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Recent Advances in Research on Fibronectin and Other Cell Attachment Proteins

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Fibronectin and other cell attachment proteins provide molecular models for beginning to unravel the complex interactions of the cell surface with the extracellular matrix. This area has been reviewed in considerable detail previously [1–10]. Our brief review will therefore be selective rather than comprehensive, and it will focus on some recent generalizations about this class of proteins, as well as on recent advances in the molecular analysis of the functions of these proteins and their receptors. We shall also present various popular or provocative hypotheses and speculations about future work in the field.

CLASSES AND SPECIFICITY OF ATTACHMENT PROTEINS

An emerging generalization about this class of proteins is that they are composed of separable functional regions, each specialized for specific binding activities (Fig. 1). Each appears to have one or more regions essential for binding to the cell surface. Fibronectin (Fig. 2) interacts with the fibroblast cell surface primarily through a region termed the cell-binding or cell-recognition site; recent molecular analysis of the function of this site will be discussed below. Although this site appears to be required for cell interactions with fibronectin [11,12], interactions at a heparin-binding domain may provide substantial strengthening of this interaction [13]. In addition, neuronal cells may be capable of interacting with a heparin-binding domain elsewhere in the molecule in the process of axonal elongation; fibronectin-independent interactions with extracellular materials such as heparan-sulfate-containing molecules appear to be important in some aspects of this process [14,15].

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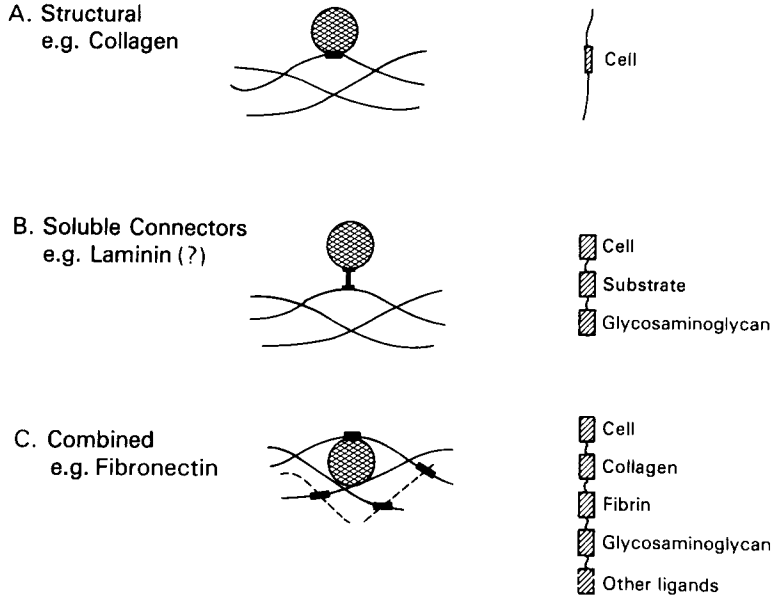

Fig. 1. Classes of cell attachment proteins. A provisional classification of molecules mediating cell interactions with extracellular materials is shown. Class A involves direct interactions between the plasma membrane and a structural molecule such as collagen. There are specific sites on the collagen that interact with cells. Class B postulates soluble intermediary or connector molecules, which contain sites for binding to the plasma membrane of cells and for binding to a substrate molecule, eg, to a collagen. Attachment proteins often contain a third site for binding to glycosaminoglycans and/or proteoglycans. Class C postulates a molecule that is both structural and a cross-linker. For example, fibronectin can be the major structural component of matrices, yet can also mediate a series of cross-linking and binding interactions.

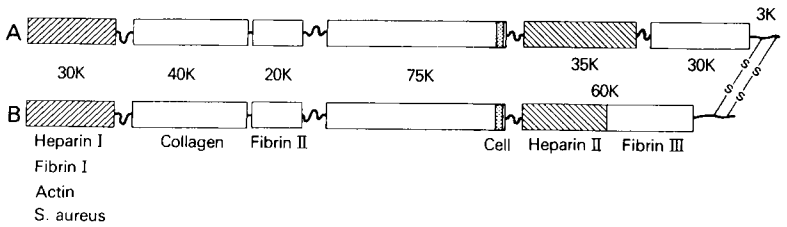

Fig. 2. Functional domain structure of fibronectin. Fibronectin is a molecule composed of two or more disulfide-linked subunits. Each chain contains a similar linear sequence of modular domains, which can be separated by proteolytic cleavage. The apparent sizes of these domains in human plasma fibronectin are indicated by the numbers (K = kilodaltons). The ligands that they bind are indicated at the bottom. Note that there are two domains for binding to heparin and three for fibrin, which appear to differ in affinity.

Cells interact with proteolytic fragments of laminin originating from the center of the molecule, but also with more peripheral fragments containing globular regions [16] (Fig. 3). The cell-interaction site of laminin may therefore be less well-defined than in fibronectin; more studies are needed to define the exact locus of this activity. As for fibronectin, laminin may also interact most effectively with neuronal cells by means of a heparin-binding domain at the end of the long arm of the molecule [16a].

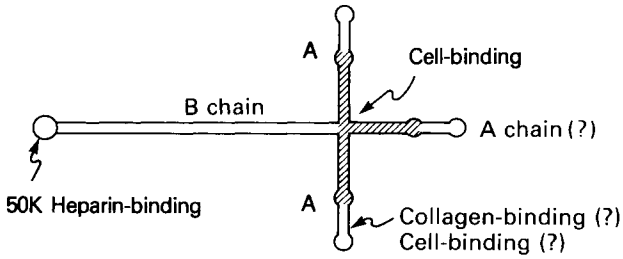

Fig. 3. Overall structure and functional organization of laminin. Laminin is a cruciform glycoprotein 1 million daltons in size, composed of two types of chain, termed A and B. There are two or three A chains and one B chain linked by disulfide bonds. A 50,000-dalton, globular heparin-binding domain exists at the end of the long arm of the cross-shaped molecule. Cell-binding and collagen-binding regions have been postulated to exist in the short-arm region. It is not yet certain whether the B chain extends the entire length of the protein or whether the central short arm is a distinct A chain.

The plasma protein termed serum spreading factor or vitronectin also appears to contain a localized cell-binding domain, which is located near the amino terminus of the protein [16b].

Cell-attachment proteins are also thought to contain at least one additional domain involved in attachment to another extracellular molecule. For example, fibronectin contains domains for binding to collagen and fibrin (Fig. 2) (see reviews listed in the Introduction) and laminin is postulated to contain binding regions for type IV collagen at the ends of the short arms [17,18] (Fig. 3). It will be important to determine whether collagen-binding or other types of specific substrate-binding domains are present in all attachment factors, eg, in serum spreading factor.

Finally, a striking general finding has been the presence of one or more glycosaminoglycan-binding sites in these molecules (Fig. 1). Fibronectin contains two heparin-binding sites in these molecules (Fig. 1). Fibronectin contains two heparin-binding domains, each of which can function under physiological conditions [9,10] (Fig. 2). Laminin contains a globular heparin-binding region at the end of the long arm [19] (Fig. 3). Serum spreading factor (vitronectin) also binds to heparin, although native molecules may only bind well at a non-physiological, slightly acidic pH; this binding activity has been used to affinity purify this protein [20,21]. Finally, chondronectin is reported to bind to chondroitin sulfate [22].

The function of these glycosaminoglycan-binding sites on attachment proteins is not known with certainty; nor is it known whether all attachment proteins will contain similar sites. One speculation is that heparin-binding sites serve to increase the affinity of the interaction of these molecules with the cell surface by binding to heparan sulfate proteoglycan in the plasma membrane. Another possibility is that an intrinsic part of the function of these proteins is to interact with extracellular glycosaminoglycans (proteoglycans), just as they bind to collagen or other non-proteoglycan structural proteins.

FUNCTIONAL ANALYSIS

These functional domains in attachment proteins are thought to be used in different combinations to perform different functions. The interaction analyzed in greatest detail is fibronectin-mediated attachment of fibroblasts to a collagen substrate

coated onto a tissue culture dish. A large monomeric fragment of fibronectin remains able to mediate cell attachment to collagen, but the separated cell- and collagen-binding domains cannot [11,23]. This model requires further testing, ie, by examining whether the isolated fragments can be recombined to restore attachment activity.

One approach to creating such recombinant protein molecules is shown in Figure 4. Purified fragments are derivatized covalently with biotin using its N-hydroxysuccinimide ester, which covalently links biotin to amino groups in the proteins. Ideally, there should be only one such biotin molecule per fragment. The biotin-derivatized molecules can then be cross-linked by avidin, which binds with extremely high affinity to biotin.

An experiment demonstrating the feasibility of this method is shown in Figure 5. Controls, including a mixture of biotin-derivatized cell- and collagen-binding fragments without avidin cross-linking, show little attachment activity. After they are linked together to form a complex by avidin, the isolated fragments can once again mediate cell attachment to collagen (Fig. 5). In other, more preliminary experiments, this method was found to depend heavily on the ability to produce fragments containing only limited amounts of covalently attached biotin; presumably, an excess of biotin labeling can lead to excessive cross-linking of fragments. This approach may eventually prove useful for creating a series of other recombinations, even between domains isolated from different adhesion proteins, in order to test the role of cooperative interactions between each of the functional domains.

SPECIFICITY OF ADHESIVE PROTEIN FUNCTION

The degrees of specificity of different attachment proteins for different cell types remains to be established comprehensively. In general, however, there is as yet surprisingly little evidence that any given normal cell type requires any specific attachment protein. For example, fibroblasts have now been reported to interact directly with a number of attachment molecules (Fig. 6), including fibronectin [1–10], collagen [24,25], laminin [26,27], and serum spreading factor [20,21,28]. This concept of a lack of absolute specificity should be tempered by the likelihood of differences in affinity for different attachment proteins [eg, 26]. In fact, laminin has been reported to have a deleterious effect on replication of some fibroblasts [29]. Nevertheless, absolute specificity for only one attachment protein does not appear to exist for fibroblasts.

Normal, untransformed epithelial cells also appear to lack a requirement for interaction with only one attachment protein such as laminin (Fig. 6). For example, epidermal cells can attach and spread on substrates of laminin, fibronectin, and epibolin [30]. Hepatocytes attach readily to fibronectin, laminin, and collagen [31,32]. Endothelial cells attach to laminin and fibronectin [33,34]. Finally, corneal epithelial cells appear to bind laminin and at least two types of collagen directly to their cell surfaces [35]. Quantitative specificity may exist, however, for certain epithelial tumor cells that express unusually large numbers of functional laminin receptors on the plasma membrane, some of these lines attach much less effectively to fibronectin [36]. In general, however, epithelial cells also do not appear to have an absolute requirement for only one attachment protein such as laminin.

This lack of absolute specificity for a specific attachment protein suggests that cells are capable of interacting with multiple factors, and that a crucial parameter,

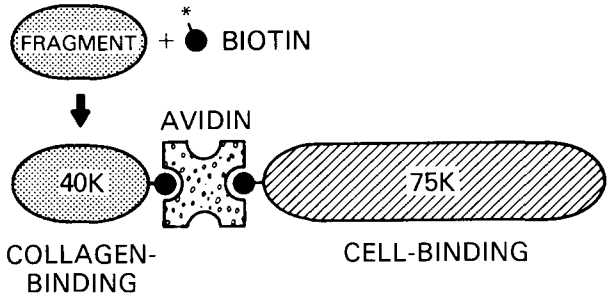

Fig. 4. A general method for producing recombinant protein molecules. Polypeptides, eg, polypeptide fragments of fibronectin, are derivatized by biotin using the N-hydroxysuccinimide ester of biotin. Fragments such as the 40,000-dalton collagen-binding domain and the 75,000-dalton cell-binding domain can then be cross-linked into a complex using avidin.

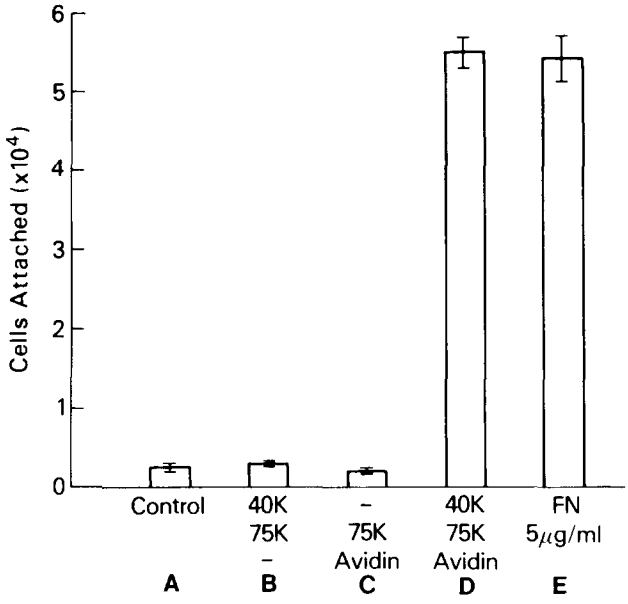

Fig. 5. Reconstitution of cell-to-collagen binding activity using the biotin-avidin system. Collagen-binding (40K) and cell-binding (75K) fragments of fibronectin were derivatized with biotin, then assayed for activity in mediating attachment of Chinese hamster ovary (CHO) cells to type I collagen spread as a substrate on 35-mm plastic petri dishes (Falcon). A) The control received no added proteins. B) A mixture of 10 μg/ml of 40K collagen-binding domain and 20 μg/ml 75K cell-binding domain. C) The cell-binding domain (20 μg/ml) cross-linked with 15 μg/ml avidin in the absence of collagen-binding domain; in other experiments, the collagen-binding domain plus avidin was also devoid of cell-attachment activity (data not shown). D) A mixture of 10 μg/ml collagen-binding domain, 20 μg/ml cell-binding domain, and 15 μg/ml avidin. E) Intact fibronectin at 5 μg/ml. In this particular experiment, the collagen-binding domain was bound first to the collagen, followed by incubation with the avidin, then with the cell-binding fragment. Similar reconstitution is obtained if all components are mixed together first, although the specific activity appears to be slightly lower (unpublished data).

therefore, is quantitative regulation by the organism of amounts of an attachment protein in the local microenvironment of a cell. Although additional contributions from regulation of the quantities of receptors for different attachment proteins is also likely, the most crucial regulatory event may be the rate of biosynthesis and deposition of attachment proteins at specific sites. Future research should elucidate the relative importance of these factors and how regulation of the synthesis and controlled deposition of matrix proteins occurs.

THE FIBRONECTIN GENE AND ITS PROTEIN PRODUCTS

Recombinant DNA clones corresponding to the fibronectin gene and its mRNA have been characterized from several species [37–44]. Analysis of recombinant genomic DNA clones spanning the chicken fibronectin gene reveals an enormous, highly complex gene: there are at least 48 exons (coding regions as determined by R-loop analysis) covering 48 kilobases [38] (Fig. 7). The exons are generally small and similar in size, averaging 150 base pairs in length. This size of coding unit closely matches the size of two of the three repeating homologous units identified within the known protein sequence of fibronectin [45] (Fig. 8). Another type of homologous unit in the protein structure is exactly twice the size of the average exon, suggesting that many of the latter repeating structures may often be encoded by two exons each (compare [38] with [40,41], and [44], as well as Figs. 7, 8). This prediction is readily testable by sequencing of the gene, which is under way in several laboratories, especially those with rat and human clones [40,41].

Earlier work had indicated the presence of apparent polypeptide differences between the various protein subunits of fibronectin [46–51]. Specifically, protease-mapping studies revealed puzzling differences at an internal polypeptide site located near the carboxy terminus of the two subunits of plasma fibronectin [46–48], and even more striking differences in biological activity and subunit structure between the cellular and plasma forms of fibronectin [49–52]. One study identified as many as three difference regions between cellular and plasma fibronectins [50]. Another study was able to obtain a monoclonal antibody that recognized a quantitative difference in immunological reactivity to a site near the carboxy terminus [51]. Nevertheless, detailed immunological and peptide-mapping comparisons of cellular and plasma fibronectins from different species show strong similarities within a species and considerable evolutionary drift between species [53] (but see also [54]). These peptide analysis data have been interpreted as indicating that the two major forms of fibronectin are derived from only one gene [53].

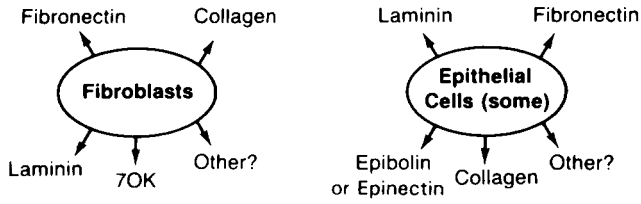

Fig. 6. Multiple interactions at the cell surface. Fibroblasts and epithelial cells have been reported to interact with a number of attachment proteins as indicated. See the text for discussion.

Analysis of genomic DNA by Southern hybridization provides independent evidence for the existence of only one gene for fibronectin [40, 44]. Although this conclusion will remain tentative until clones from everywhere in the gene are tested, the present combination of DNA and protein information argues strongly for the existence of only one functional gene (although highly diverged genes producing somewhat related products might still exist).

Recent cDNA cloning studies have demonstrated the existence of more than one mRNA that encodes fibronectin [41–44]. These findings can explain the existence of more than one fibronectin subunit. Since there is probably only one fibronectin gene, the simplest interpretation is that cells can differentially process the fibronectin mRNA precursor to produce distinctive mRNA species [41–44]. There appear to be at least two mechanisms for generating this mRNA diversity (Fig. 9).

One simple mechanism for differential splicing is to remove an entire exon encoding a difference region (Fig. 9: I). In fibronectin, one mRNA species corre-

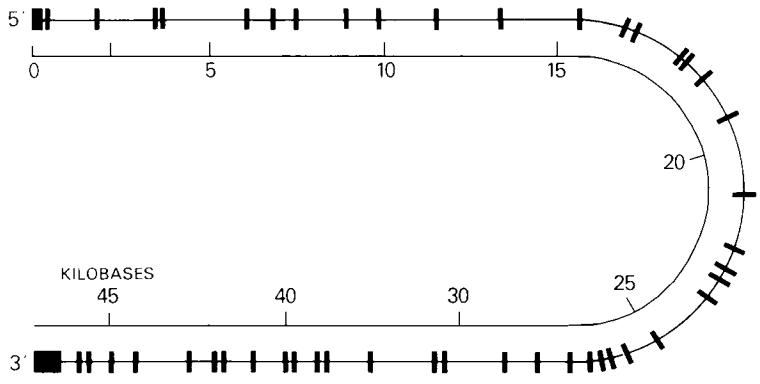

Fig. 7. Structure of the fibronectin gene. The overall organization of the chicken fibronectin gene is depicted schematically. The coding regions (exons—indicated by the heavy vertical bars) and intervening sequences (introns—indicated by the thin horizontal line) are indicated schematically based on R-loop analyses. See text and reference 38 for discussion.

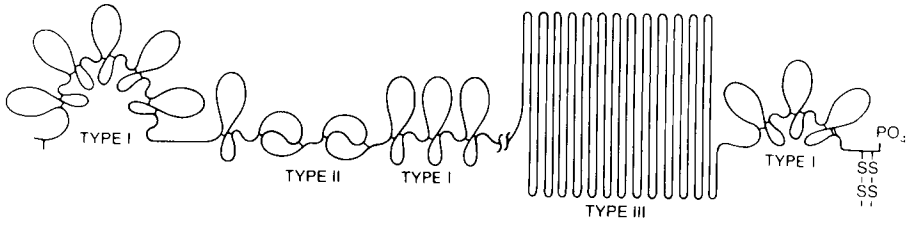

Fig. 8. Current model of the structure of fibronectin. The three types of homologous repeating unit in the amino acid sequence of fibronectin are depicted as loop structures based on both protein and DNA-sequencing data [40–43,45]. Type I units contain at least two intrachain disulfide bonds linking a double-loop structure of roughly 4,500 to 5,000 daltons. The type II unit has been identified as yet only in the collagen-binding domain, and also contains two intrachain disulfide bonds. Type III structures are double the size of the other two types, and they exist without intrachain disulfide bonds. There is a phosphate group close to the carboxy terminus of bovine plasma fibronectin. Other phosphate groups may exist in other fibronectins, but they have not been mapped precisely to date. Sequencing of the molecule is not complete, so it is not possible to rule out the existence of other types of homologous units to date.

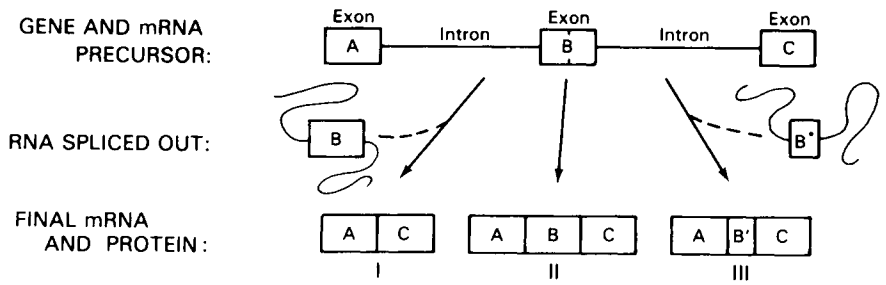

Fig. 9. Possible mechanisms for producing different fibronectin mRNA molecules from one gene. The gene would contain all of the possible coding information, of which only three exons are shown. Simple processing, in which only introns are removed, would yield the final product shown in II, with all three coding regions present in a mRNA and its protein product. Splicing out of exon B would yield the truncated mRNA and protein shown in I. An alternative splicing mechanism in which only part of an exon is removed yields the unusual mRNA shown in III, in which exon B is split into a portion that is spliced out (B*) and the remainder that is retained in the final mature mRNA (B'). See text and references 41–44 for discussion.

sponding to a type III homology unit is present in a cellular fibronectin and absent from plasma fibronectin from the liver [42,43] (mechanism I versus II in Fig. 9). This intriguing "extra domain" contains a number of hydrophilic amino acids whose function is as yet unknown.

A second, fascinating mechanism that has been discovered involves alternative splicing within an exon, so that only part of the exon is present in one or more messages [41] (Fig. 9, III). This type of splicing mechanism has been reported for liver mRNAs, suggesting that the previously described differences in polypeptide structure between chains are due to such highly unusual intraexonic splicing differences [41,44].

These two alternative splicing mechanisms provide distinct ways in which to produce distinct proteins from the same gene. Since other difference regions may occur to differentiate cellular from plasma fibronectins [50], it is possible that a whole set of alternative splicing sites may exist, and that one fibronectin gene might produce a number of related, but structurally and functionally unique, polypeptides. A major challenge for the future will be to determine the biological consequences of these difference regions.

RECEPTOR FOR CELL ATTACHMENT PROTEINS

Cell adhesion proteins must interact with the plasma membrane in some manner to mediate adhesive interactions. They could act by being intrinsic membrane proteins that aggregate with similar proteins on other cells [eg, 55] or that bind to the extracellular matrix [eg, 56,57]. Alternatively, they could be soluble proteins binding to intrinsic membrane protein receptors. Although the simplest model is that there is only one type of receptor for each attachment protein, the possibility of several types of receptor for one attachment protein remains, especially for proteins such as fibronectin that can interact with many different types of cells with relatively low affinity. Conversely, it is even possible that several extracellular molecules could share the same receptor, although they would probably interact with differing affini-

ties. In fact, recent experiments with a synthetic peptide inhibitor suggest that a shared platelet receptor mechanism may be involved in the binding of fibrinogen, fibronectin, and von Willebrand factor [58]. Similar overlapping receptor functions may also exist for fibroblast interactions with fibronectin, serum spreading factor, and collagen.

THE LAMININ RECEPTOR

A disulfide-linked glycoprotein complex containing subunits with apparent molecular weight 68,000 has been identified in carcinoma and sarcoma cells, as well as in myoblasts and muscle tissue [59–61]. This protein is reported to bind laminin with high affinity ($K_D = 2 \times 10^{-9}$M). This putative receptor can be isolated by affinity chromatography on laminin-agarose columns, and its activity can be reconstituted on nitrocellulose filters or in liposomes [59–61]. Monoclonal antibodies against this receptor block the attachment of carcinoma cells to laminin, suggesting that it is crucial for cell interactions with laminin [62]. Preliminary evidence suggests that this protein may also be a transmembrane molecule that interacts with actin [63], although the data are as yet only very circumstantial, since it has not yet been shown that the receptor and actin do not undergo mutual non-specific interactions; more rigorous tests of this intriguing hypothesis in the future will be of considerable interest. It will also be important to determine whether all cells that attach to laminin use the same type of receptor, and whether receptor numbers are regulated during differentiation and as cells become malignant.

THE FIBRONECTIN RECEPTOR

The mechanism by which fibronectin binds to cells remains obscure. The affinity of fibronectin to cells, unlike that of laminin, appears to be relatively low; substantial binding was previously observed only with fibronectin-coated beads [eg, 64] or aggregates of cellular fibronectin [65]. More recently, plasma fibronectin labeled with ^{125}I was shown to bind to monolayers of fibroblasts in a cumulative manner [66]. Fibronectin continued to bind over time, and it became covalently linked to the extracellular matrix by disulfide bonds. Detergent extraction experiments suggested, however, that over half of the initial binding was to the matrix rather than to detergent-extractable integral membrane proteins [66]. This result is not surprising, since fibronectin is known to bind to pre-existing fibronectin fibrils [67].

One complication in fibronectin-binding studies is the possibility that the iodination procedure destroys the ability of fibronectin to interact with cells [68]. Even after using alternate labeling procedures, direct binding studies to cells in suspension is difficult with low concentrations of fibronectin. Such binding has been reported to be stimulated by incubation at 4°C, perhaps because the fibronectin is more likely to form multivalent aggregates [69].

Very recently, it has been possible to demonstrate direct, saturable binding of fibronectin to cells in suspension under physiological conditions; the key elements appear to be to use more physiological fibronectin concentrations and culture medium, as well as higher cell concentrations—all conditions to optimize the analysis of a low-affinity receptor [69a]. Binding of fibronectin to cells is saturable and specific, with only a moderate estimated binding affinity ($K_D = 8 \times 10^{-7}$M); there are substantial

numbers of receptors per cell (5×10^5). This receptor, like the reported biological function of the receptor [70], becomes much more sensitive to trypsin after chelation of divalent cations by EDTA [69a]. The modest affinity of this receptor explains why it has not been simple to isolate such a receptor by affinity chromatography.

The molecular identity of the receptor is still unknown. Three possibilities at present are glycoproteins with estimated molecular weights of 140,000 [71–73; 73a,b] and 47,000 [70,75–77] and certain gangliosides [78–81]. A protein complex of glycoproteins averaging 140,000 in apparent molecular weight on SDS gels appears to be necessary for cell attachment to fibronectin. In earlier immunological and biochemical experiments by Buck and co-workers, a complex of proteins in this size range was implicated in the attachment of hamster cells to culture dishes in vitro [82]. This complex was isolated in detergent and purified by chromatography, but not further characterized. Another complex of glycoproteins in another species (chicken) has been identified by monoclonal antibodies, and it contains glycoproteins of similar size [71–73; 73a,b]. There is as yet no formal proof that the mouse and chicken molecules are immunologically or functionally equivalent, although they appear similar.

Immunological localization studies show that the 140,000-dalton antigen is present in close proximity to some microfilament bundles [72], but it is in especially close association with fibronectin fibrils in adhesive sites on the ventral surfaces of cells [73a,b]. In comparisons with the immunofluorescence patterns of several cytoskeletal proteins and fibronectin, the 140,000-dalton protein appeared to co-localize most strikingly with fibronectin located in attachment sites, but not with extracellular matrix fibrils of fibronectin located far from the cell body. These results suggest a spacial relationship between the 140,000-dalton integral membrane protein complex and fibronectin [73a,b].

Biologically, the 140,000-dalton protein complex has been implicated in myoblast attachment to gelatin [71,72] and in fibroblast attachment to fibronectin [73; 73b]. Since myoblast attachment to gelatin is thought to require fibronectin [83], the former effects could also be due to inhibition of fibronectin function. Our results, however, do not show 100% inhibition of cell attachment to fibronectin by the monoclonal antibody. In fact, the monoclonal antibody itself was discovered to be capable of serving as an attachment protein when it became adsorbed to the plastic substrate [73b]. This result is consistent with the expected activities of an antibody directed against the fibronectin receptor or a closely associated molecule, which would be inhibitory when added in solution to cells, yet would become a positive mediator of adhesion if located on a substrate in a position to mimic an attachment protein binding to its receptor. Of course, if fibronectin is already bound to the cell surface, anti-fibronectin antibodies adsorbed to substrates can bind this fibronectin to mediate adhesion [74].

Biochemically, the 140,000-dalton complex has been characterized using material isolated by two apparently similar monoclonal antibodies, ie, antibodies against the CSAT antigen by Horwitz and co-workers [72] and JG22E antibodies related to the JG22 antibodies of Greve and Gottlieb [71,73b,84]. The antigen isolated from chicken by both antibodies contains 3–4 distinct proteins, which can be shown to be unique proteins by peptide mapping [84]. They appear to exist as a noncovalent complex; eg, they migrate as a unit in sucrose gradients [84,84a]. Whether this

protein complex directly binds to fibronectin and serves as its receptor remains to be determined; however, very recent results from human cells indicate that a similar-sized set of proteins does bind to fibronectin [84b].

A second leading candidate for the fibronectin "receptor" is a glycoprotein with an apparent molecular weight of 47,000 as estimated by SDS gels. This protein was initially identified by chemical cross-linking experiments designed to identify membrane proteins located in very close proximity to substrate-attached fibronectin [75]. This protein was reported to be distinct in size from actin and to be a ricin-binding glycoprotein located on the external surface of cells; it was labeled anomalously poorly by lactoperoxidase-mediated iodination. It is possible that substantial amounts of the antigen in this size range may be intracellular [76; R.C. Hughes, personal communication], although this point remains to be examined further.

A cell surface glycoprotein of similar size has been reported by others on baby hamster kidney (BHK) cells, and its presence correlates with the presence of biological "receptor" activity; ie, proteolytic treatments that affect this protein also affect the ability of cells to attach and spread on a fibronectin-coated substrate [70, but also see 77]. This relatively protease-resistant molecule has an apparent molecular weight similar to the protein identified by cross-linking, but it may not be identical; direct comparisons concerning this point are obviously needed.

Antibodies against preparations containing these 47,000-dalton proteins can partially inhibit cell-to-substrate adhesion [70,76]. It is of obvious importance to determine the relationships of these proteins, as well as whether monospecific antibodies against either this component *or* the 140,000-dalton complex can each cause similar and parallel effects on cell adhesion to fibronectin, or whether these molecules are responsible for different biological steps in the interaction of cells with fibronectin.

A third candidate molecule for a "receptor" function is the class of gangliosides with larger numbers of sialic acid residues, eg, G_{T1b}, which contains three sialic acids. Charged gangliosides are competitive inhibitors of fibronectin-mediated adhesion in a series of in vitro assays for fibronectin function, and their activity resides in the oligosaccharide moiety [78,79].

Moreover, a somatic cell variant that lacks such cell surface gangliosides is defective in its interactions with its own secreted fibronectin, and a reconstitution of gangliosides in these cells restores the capacity of the cells to organize secreted fibronectin into a fibrillar matrix [80]. Recent experiments suggest that the reconstituted gangliosides and fibronectin are concentrated at similar regions, and that even exogenously added fibronectin is also reorganized into fibrils by the cells into which gangliosides are reconstituted [81]. These findings strongly suggest that certain gangliosides can function in the binding of fibronectin to the cell surface, although it is not clear how the binding occurs or whether these molecules are actually direct receptors for fibronectin.

The fibronectin "receptor" is therefore a surprisingly elusive and complex entity. Although there is no definitive evidence as yet for such a hypothesis, it is possible that there is more than one molecule on the cell surface that can bind to fibronectin and serve as a "receptor." If there is more than one such molecule, it seems likely that each should have a similar low affinity for fibronectin, or that one should be present in much larger numbers than others; otherwise, the direct binding studies should have shown more than one class of binding site. A second hypothesis

is that at least two of the candidates for the receptor are part of a single "receptor" mechanism. One of many possibilities might be the following model: one molecule may be the initial recognition moiety, eg, the glycoprotein of 47,000 or of 140,000 daltons, which is dependent on the presence of a collar of ganglioside-like lipid molecules in tight association for maintaining optimal activity, and which also requires the members of the 140,000-dalton integral membrane protein complex to stabilize or transduce the binding signal during cell spreading on substrates. This type of model will be testable once specific inhibitors are available for each of these molecules, and if fibronectin binding can be analyzed in artificial in vitro systems.

THE COLLAGEN RECEPTOR

As reviewed earlier, epithelial and fibroblastic cells can bind directly to collagen. Several putative integral membrane protein receptors for various types of collagen have been identified in a variety of cell types. Although definitive comparisons of these proteins remain to be completed, there may be at least three types of cellular receptor for collagen in different cell types [56,57,85–89]. The published properties of the various putative collagen receptors that have been identified to date are compared in Table I.

Some of the collagen-binding molecules appear to vary in estimated binding affinities and molecular weights. For example, mouse 3T3 fibroblasts have approximately 5×10^5 collagen-binding sites per cell with very high affinity ($K_D = 1.2 \times 10^{-11}$ M), which bind type I collagen or its isolated α_1 and α_2 chains [85–87]. In addition, these cells have a molecule of 47,000 daltons (colligin) that binds to both gelatin and native type IV collagen [57]; the relationship of these two receptor moieties from the same type of cell has not been resolved. A 47,000-dalton gelatin-binding protein (colligin) is also present in parietal endoderm, embryonal carcinoma, and hepatoma cells; this protein appears to be distinct from the 47,000-dalton glycoprotein that is cross-linked to fibronectin [57]. In contrast, platelets have a protein with subunits of 65–75,000 daltons, they bind collagen with a significantly lower affinity ($K_D = 2 \times 10^{-8}$ M), and there are only 2×10^4 copies per platelet, which are smaller than fibroblasts and have less plasma membrane surface area [88,89].

These putative receptors are beginning to be characterized. Anchorin CII (a protein from chondrocytes that binds type II collagen) and colligin (47,000 daltons) are reported to be glycoproteins; the former contains substantial amounts of mannose, while the latter contains fucose [56,57]. Anchorin CII is hydrophobic and can be inserted into liposomes, where it binds type II collagen under physiological salt conditions [56].

It is obvious that comparisons of the different putative receptors are needed to establish definitively that distinct collagen receptors exist. The specificity of each of these molecules for native and denatured collagen chains of types I–IV, and the newer types still requires careful examination, especially since the detergent-isolated anchorin CII molecule does not show specificity [56]. It is not clear whether this lack of specificity is a problem of the isolation procedure or reflects a more general property; ie, it must be established whether there are unique receptors for each of the collagen types or of certain subsets, or whether only a couple of multi-purpose collagen receptors will suffice for cell interactions with all types of collagen.

TABLE I. Properties of Putative Collagen Receptors

Nomenclature	Collagen binding sites	Platelet's adhering protein	Collagen α_1(I) receptor	Anchorin CII	Colligin
Cell type	Mouse 3T3 fibroblasts	Bovine platelets	Human platelets	Chick chondrocytes	Mouse endoderm cells, etc
Collagens bound	Native types I–III	Type I	α_1(I) chain, fibrillar type I	Native types I–III, V, and M	Gelatin and native type IV; others not tested
Affinity	$K_D = 1.2 \times 10^{-11}$M	?	$K_a = 5 \times 10^7 M^{-1}$		
Sites per cell	5×10^5				
Isolated by—		Type I collagen-agarose affinity columns	Gel filtration, α_1(I)-agarose or type I-agarose affinity columns and preparative electrophoresis	Type II collagen-agarose affinity columns	Gelatin-agarose or native type IV collagen-agarose affinity columns
Molecular weight					
Native	?	300,000	?	?	?
Subunit	?	75,000	65,000	31,000	47,000
Isoelectric point		?	?	6.1	7.5–8.0
References	85–87	88	89	56	57

The functions of these "receptors" for collagen might include (a) cell adhesion to collagen and the maintenance of stable tissue organization, (b) function as the initiation sites for collagen fibrils as they are extruded by cells, and (c) functions as motility-related sites of collagen remolding. The purification of these various collagen receptors should permit a detailed analysis of cell interactions with this most abundant structural protein. In addition, it should permit an evaluation of the specificity of this receptor in terms of interactions with other matrix components.

DUALISTIC NATURE OF ADHESIVE PROTEIN FUNCTION

If an adhesive protein binds to cells via a receptor, a theoretical prediction based on this fact is that the protein could display positive or negative activities, depending upon its location and concentration (Fig. 10). In general, an attachment protein is active if it is pre-adsorbed to a substrate, non-bound protein is washed away, and the substrate-adsorbed fraction is tested for activity [1–10]. Even after blocking of non-specific binding sites on the substrate with native or heat-denatured bovine serum albumin in high concentrations, the attachment proteins remain fully active in mediating cell attachment and spreading on substrates (Fig. 10: I). If present in solution at high concentrations, however, attachment proteins could theoretically saturate all cell surface receptors. There would then be insufficient numbers of free receptors to bind to substrate-adsorbed attachment proteins (Fig. 10: II). A further prediction is that this relationship would be competitive; ie, high concentrations of substrate-adsorbed adhesive protein might be able to compete for free receptors more efficiently than a fixed amount of soluble protein.

These predictions are fulfilled for fibronectin [90]. High concentrations of plasma fibronectin almost completely inhibit fibroblastic cell adhesion to a fixed amount of fibronectin on the substrate in dose-dependent fashion. Moreover, increasing the amount of fibronectin on the substrate can compete with this inhibitory activity and permit attachment [90]. With hepatocytes, a more transient, partial inhibition has

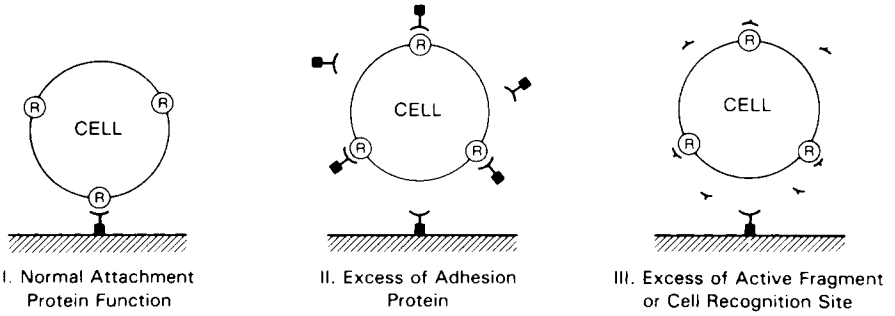

Fig. 10. Dualistic nature of adhesive-protein function. I) Attachment proteins can function after binding to a surface and remain bound and active even after extensive washing. II) Theoretically, an excess of the same adhesion protein could become a competitive inhibitor for cell attachment to the substrate-adsorbed protein if it saturates its receptor on the cell surface. Attachment of such receptor-bound ligand is prevented by coating nonspecific adsorption sites on the substrate by an excess of heat-denatured bovine serum albumin. III) A fragment of the adhesion protein containing the site recognized by the cell surface or even a synthetic peptide recognition site could also inhibit the function of the receptor competitively. See text for specific experiments.

been found [91]. Platelet aggregation is also inhibited by an excess of plasma fibronectin [92].

A similar inhibition of fibronectin-mediated adhesion is found with the cell-binding domain of fibronectin, which is even more active than intact fibronectin on a molar basis (Fig. 10: III). The concentrations of this fragment required for complete inhibition are close to those required for maximal inhibition of the binding of labeled fibronectin to these cells; the same type of increase in molar activity of fragments for competitively inhibiting binding is also seen in this physical binding system [90; Akiyama and Yamada, submitted]. A substantially greater inhibition of hepatocyte attachment by an unfractionated mixture of proteolytic fibronectin fragments compared to the intact protein has also been reported [91]. These apparent increases in molar activity of fragments of the protein suggests that cleavage may expose a binding region in a more favorable conformation for binding. It should be possible to test this idea by direct binding assays using labeled, purified fragments of fibronectin.

Since activity is retained in polypeptide fragments of an adhesion protein such as fibronectin, even smaller fragments of the adhesion protein that still retain a crucial recognition signal might be active (Fig. 10: III). Previous studies of Pierschbacher and Ruoslahti [93] showed that a 3,400-dalton synthetic peptide still retained fibronectin's cell attachment activity. Recent studies show that competitive inhibitory activity is even present in small peptides of fibronectin ranging from four to ten amino acids in length [90,94, accompanying paper, see Yamada and Kennedy, this issue]. Molar effectiveness of the synthetic peptides appears to vary depending on the assay system; the estimated affinity for inhibition of normal rat kidney (NRK) cell attachment is 6×10^{-4} M for a tetrapeptide [94], but it is much better for a heptapeptide inhibiting BHK cell attachment—approximately 5×10^{-5} M [90]. For comparison, half-maximal inhibition of cell attachment by intact plasma fibronectin in BHK cells occurs at approximately 2×10^{-5} M [90]. These discrepancies in the two published studies are probably due to differences in assays and the sizes of peptides; for example, the amount of fibronectin adsorbed on the substrate has striking effects on the ability of a peptide to competitively inhibit adhesion [90].

Nevertheless, the overall rankings of peptides according to their relative activities are in good agreement in comparisons of work from two different laboratories using different assay systems [90,94, accompanying paper, see Yamada and Kennedy, this issue] (Fig. 11). The minimal requirement for activity is the tetrapeptide sequence

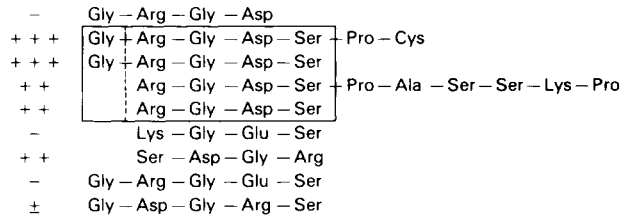

Fig. 11. Sequence requirements for function of cell-recognition region of fibronectin. The amino acids required for competitive inhibition of fibronectin-mediated cell attachment and spreading of fibroblasts are indicated by the box. Synthetic peptides containing the pentapeptide sequence were most active, but substantial activity was present in the tetrapeptide sequence and the reverse sequence. Activity is lost if the charged amino acids are replaced by different amino acids of the same charge or if key orientations are disrupted. For discussion and details, see text [based on references 90, 94, 95, and accompanying paper, see Yamada and Kennedy, this issue].

Arg-Gly-Asp-Ser [94], although the requirement for the serine does not appear to be stringent [95]. Further work will be required to evaluate the exact requirements for activity, eg, whether there can be minor rearrangements of the sequence. For example, in the BHK-assay system, adding a glycine residue prior to the arginine to produce the sequence Gly-Arg-Gly-Asp-Ser substantially increases biological activity [accompanying paper, see Yamada and Kennedy, this issue].

The most surprising recent result is that the reverse sequence, ie, Ser-Asp-Gly-Arg, is similar in activity to the forward tetrapeptide sequence [see accompanying paper, Yamada and Kennedy, this issue]. This unexpected result suggests that the recognition site on the cell surface can deal with some ambiguity, and that the crucial information resides in the amino acid side chains rather than in the direction of the peptide backbone. Since the bond angles would differ, however, it also appears likely that "wobble" is occurring to permit a fit with similar affinity. Other switches in amino acid positions or presence of the "reverse" sequence in a longer peptide did not retain activity, suggesting that the recognition signal may involve a positive and a negative charge in a specific spacial arrangement modulated by a more carboxy-terminal amino acid and other nearby sequences [see accompanying paper, Yamada and Kennedy, this issue]. Interestingly, the reverse sequence has been found in only two types of protein as yet, type II histocompatibility antigens and an amylase precursor. In contrast, the forward sequence is present in several proteins, some of which may use the sequence as an attachment signal [94].

These findings suggest that fibronectin utilizes a recognition sequence of 3–5 amino acids in its binding to the cell surface. There are several important questions that remain. It remains to be determined whether this sequence is unique to fibronectin and to systems that mimic this interaction. That is, it is not yet clear whether this signal is a specific signal used only for cell attachment. Preliminary results suggest that cell attachment to serum-spreading factor (vitronectin) and native collagen may use a similar signal [K. Nagata, K. Yamada, and D. Kennedy, unpublished preliminary work], and it is therefore crucial to know whether these molecules use the same cell surface receptor.

Second, it is not clear how much effect modifying sequences play in modulating the activity of this recognition sequence. A priori, the presence of a crucial biological signal in only a simple tetrapeptide (forward or backward) or a pentapeptide sequence appears surprising, and substantial regulation of its exposure and of the local polypeptide conformation by adjacent sequences may prove to be important for specificity, as well as preventing activity of other proteins that would contain this sequence. It should be noted that most proteins are not yet sequenced, and that it would seem likely that many more proteins contain this sequence; it would be surprising if all use it in a similar fashion.

Third, it is crucial to understand how this sequence is recognized by the cell surface. The localization of binding activity to a short, simple peptide sequence indicates that fibronectin is bound by some moiety on the cell surface, rather than that a polypeptide-binding pocket of fibronectin binds to a specific target on the cell. The postulated cell surface receptor then becomes quite interesting to analyze, since it must presumably be able to recognize this simple sequence. This direction of the recognition event differs from that of other domains in fibronectin, which are thought to contain conformationally specific binding sites for target molecules such as heparin and collagen. It is also important to learn whether this recognition occurs by a single

receptor or by several receptors with overlapping activities. Finally, it is possible that recognition of this sequence is only the first step in fibronectin's binding to cells, and that other signals may be needed for full binding.

The existence of synthetic peptides with biological activity provides a new approach to evaluating the in vivo significance of a recognition signal. Microinjection of such peptides to determine which biological processes are dependent on the signal should provide information complementary to inhibition data obtained from antibody microinjection experiments. The fibronectin recognition signal appears to be required for gastrulation of embryos and for migration of neural crest cells later in development, since these events are specifically inhibited by fibronectin recognition peptides [96]. Other recognition sequences may be found that are specific for other systems. For example, slime mold morphogenesis is inhibited by synthetic peptides from the protein discoidin with a sequence that is similar to, but distinct from the fibronectin recognition sequence [97].

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