the tyrosine phosphorylation of Raf may be mediated by Jak2 (Xia et al., 1996) or possibly a member of the Src kinase family (Fabian et al., 1993). Once activated, Raf phosphorylates and activates the dual specificity MAP kinase kinases, often referred to as MEKs, which in turn activate the MAP kinases, ERK-1 and ERK-2. Activated ERKs can then translocate into the nucleus and phosphorylate several transcription factors, which influences their activity toward target genes. ERKs also activate several cytoplasmic targets, including other kinases. Details of the regulation of MAPKs and their target proteins are the subject of several recent reviews (Robinson and Cobb, 1997; Treisman,

1996). The relationship between Ras, Raf, MEK, and ERK is shown in figure 3.

The distal portion of the Ras-MAPK pathway may have an important function in transmitting cytokine signals for survival, proliferation and differentiation downstream from Ras in hematopoietic cells. Using the IL-3-dependent cell line 32Dcl3, Cleveland et al. (1994) showed that v-Raf, a retroviral form of Raf that is constitutively active, suppressed apoptosis and promoted the growth of these cells in the absence of IL-3. Similar results have been described more recently in the human cytokine-dependent cell line, TF-1 (Chao et al., 1997) and are consistent with the work in which transfection of TF-1 cells with an activated allele of Ras produced cytokine-independence (Maher et al., 1996). In regard to the suppressive effect of Raf on apoptosis, it is interesting to note that Bcl-2, an important regulator of apoptosis, may interact directly with Raf (Wang et al., 1994, 1996). In contrast to these findings, other work has shown that Raf-1 and MAPK activities were induced during monocytic differentiation of human myeloid leukemia cell lines (Kharbanda et al., 1994). Consistent with this result is the recent observation that constitutively active MEK mutants promote megakaryocytic differentiation of the erythroleukemia cell line, K562 (Whalen et al., 1997). Thus, the Raf-MAPK pathway can promote cell growth and survival in early progenitor cells but may ultimately contribute to the acquisition of the differentiated phenotype.

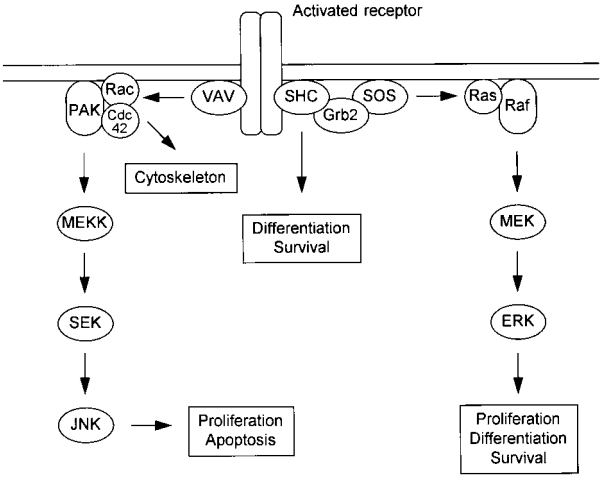

FIG. 3. Activation of small G-protein/MAPK pathways downstream of cytokine receptors. Binding of a cytokine to its receptor induces receptor oligomerization and activation of associated tyrosine kinases (kinases not shown for clarity). Tyrosine phosphorylation of the receptor creates high-affinity binding sites for effector molecules with SH2 domains, including several involved in the regulation of small G-proteins downstream. Recruitment and tyrosine phosphorylation of Shc promotes association with the Grb2/SOS guanine nucleotide exchange complex for Ras. Once activated, Ras can interact with multiple downstream effectors, including the serine/threonine kinase Raf. Activated Raf leads to the activation of ERK via the intermediate dual-specificity kinase MEK. Activated ERKs can translocate to the nucleus and phosphorylate several transcription factors. Depending upon the cellular context, this pathway has been shown to influence proliferation, differentiation and survival in hematopoietic cells. Some studies suggest that Shc may represent a branch point in the signaling pathway, promoting effects on differentiation and survival that are independent of Ras. Many cytokines are also known to induce the tyrosine phosphorylation of Vav, which stimulates its guanine nucleotide exchange activity for the Rho family GTPases, Rac and Cdc42. Activation of these small GTPases directly influences cytoskeletal architecture and stimulates a parallel MAPK pathway to the nucleus. This pathway terminates in the activation of the c-Jun N-terminal kinase (JNK) and involves the kinase intermediates PAK (for p21-activated kinases, which are activated directly by Rac and Cdc42) as well as MEKK and SEK (analogous to Raf and MEK, respectively). Although JNK has been linked to proliferation, apoptosis and the stress response in other systems, its role myeloid growth regulation is just beginning to emerge. Note that the pathway from Vav to Cdc42/Rac/JNK is not an exclusive one; connections between Ras and Rac/Cdc42 as well as crossover between several of the downstream kinases have also been observed.

One important group of targets for MAPK in hematopoietic cells may be the GATA family of transcription factors. Both GATA-1 and GATA-2, which have been shown by gene targeting to be essential for hematopoiesis, are phosphorylated by MAPK (Towatari et al., 1995; Crossley and Orkin, 1994). Stimulation of hematopoietic progenitor cells with IL-3 led to a rapid enhancement of GATA-2 phosphorylation. Using a dominant-interfering mutant of MEK, the phosphorylation event was shown to be dependent on MAPK (Towatari et al., 1995).

C. Other Src Homology 2 Effectors

1. Vav: alternative route to small G-protein activation. The vav proto-oncogene encodes a 95 kDa protein with several motifs associated with growth-regulatory signal transduction. These include two C-terminal SH3 domains flanking a single SH2 domain, as well as a central domain homologous to the Dbl family of guanine-nucleotide exchange factors for small GTPases of the Rho family (reviewed by Cerione and Zheng, 1996). Vav also contains putative nuclear translocation signals, suggesting a nuclear role for this protein as well. Early studies showed that Vav expression is restricted primarily to hematopoietic cells of both myeloid and lymphoid lineages, suggestive of a specific function in hematopoiesis (Katzav et al., 1989). Consistent with this view, Vav tyrosine phosphorylation has been observed in response to several cytokines (Evans et al., 1997; Platanias and Sweet, 1994; Matsuguchi et al., 1995) and in activated T and B-cells (Bustelo and Barbacid, 1992; Bustelo et al., 1992; Margolis et al., 1992). Lymphocytes lacking Vav show significant defects in antigen receptor signaling (Fischer et al., 1995; Tarakhovsky et al., 1995), confirming an important role for Vav in lymphocyte maturation and antigen responsiveness.

The role of Vav in myeloid differentiation is more controversial. Vav was not required for the generation of erythroid or myeloid lineages when assessed by in vitro colony assay of *vav*-deficient embryonic stem cells (Zmuidzinas et al., 1995; Zhang et al., 1994). However, suppression of Vav expression in embryonic stem cells with Vav antisense vectors did affect myeloid development in vitro (Wulf et al., 1993). These conflicting outcomes may be partially explained by the recent discovery of a second *vav*-related gene (*vav*-2), which may

serve a partially redundant function in the development of myeloid cells (Henske et al., 1995). Whereas Vav-2 expression would not be influenced in the Vav knockout cells, the antisense RNA could suppress expression of Vav, Vav-2, and possibly other undiscovered homologs of this gene family, leading to the observed effects on myelopoiesis.

The specific biochemical function of Vav is also somewhat controversial. Initial reports suggested that Vav acts as a guanine nucleotide exchange factor for Ras (analogous to the Grb2/SOS complex described in Section V.B.2.) despite its closer sequence homology to Dbl and other exchange factors for Rho-family GTPases (Gulbins et al., 1993, 1994a,b). However, more recent work did not reproduce these findings and showed that the morphology of Vav-transformed fibroblasts is quite distinct from that of Ras-transformed cells (Khosravi-Far et al., 1994; Bustelo et al., 1994). The presence of a Dbl-homology domain in Vav suggests that exchange activity toward the Rho family of GTPases is a more likely possibility. The Rho family of GTPases regulates both cytoskeletal architecture as well as a parallel MAPK pathway to the nucleus, which terminates in the activation of the c-Jun N-terminal kinase (JNK; reviewed by Vojtek and Cooper, 1995; Tapon and Hall, 1997). Two recent reports have shown that tyrosine phosphorylation of Vav stimulates its exchange activity for the Rho family members Rac and Cdc42 (but not Ras); this effect correlated with the activation of JNK downstream (Han et al., 1997; Crespo et al., 1997). These results suggest that a Ras-independent signaling pathway may exist, linking multiple cytokine receptors (as well as lymphocyte antigen receptors) to the Rho/JNK cascade via tyrosine phosphorylation of Vav (fig. 3). Interestingly, a recent study has shown that EPO, TPO, and IL-3 can induce JNK activation (Nagata et al., 1997). The contribution of the Rho family GTPases and their downstream effectors to myeloid growth, survival and differentiation is an important area for future investigation.

2. Stat transcription factors. In addition to Ras and related proteins, a second major pathway has emerged in recent years as a critical part of cytokine signal transduction. This pathway involves a group transcription factors with SH2 domains that are directly activated by tyrosine phosphorylation. Collectively termed Stats (for signal transducers and activators of transcription), these transcription factors are activated by essentially all known cytokines as well as some growth factors. The current model of Stat activation involves recruitment of the monomeric factor to an activated, tyrosine-phosphorylated cytokine receptor by the Stat SH2 domain. The Stat is then phosphorylated by activated tyrosine kinases associated with the receptor (e.g., Jak kinases, see Section IV.A.). Tyrosine phosphorylation induces Stat dimerization, release from the receptor, nuclear translocation, binding to specific DNA elements and transcriptional activation. An explosion of research has occurred in the Jak-Stat field and is the subject of many comprehensive reviews (Watanabe and Arai, 1996; Ihle, 1996; Schindler and Darnell, 1995; Ihle et al., 1995). Recent data implicating Stat factors in myeloid differentiation will be the focus of this section.

Some of the most striking evidence implicating Stat signaling in differentiation comes from studies of IL-6, a multifunctional cytokine that induces macrophage differentiation in the murine myeloid leukemia cell line, M1. As described above, the receptor for IL-6 shares the gp130 signal-transducing subunit with LIF, CNTF, OSM, IL-11 and CT-1. Several Jak kinases constitutively associate with gp130, and their activation in response to IL-6 leads to the activation of Stat1 and Stat3. (For a review of IL-6/gp130 signaling, see Hibi et al., 1996.) Using a panel of chimeric growth hormone (GH) receptor/gp130 chimeras, the region of gp130 required for the activation of Stat3 was shown to be essential for the generation of signals for growth arrest and macrophage differentiation (Yamanaka et al., 1996). These biological effects correlated with down-regulation of c-*myc* and c-*myb*, which is required for differentiation to occur. Another significant aspect of this study is that it showed that homodimerization of gp130 alone is sufficient to generate all of the signals produced by IL-6, implying that the unique component of the IL-6 receptor complex functions solely to confer binding affinity and specificity and does not contribute to downstream signaling.

More direct evidence implicating Stat3 in myeloid differentiation comes from studies with dominant-negative mutants of Stat3 (Nakajima et al., 1996). These mutants were produced by substitution of the Stat3 tyrosine phosphorylation site with phenylalanine or by mutation of residues critical for DNA binding. When these mutants were introduced into M1 myeloid leukemia cells, the cells no longer responded to IL-6 with growth arrest and terminal differentiation. In addition, suppression of endogenous Stat3 activation by this mutant also inhibited IL-6-induced repression of c-*myc* and c-*myb* gene expression. Another striking feature of this study is that M1 cells transfected with Stat3 dominant-negatives demonstrated enhanced growth in response to IL-6 treatment. This finding suggests that the receptor is able to generate independent signals for proliferation and differentiation and that the differentiation signal is dominant, at least in this model system. In a related study, similar dominant-negative mutants of Stat3 were shown to block macrophage differentiation of M1 cells in response to both IL-6 and LIF (Minami et al., 1996). This result suggests that differentiation-related signals from both cytokines are transmitted through the shared gp130 subunit of their respective receptor complexes.

A final aspect of the Stat3 signaling pathway concerns the identity of the tyrosine kinase that is responsible for the differentiation effect. Although activation of Stat3 by

Jak kinases is likely to occur, a case can be made for the contribution of other kinases as well. For example, activation of Fps/Fes tyrosine kinases has been shown to induce terminal differentiation in several myeloid contexts (see Section IV.C.). A recent report has shown that treatment of mature granulocytes with GM-CSF leads to the rapid activation of both Jak2 and Fes, leading to the tyrosine phosphorylation of Stat1 and Stat3 (Brizzi et al., 1996). In addition, we have observed that c-Fes potently activates Stat3 when the proteins are coexpressed in the human cell line, 293T (Nelson et al., 1997). Fes also activated Stat3 after coexpression in Sf-9 insect cells, strongly suggesting that the activation mechanism is direct and does not require activation of Jaks or other endogenous tyrosine kinases. Because Fes has been linked to signal transduction by IL-6 and GM-CSF, these results suggest that Fes may contribute to Stat3 activation as part of a differentiation signaling cascade.

In addition to Stat3, a strong correlation has been established between the activation of Stat5 and differentiation of myeloid leukemia cell lines (Woldman et al., 1997). In these experiments, induction of monocytic differentiation in human U937 cells with phorbol ester, retinoic acid, or 1α,25-dihydroxy-vitamin D_3 induced strong DNA-binding activity of Stat5; similar results were observed after chemically-induced differentiation of HL-60 promyelocytic leukemia cells. Although supershift analysis provided strong evidence for the activation of Stat5 in these studies, tyrosine phosphorylation of Stat5 was not reported. In the same study, Stat5 activation was also observed in primary cultures of chicken myeloblasts after differentiation to macrophages with chicken myelomonocytic growth factor. (The biological activity of this chicken cytokine resembles that of mammalian GM-CSF and CSF-1.) In this case, potent tyrosine phosphorylation of chicken Stat5 was observed. Although these results implicate Stat5 in some CSF-mediated differentiation responses, other work has clearly established a role for Stat5 in CSF-induced proliferation (Mui et al., 1996). Further work will be required to understand why Stat5 may contribute to differentiation in some cellular contexts and proliferation in others.

An important question in the area of Stats and myeloid differentiation concerns the identification of target genes that are ultimately responsible for growth arrest and differentiation. Treatment of M1 cells with IL-6, which leads to Stat3-dependent differentiation as described earlier in this section, has been shown to modulate several pathways directly linked to cell-cycle progression, including suppression of Rb phosphorylation (Resnitzky et al., 1992) and E2F DNA-binding activity (Melamed et al., 1993), induction of the cyclin-dependent kinase inhibitor p21/WAF-1/CIP-1 (Steinman et al., 1994), and suppression of G_1 cyclin levels (Levy et al., 1994). It will be of interest to determine whether these cell-cycle related genes are directly affected by Stat3 or other Stat factors as a function of differentiation.

3. Terminating the signal: hematopoietic cell phosphatase. Negative regulation of the tyrosine phosphorylation-dependent signals generated by activated cytokine receptors involves the hematopoietic cell protein-tyrosine phosphatase, (HCP; also known as SH-PTP1; reviewed by Tonks and Neel, 1996). HCP consists of two N-terminal SH2 domains and a C-terminal catalytic domain. The essential role for HCP in the negative control of hematopoiesis is dramatically evident in the phenotype of motheaten, a naturally occurring knockout of the murine HCP locus (Tsui et al., 1993; Shultz et al., 1993). Motheaten mice die within several weeks of birth and exhibit overproduction of macrophages, EPO-independent erythropoiesis, and excessive proliferation of lymphoid lineages (Van Zant and Shultz, 1989).

Direct interaction with HCP has been demonstrated for several hematopoietic growth factor receptors, including those for SCF, EPO, and IL-3 (Klingmüller et al., 1995; Yi et al., 1995; Yi and Ihle, 1993). Recruitment of HCP to the activated receptor complex involves the SH2 domains of HCP and tyrosine phosphorylation sites on the receptor. In the case of the EPO-R, HCP is required for dephosphorylation and inactivation of Jak2. The HCP SH2 domain binding site falls within a receptor region that is deleted in a form of inherited erythrocytosis (Klingmüller et al., 1995). Thus, the loss of the SH2 docking sites for HCP results in a persistent signal for erythropoiesis. Other studies in which mice bearing kinase-defective alleles of c-*kit* (the SCF receptor) were crossed with *motheaten* heterozygotes clearly indicate that HCP negatively regulates SCF signaling and show that excessive signaling from Kit contributes significantly to the *motheaten* phenotype (Paulson et al., 1996; Lorenz et al., 1996).

VI. Phorbol Esters and Protein Kinase C

The focus of this review so far has been on the factors that regulate myeloid growth and differentiation under physiological conditions. In addition to cytokines and other hematopoietic growth factors, many pharmacological agents have been shown to produce potent effects on differentiation, especially of myeloid leukemia cell lines. One of the first examples of this was the observation of granulocytic differentiation in HL-60 promyelocytic leukemia cells (Collins et al., 1977) after treatment with the organic solvent dimethylsulfoxide (Collins et al., 1978) or with retinoic acid (Breitman et al., 1980). These findings were highly significant because they suggested that the differentiation block associated with leukemia could be reversed with the appropriate pharmacological agent. Such a therapeutic strategy represents an attractive alternative to the use of more conventional cytotoxic chemotherapy. The promise of this original finding has been realized to some extent in the use of all-trans-

retinoic acid for the treatment of acute promyelocytic leukemia (Degos et al., 1995). Other pharmacological agents with differentiation-inducing activity have led to the discovery of signaling pathways that may contribute to physiological control of differentiation. One important group of compounds in this regard are the phorbol esters, which are discussed in more detail in this section.

Phorbol esters such as phorbol 12-myristate 13-acetate (PMA) are potent inducers of differentiation in several myeloid leukemia cell lines. The promyelocytic leukemia cell line HL-60 differentiates into macrophages in response to the PMA treatment (Rovera et al., 1979; Huberman and Callaham, 1979), as do the monocytic cell lines THP-1 and U-937 (Ways et al., 1987). Interestingly, the more immature progenitor line K-562 differentiates along the megakaryocytic pathway in response to PMA (Koeffler et al., 1981). These effects of phorbol esters are mediated by protein kinase C.

PMA and other phorbol esters are believed to mimic the effects of diacylglycerol, which is produced under physiological conditions by the actions of membrane-associated phospholipases (reviewed by Lee and Rhee, 1995). Several major classes of phospholipases are known. These include the β-isoforms, which are coupled to G-protein-linked receptors, as well as members of the γ-class, which contain SH2 domains and are activated downstream of protein-tyrosine kinases. The downstream targets of diacylglycerol and phorbol esters are members of a large family of Ser/Thr protein-kinases known collectively as protein kinase C (PKC; reviewed by Jaken, 1996; Newton, 1995). Binding of these lipid activators to the N-terminal regulatory region of PKC induces significant conformational changes that are responsible for translocation of PKC to cellular membranes and activation of the kinase. After targeting to membranes and activation, PKC is proteolytically cleaved and rapidly cleared from the cell.

Many studies have correlated the expression of various isozymes of PKC with PMA-induced differentiation of HL-60 cells (Tonetti et al., 1994, and references cited therein). Other work has extended these observations to show that PKC activation is required for differentiation in response to phorbol ester treatment. For example, a variant of HL-60 cells has been described that lacks the expression of PKC-β and is resistant to differentiation by PMA but not by other inducers that stimulate alternative pathways (Tonetti et al., 1992; Homma et al., 1986). Differentiation competence can be restored in these cells by pretreatment with agents that induce PKC-β expression (Yang et al., 1994) or by transfection of the cells with a PKC-β expression vector (Tonetti et al., 1994).

More recent studies by Rossi, et al. (1996) suggest that the level of PKC activation may control lineage determination. These studies employed avian multipotential progenitor cells transformed with the E26 avian leukemia virus (Myb-Ets-transformed progenitors or MEPs) (Graf et al., 1992). MEPs can give rise to multiple myeloid lineages, some of which can be selectively determined by PMA in a dose-dependent manner. These effects can be attributed to differential activation of PKC. High concentrations of PMA (100 nM) cause a dramatic down-regulation of PKC activity and induce a myelomonocytic phenotype. Thus the effect of a high concentration of PMA is actually less efficient PKC signaling overall, because of relocalization and proteolysis. Similarly, very low concentrations of PMA (1 nM) also induce myelomonocytic differentiation. As with very high concentrations, low PMA levels result in a low but constant amount of PKC activity. In contrast, intermediate concentrations of PMA (20 nM) that permit sustained activation of PKC without down-regulation led to eosinophilic differentiation. Experiments with MEPs transfected with PKC isozymes also support this model (Rossi et al., 1996). In this case, the exogenous PKC is not susceptible to down-regulation. In the absence of PMA treatment, low basal PKC activity is now sufficient in the transfected cells to drive myelomonocytic differentiation. Addition of intermediate (20 nM) or high (100 nM) concentrations of PMA drives eosinophilic differentiation, because a high sustained level of PKC activity is maintained in the absence of down-regulation. These results imply that thresholds of PKC activity exist that control lineage determination. Below the first threshold, differentiation is not observed. Above the first threshold, myelomonocytic differentiation is favored, whereas levels above the second threshold push the cells toward the eosinophilic phenotype.

The MEPs system has also offered some insight into the signaling pathways in which PKC may participate. One possibility is that PKC isozymes activate Raf-1, a known target for PKC in murine hematopoietic cells (Carroll and May, 1994). Consistent with this finding is the observation that both v-Raf and v-Ras are able to induce MEP cell differentiation (Graf et al., 1992). As described in Section V.B.5., Ras activates Raf, which in turn activates the MAPK pathway downstream. Relevant targets for this pathway may include GATA-1 and C/EBPβ, two lineage-specific transcription factors that are essential for normal hematopoietic differentiation (Orkin, 1995, 1996). GATA-1 is highly expressed in the MEPs and to some degree in eosinophils but not in myeloblasts. In contrast, C/EBPβ is expressed in myeloblasts and eosinophils, but not in MEPs. Interestingly, treatment of MEPs with PMA induces down-regulation of GATA-1 and rapid induction of C/EBPβ (Kulessa et al., 1995), consistent with determination of the eosinophilic lineage. Importantly, C/EBPβ is dependent upon MAPK for nuclear translocation and transcriptional activation (Kowenz-Leutz et al., 1994; Katz et al., 1993). Thus, these and other hematopoietic transcription factors may represent convergent endpoints for differentiation signaling pathways that activate PKC as well as Ras.

VII. Conclusions

The work reviewed here provides a glimpse into the complex network of signaling pathways that regulate the growth, differentiation, and function of hematopoietic cells. Despite the vast amount of information that has been reported in recent years regarding these and related signaling networks, many challenges remain. Discovery of novel signal transduction molecules will continue to be an important goal. As additional components and biologically relevant partnerships are described, the next challenge will be to understand how multiple signaling pathways act together to produce a given biological fate—e.g., growth, differentiation, or apoptosis. Current biochemical approaches for understanding signal transduction often require a somewhat reductionist view of the process, studying one or a few protein-protein interactions, phosphorylation reactions, or other signal transduction events in isolation. However, one theme that is clear from the work cited here is that cytokine receptors can activate multiple signaling pathways simultaneously. The next level of investigation will require development of new approaches to observe multiple signaling events over time to understand how they are integrated into a single biological response. Such an approach may provide new clues to the enigmatic question of how the same signal transduction pathways can produce seemingly opposite responses in different target cells.

Acknowledgments. Work from the author's laboratory is supported by grants from the National Institutes of Health (CA-58667), the American Cancer Society (BE-245), and the Nebraska Department of Health.

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