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EXTRACELLULAR MATRIX MOLECULES AND THEIR RECEPTORS: Functions in Neural Development

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INTRODUCTION

The development of neurons and virtually all other cell types in the organism depends upon interactions with molecules in their environment. Studics of individual cell types have revealed tremendous diversity in the molecules that regulate the development of cells in the nervous system. These include chemotropic and trophic factors [e.g. nerve growth-factor (NGF) and brain derived neurotrophic factor (BDNF)], cell adhesion molecules [e.g. the neural cell adhesion molecule (NCAM) and N-cadherin], and molecules secreted into the extracellular matrix (ECM) [e.g. laminin (LN) and fibronectin (FN)]. Each class of molecule has now been shown to influence major steps in the development of the nervous system, including neuronal survival, determination, and migration; axonal growth and guidance; synapse formation; and glial differentiation. As molecules in the ECM influence all of these events and can be used to illustrate many of the principles derived from studies of the other classes of molecules, this review focuses upon constituents of the ECM and their receptors. The role of the ECM in neural development has recently been reviewed in this series (Sanes 1989). This review focuses on cellular and molecular themes not emphasized in the previous one and includes examples of recent work on nonneural systems that illustrate probable future directions for research in the nervous system.

Recent reviews on the composition and function of the ECM and its receptors include those of Hynes (1990), Hemler (1990), Kishimoto et al (1989), Plow & Ginsberg (1989), Burgeson (1988), Buck & Horowitz (1987), McDonald (1988), Ruoslahti (1988, 1989), Fessler & Fessler (1989), and Erickson & Bourdon (1989). Reviews focusing on aspects of ECM function in neural development include those by Lander (1989), Sanes (1989), and Edgar (1989). Because of space limitations, only representative examples and references are cited in this review.

COMPOSITION AND FUNCTION OF THE EXTRACELLULAR MATRIX

The ECM was originally defined morphologically as a cellular material with structure visible in the electron microscope (e.g. fibrils or basal lamina). It is now defined more broadly to include secreted molecules that are immobilized outside cells, even if they lack organization that is detectable in the microscope. Major constituents identified initially in the ECM included collagens, noncollagenous glycoproteins, and proteoglycans. Recent work has revealed a surprising diversity in these molecules. The number of distinct collagens is now greater than

12; the number of adhesive glycoproteins is constantly growing. Multiple genes and differential splicing further add to diversity in both classes of ECM glycoproteins (cf. Ehrig et al, sequences of the core proteins for several proteoglycans has shown that this class of molecules is best considered as a group of diverse glycoproteins with functions mediated both by their protein cores and by their carbohydrate side chains (cf. Ruoslahti 1989, Saunders et al 1989, Clement et al 1989). Recent work also indicates that several cell-cell adhesion molecules interact with or have forms that are immobilized in the ECM (e.g. NCAM, myelin-associated glycoprotein) (Sanes et al 1986, Fahrig et al 1987). Finally, the ECM has also been shown to bind to and regulate the activity and stability of several growth factors, most notably basic-fibroblast growth factor (FGF) and transforming growth factor (TGF)-β (cf. Rifkin & Moscatelli 1989, McCaffrey et al 1989).

DOMAINS IN EXTRACELLULAR MATRIX PROTEINS

The collagens and other ECM glycoproteins are molecular giants, in some cases spanning distances of several hundred nanometers. Primary sequence analysis has revealed that they are chaemeric proteins, assembled by exon shuffling. Multiple functional domains have been identified in individual ECM glycoproteins such as FN or LN (Figure 1). These include binding sites for cells, other ECM glycoproteins, glycosaminoglycans, and glycolipids (e.g. Mann et al 1989). Some ECM glycoproteins and proteoglycans have also been shown to bind growth factors and proteases (cf. Rifkin & Moscatelli 1989,Silverstein et al 1986). With most of the ECM glycoproteins, it has been possible to localize specific functions, such as cell binding, to specific domains, sometimes as short as 3–20 amino acids (cf. Pierschbacher & Ruoslahti 1984,Wayner et al 1989,Guan & Hynes 1990,Tashiro et al 1989). One of these short peptide sequences, RGD, functions as a cell attachment site in several different ECM glycoproteins, including FN (see Table 1). The demonstration of a specific function for a particular structure in one protein has raised the possibility that this structure functions similarly wherever it is found. By this criterion, many of the domain structures in those ECM proteins that have been sequenced are potentially adhesive. Most prominent among these domains is the fibronectin type III repeat, one of which contains the sequence RGDS as the major cell attachment site in fibronectin (Pierschbacher & Ruoslahti 1984). Multiple copies of this motif are found in FN, tenascin, and thrombospondin (Figure 1). Currently, two of the 18 such domains in FN, one of seven in tenascin, and one of six in thrombospondin have been shown to include cell attachment sites (Wayner et al 1989,Guan & Hynes 1990,Spring et al 1989,Lawler et al 1988). It seems likely that more of these domains will prove to have adhesive functions when tested with appropriate cells. FN type III domains are differentially spliced in both FN and tenascin (cf. Hynes 1985,Spring et al 1989). In the case of FN, one of the differentially spliced type III domains has been shown to contain a cell attachment site recognized by both neural crest cells and sensory neurons (cf. Dufour et al 1988,Humphries et al 1986,1988).

A second major domain implicated in cell adhesion, the immunoglobulin domain, has recently been identified in the major HeS-proteoglycan, percalin, which contains multiple copies of this structural motif (Noonan et al 1988). Similar domains have been found in several Ca^{2+}-independent neural cell adhesion molecules, including NCAM, L1, contactin, neuroglian, and fasciclin II (cf. Harrelson & Goodman 1988). Intriguingly, most of these cell adhesion molecules also contain several FN type III repeat motifs. Even an ECM receptor subunit, integrin β_4 contains three FN type III repeats in its cytoplasmic domain (Suzuki & Naitoh 1990). Therefore neither the immunoglobulin nor FN type III domains distinguish cell membrane-associated adhesion molecules from adhesive glycoproteins found in the ECM.

Individual proteoglycans have recently been found to contain several other domains that suggest adhesive or mitogenic functions, including putative hyaluronic acid binding domains, lectin binding domains, leucine-rich repeats, and epidermal growth factor (EGF) repeats (cf.

Ruoslahti 1989). For example, the very large (2389 amino acids) chondroitin sulfate proteoglycan named versican, identified originally as a secretory product of fibroblasts, is identical to glial hyaluronic acid-binding protein, which has been isolated from brain white matter (Perides et al 1989, Zimmerman & Ruoslahti 1989). In addition to a functional hyaluronate-binding domain, versican contains two EGF repeats, a lectin-like domain, and a complement-regulatory protein-like repeat (Figure 1). All of these domains are also found on one or more transmembrane cell surface glycoproteins that function as receptors (cf. Ruoslahti 1989). Thus, structural analyses detect few motifs that distinguish cell-surface-associated and ECM-associated adhesive glycoproteins.

Not all domains in ECM constituents are believed to function in cell adhesion. In particular, domains homologous to the EGF precursor, named EGF repeats, are found in LN, entactin, thrombospondin, and tenascin, thereby raising the possibility that ECM molecules may have interactions with cells similar to those mediated by EGF and its receptor (cf. Engel 1989).

STRUCTURE AND FUNCTION OF ECM RECEPTORS: INTEGRINS

Progress has been so rapid in identifying receptors for the ECM that the field is very fluid. Many receptor classes identified on nonneural cells have not yet been found in the nervous system, although it seems likely that they will eventually be found there. At this point, neurons appear to use the same general classes of receptors as nonneural cells, so studies on the latter are useful in understanding the functions of these receptors in the nervous system. In this section, we first consider the major superfamily of ECM receptors, the integrins (listed in Table 1), and then consider other receptors likely to be important in mediating interactions with the ECM.

Integrin receptors are noncovalently associated heterodimeric glycoprotein complexes expressed on the surfaces of neurons and nonneural cells that appear to be the primary cellular receptors for many ECM constituents, including LN, FN, thrombospondin, and several collagens (see Table 1; cf. Hemler 1990). With assays of attachment or neurite outgrowth in vitro, antibodies to the integrins, notably the CSAT antibody that binds to the chicken integrin β_1 subunit, appear sufficient to disrupt virtually all interactions of neurons with both purified ECM glycoproteins and complex extracellular matrices (cf. Bozyczko & Horwitz 1986,Cohen et al 1986,Tomaselli et al 1986). Thus there is direct evidence that functional integrins are required for binding of neurons to the ECM. Injections into developing avian embryos of antibodies to the integrin β_1 subunit have dramatic inhibitory effects on migration of several cell types, including cranial neural crest cells and myoblasts populating limb masses (Bronner-Fraser 1986,Jaffredo et al 1988). Thus this class of receptors also has important functions in vivo.

As illustrated in Table 1, the integrins are a large receptor family (see also Hemler 1990). As shown in Figure 2, integrin receptors are transmembrane heterodimers composed of one α chain noncovalently associated with a β subunit. Multiple genes encode families of both α and β subunits. Ligands for almost all of the individual heterodimers have now been identified with function-blocking monoclonal antibodies and affinity chromatography on purified ECM glycoproteins (see Table 1). At present, there are six (and probably more) distinct, but homologous, β subunits (β_1–β_5, β_p), each of which can associate with one or more of at least 12 α subunits. By convention (Hynes 1987), integrins have been classified into subfamilies according to the β subunit that is shared among several different heterodimers. Originally, groups of α subunits were thought to associate with one particular β subunit. It has recently become clear, however, that some α subunits can interact with more than one β subunit (cf. Cheresh et al 1989b, Kajiji et al 1989,Hemler et al 1989,Holzmann & Weissman 1989). Nevertheless, it is still useful to refer to integrin β-class subfamilies.

Integrins are expressed by neurons and glial cells in both the central and peripheral nervous systems (cf. Bozyczko & Horwitz 1986, Cohen et al 1987, Tomaselli et al 1986, Hall et al 1987, Pesheva et al 1988, Letourneau & Shattuck 1989, Tawil et al 1990). In particular, β_1-class integrins have been shown to mediate neuronal attachment and process outgrowth in response to several ECM proteins, including LN, FN, and several collagens. Of the eight known β_1 integrins (Table 1), five (α_1, α_3, α_5, α_6, α_{VN}) have been identified in either primary neurons or neuronal cell lines (Tomaselli et al 1988a,b, 1989, and unpublished, Turner et al 1989, Ignatius & Reichardt 1988, Ignatius et al 1990, Toyota et al 1990, Vogel et al 1990). Strong indirect evidence for the expression of α_4 by peripheral neurons comes from studies of their interactions with the alternatively spliced IIICS domain in fibronectin (Humphries et al 1988, Guan & Hynes 1990). β_3 integrins have also been described on retinal neurons (K. M. Neugebauer and L. F. Reichardt, unpublished). Members of the β_2 family, the lymphocyte integrins, have yet to be identified on neural cells. β_2 integrins are unlikely to play a prominent role in the nervous system, however, since mutations in the β_2 subunit have no obvious effects on the development or function of the nervous system (cf. Kishimoto et al 1989). Although which of the recently discovered β subunits (β_4, β_5, β_p) are expressed in the nervous system is not yet clear, those found on epithelial cells, β_4 and β_5, seem likely to be present there. A major challenge for the future is to catalog the integrin α and β subunits expressed in different neural cells and to identify the functions of individual receptors in mediating the interactions of these different cells with ECM-bound and other ligands.

The integrins have several important features to consider in understanding their demonstrated and potential functions in the nervous system. First, as shown in Table 1, their ligands include not only virtually all described ECM constituents, but also cell surface molecules. In the β_1 family, $\alpha_4\beta_1$ has been shown to interact directly with FN and with an integral membrane glycoprotein ligand, VCAM-1, which contains several extracellular immunoglobulin-like domains (Elices et al 1990). Based upon their distributions, it has been suggested that other members of this family, notably $\alpha_2\beta_1$ and $\alpha_3\beta_1$, may also have cell surface ligands. Such ligands seem likely to be particularly important in the central nervous system, where many ECM constituents that are present in the peripheral nervous system are largely absent. Secondly, both the α and β subunits have been shown to be important in determining ligand binding properties of integrin heterodimers (cf. Cheresh et al 1989). As a consequence, tremendous potential functional diversity is encoded by the integrin family. Since many subunits have only been discovered in the past year, additional subunits will very likely be discovered in coming years, adding further to diversity of these receptors. Third, the integrins include both heterodimcrs that recognize many ligands, e.g. $\alpha_3\beta_1$, which binds LN, FN, and collagen, and heterodimers that bind only one ligand, e.g. $\alpha_6\beta_1$ and $\alpha_5\beta_1$, which are specific for LN and FN, respectively. Thus, some heterodimers are likely to play very general roles whereas others play much more specific roles in regulating development of neurons and other cells in the nervous system. Finally, cell-specific factors have recently been shown to modify the ligand-binding specificities of two integrins. Both $\alpha_2\beta_1$ and $\alpha_3\beta_1$ heterodimers exhibit different ligand-binding specificities in different cell types (Languino et al 1989,Elices & Hemler 1989,Gehlsen et al 1989). The $\alpha_2\beta_1$ receptor mediates binding to collagen wherever it is expressed but mediates binding to LN in only a subset of cells. The ability of the $\alpha_3\beta_1$ receptor to bind collagen is also regulated by cell-specific factors. The molecular bases for these surprising results are not yet understood.

Structurally, both integrin α and β subunits are transmembrane glycoproteins. Primary sequence analysis of the five sequenced β subunits indicates that each has a large N-terminal extracellular domain, containing a fourfold repeat of a 40-amino-acid cysteine-rich segment, a single transmembrane domain, and, except for β_4, a short cytoplasmic domain of ca. 40 amino acids (Figure 2; cf. Tamkun et al 1986,Fitzgerald et al 1987,Suzuki et al 1990). The integrin β_4 subunit has a very large cytoplasmic domain containing more than 1000 amino acids (Suzuki

& Naitoh 1990). The β-subunit cytoplasmic domains contain potential phosphorylation sites for tyrosine and serine/threonine kinases. Differential splicing has been shown to result in β_3 and β_4 subunits with two different cytoplasmic domains (van Kuppevelt et al 1989,Suzuki & Naitoh 1990). It is not yet clear whether these are both functionally significant. As is discussed below, the cytoplasmic domains appear to mediate interactions with cytoskeletal elements and are required for the adhesive functions of integrins on cells (Solowska et al 1989,Hayashi et al 1990).

Primary sequence analysis of the 11 sequenced α subunits indicates that each also has a large N-terminal extracellular domain, a single transmembrane domain, and a short cytoplasmic domain (see Figure 2; cf. Hemler 1990). The N-terminal half of each α sequence encodes seven repeats of an approximately 50-amino-acid domain, three or four of which, depending on the subunit, contain putative divalent cation binding sites. Cross linking with small peptide ligands has shown that the ligand-binding site is located in the vicinity of the first three divalent cation-binding sites in the vitronectin receptor, $\alpha_{VN}\beta_3$ (Smith & Cheresh 1990). Insertions of ca. 200 amino acids are present in the leukocyte family of integrins ($\alpha_{LFA\text{-}1}$, $\alpha_{Mac\text{-}1}$, α_{gp150}) and at least two members of the β_1 family (α_1 and α_2) (cf. Kishimoto et al 1989,Takada & Hemler 1989,Ignatius et al 1990). These “interactive” or I domains are inserted between the second and third repeats within a putative ligand binding site defined by inferred homology to $\alpha_{VN}\beta_3$. These I domains are similar to I domains in several collagen-binding ECM proteins, such as von Willebrand’s factor, thereby suggesting the possibility that they mediate interactions of the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ receptors with collagen. Differential splicing has been shown to insert an exon in the putative ligand-binding region of the *Drosophila* integrin α subunit, PS2 (Brown et al 1989). To date, there is no evidence for differential splicing of vertebrate integrin α subunits.

In the electron microscope, integrins appear as long, double rods extending ca. 20 nm from the membrane with globular domains at their extracellular termini that appear to mediate subunit association (Carrell et al 1985, Nermut et al 1988). Association of the two subunits is required for surface exprcssion and ligand binding (cf. Bodary et al 1989). Cross-linking studies with small peptide ligands have shown that segments of both subunits form the ligand binding site in the integrins $\alpha_{IIb}\beta_3$ and $\alpha_{VN}\beta_3$ (D’Souza et al 1988, Smith & Cheresh 1988, 1990). Subunit association of some integrins requires low concentrations of a divalent cation (Fitzgerald & Phillips 1985). The ligand-binding activity of all integrins requires much higher, millimolar concentrations of Mg^{2+} or Ca^{2+} (cf. Marlin & Springer 1987, Gailit & Ruoslahti 1988). The divalent cation dependence of integrin function has been exploited recently to isolate several individual integrin heterodimers by affinity chromatography on ECM ligands.

Integrin functions have been shown to be regulated on several levels. Both FN and LN receptors are dynamically regulated on neurons. Rat sensory neurons have been shown to lose functional FN receptors between E16 and P4 (Kawasaki et al 1986). Chick ciliary ganglion neurons lose functional LN receptors between E8 and E14 (Tomaselli & Reichardt 1988). Chick retinal neurons have been shown to lose functional FN and LN receptors between E6 and E12 (Cohen et al 1986, Hall et al 1987). All of these decreases in functional receptors correlate with the establishment of functional contacts with the targets of these diverse neuronal populations. Ablation of the optic tectum reduces the loss of functional LN receptors on retinal ganglion cells, thus suggesting that target contact is responsible for loss of receptor function (Cohen et al 1989). Although these changes seem likely partly to reflect changes in gene expression, unambiguous evidence for loss of individual subunits has not yet been obtained (see e.g. Hall et al 1987). In addition to the postulated loss of integrin subunits, evidence suggests that the affinity of LN receptors on retinal ganglion cells is reduced upon target contact (Cohen et al 1989). Whatever the detailed mechanisms, the dynamic regulation of integrin receptor function

in several distinct classes of neurons seems likely to be important in regulating changes in neuronal behavior in vivo.

Growth factors and oncogenes have been shown to regulate integrin subunit expression in nonneural systems. At least one integrin subunit, α_1, is strongly induced by NGF on pC12 pheochromocytoma cells (Rossino et al 1990). The cell adhesion molecules L1 and NCAM are also induced by NGF (cf. Prentice et al 1987). If similar inductions of integrins and CAMs occur on primary neurons, they are likely to facilitate collateral sprouting that is induced, at least partly, by increases in NGF levels in denervated fields (cf. Diamond et al 1987). Since axons of severed peripheral and central neurons have been shown to be capable of regenerating on ECM substrates (cf. Davis et al 1987), it seems very likely that they re-express functional integrin receptors. Understanding the factors that regulate expression of these receptors is potentially useful for developing conditions that promote maximal nerve regeneration.

FUNCTIONS OF OTHER ECM RECEPTOR FAMILIES

Precedents from nonneural systems and preliminary studies on neural cells suggest that nonintegrin receptor systems may also be important in mediating interactions of cells in the nervous system with several ECM constituents. Laminin is an intriguing example of a glycoprotein for which there is now evidence for interactions mediated by both integrins and other receptors. Several adhesive sites have been identified on the classic laminin isoform that contain the A, B1, and B2 chains (summarized in Kleinman & Weeks 1989, Beck et al 1990). These include RGD-containing and IKVAV-containing sites in the A chain, both of which appear capable of promoting neurite outgrowth by PC12 cells (Tashiro et al 1989). Antibodies to the IKVAV peptide inhibit PC12 interactions with intact LN, thereby indicating that this site is functional. Similar experiments need to be done by using antibodies to the RGD-containing peptide to determine whether the RGD site is also functional in intact LN. Interactions of PC12 cells with LN are mediated by two integrins, $\alpha_1\beta_1$ and $\alpha_3\beta_1$ either or both of which may recognize these sites in LN (Tomaselli et al 1990). Recent work has identified proteolytic fragments of LN that are utilized by individual integrin heterodimers. The $\alpha_3\beta_1$ and $\alpha_6\beta_1$ dimers have been shown to bind sites in the long arm of LN present in the fragment E8 (Gehlsen et al 1989, Hall et al 1990). The $\alpha_1\beta_1$ receptor has been shown to interact with a site near the center of LN's cruciform structure, present in the fragment E1 (Tomaselli et al 1990, Hall et al 1990). Based on their locations in the structure of LN, the RGD and IKVAV sites are candidates to interact with the integrins $\alpha_1\beta_1$ and $\alpha_3\beta_1$ respectively.

In addition to the integrins, a set of antigenically related proteins with M_rS of 32K, 45K, and 67K bind LN with high affinity and have been found on many cells and tissues, including chick brain (see Kleinman & Weeks 1989, Douville et al 1988). The 67K protein is reported to utilize a sequence YIGSR located in an EGF repeat in the B1 chain of LN (Graf et al 1987). The 32K protein is identical to the galactose-specific lectin CBP 35 (Woo et al 1990). Although antibodies to the 67K protein do not inhibit LN binding by neuronal cell lines (Kleinman et al 1988), they are reported to inhibit the binding of some other cell types (Graf et al 1987). Evidence that a cell surface enzyme, galactosyltransferase, functions as a LN receptor has recently been obtained (Runyon et al 1986). Antibodies to or modifiers of this enzyme, and modifications of carbohydrate substrates on LN for this enzyme, partially inhibit both initiation and extension of neurites by a neuronal cell line on LN without significantly affecting cell attachment (Begovac & Shur 1990). Similar results have been obtained in assays of avian ncural crcst ccll migration on LN (Runyon et al 1986). These intriguing observations suggest roles for both a neuronal cell surfase enzyme and LN-linked oligosaccharides in regulating growth cone motility. Finally, gangliosides and sulfatides are present on neural cells and have been shown to bind LN (cf. Roberts et al 1985). Exogenous brain gangliosides GD_{1A} and GT_{1B} can

inhibit neurite outgrowth on LN by neuroblastoma cells (Laitinen et al 1987). Still uncertain, though, is whether these gangliosides compete with receptors or have less direct effects.

The receptors that mediate neuronal interactions with the two most prominent ECM glycoproteins in the CNS—tenascin and thrombospondin—have not yet been unambiguously identified. A neuronal proteoglycan has been shown to interact with tenascin and is consequently one candidate to be a tenascin receptor (Hoffman et al 1988). Though associated with the neuronal surface, this proteoglycan is not an intrinsic membrane constituent, and thus would presumably function indirectly in attaching neurons to tenascin. A second candidate, described in nonneural cells, is an apparently novel integrin, purified by affinity chromatography on tenascin (Bourdon & Ruoslahti 1989). This receptor's function is inhibited by RGD peptides, though, and it is not certain that cell interactions with tenascin are mediated by an RGD site (cf. Spring et al 1989). Although avian tenascin contains an RGD site, this site is not conserved in all species. Moreover, mapping both adhesive and anti-adhesive sites to individual domains of tenascin has been possible by using fusion proteins and antibodies (Spring et al 1989). The most prominent adhesive site in tenascin has been mapped to a FN type III repeat distant from the repeat containing the RGD sequence; the site on tenascin that inhibits cell flattening and motility has been mapped to EGF repeats, again far from the RGD-containing site. As discussed by Spring et al (1989), convincing identification of any receptor, integrin or otherwise, that mediates either of these two activities is still lacking. Identifying the receptor that mediates the anti-adhesive activity of tenascin will be particularly interesting, because this is a novel activity for an ECM macromolecule. In the case of thrombospondin, both the integrin $\alpha_{VN}\beta_3$ and an 88 kDa intrinsic membrane glycoprotein named GPIV have been shown to function as receptors in nonneural cells (Lawler et al 1988, Tandon et al 1989). Though neural cells clearly interact with thrombospondin (O'Shea et al 1990, Boyne et al 1989), the question of which, if either, of these receptors mediates the observed interactions remains obscure.

Although their neural functions can only be addressed speculatively, several other ECM receptor classes seem likely to be important in the nervous system. Prominent among these are hyaluronic acid receptors. Hyaluronic acid is located in pathways utilized by many cells, including neural crest cells, for migration. Ligands that bind cell surface–anchored hyaluronate dramatically inhibit migration of neural crest cells (Perris & Johansson 1990). The Hermes antigen (CD44) is a transmembrane receptor that functions in lymphocyte binding and has a domain with strong homology to several hyaluronic acid-binding proteins (Goldstein et al 1989). This receptor has been shown to interact with both ECM and cell surface-associated ligands (cf. St. John et al 1990). Whether these are exclusively hyaluronic acid is not yet clear. This protein has recently been localized on both eNS glia and Schwann cells (Picker et al 1989). Although it appears not to be expressed by adult neurons, its distribution in the developing nervous system merits attention.

Proteoglycans function as both ECM constituents and receptors (cf. Ruoslahti 1989). Individual proteoglycans have important functions in cell adhesion, proliferation, and differentiation. Specific examples document that heparan-sulfate proteoglycans can function as receptors, binding cells to ECM glycoproteins, including several collagens, FN, and thrombospondin (cf. LeBaron et al 1989, Saunders et al 1989). In addition, proteoglycans have been shown to modulate the binding of other receptors to ECM proteins. As one example, a chondroitin sulfate proteoglycan with weak affinity for FN has been shown to inhibit binding of a purified integrin to FN, probably by steric hindrance (Hautanen et al 1989). Proteoglycans are widely distributed in the nervous system. Despite appreciation of their presence and probable importance, they have received comparatively little attention. With the exception of versican, which of the comparatively well-characterized nonneuronal proteoglycans are also found in the nervous system is not yet clear. Recently, new, more powerful methods for characterizing proteoglycans

have been applied to analyze proteoglycans in the brain. The results suggest that there are very large numbers of distinct proteoglycans in the mammalian brain (Herndon & Lander 1990). Perhaps the most interesting proteoglycan that has been identified in the brain is a chondroitin sulfate proteoglycan defined by the monoclonal antibody Cat-301 (cf. Zaremba et al 1989). This is an extracellular proteoglycan, present on subsets of neurons in the brain, whose expression is regulated by synaptic activity. Although no function is known for this proteoglycan, its expression pattern suggests that it may be involved in synaptogenesis.

Finally, gangliosides and other glycolipids are differentially expressed macromolecules that are prominent neuronal surface constituents. These interact directly with many ECM glycoproteins, including FN, LN, and thrombospondin (cf. Roberts et al 1985). Thus they may function directly as receptors. In addition, gangliosides have been shown to modulate the functions of integrins (cf. Stallcup 1988, Santoro 1989). The integrin heterodimer—$\alpha_{VN}\beta_3$—has been purfied in tight association with a ganglioside (Cheresh et al 1987). Gangliosides have been shown to be essential for functional reconstitution of this integrin. Hence, gangliosides may act on neurons in part by interacting with integrins.

ECM REGULATION OF CELL MIGRATION AND AXONAL GUIDANCE

The importance of extrinsic cues in guiding migrating cells and motile growth cones is now well established in both invertebrate and vertebrate model systems (cf. Harrelson & Goodman 1988, Dodd & Jessell 1988). In several systems, cues localized on cells and within the extracellular matrix appear to provide the major guides for cell movements. In the developing grasshopper limb, for example, growth cones of pioneer neurons follow trajectories restricted on one side by a basal lamina and on the other by epithelial cells (cf. Bentley & Caudy 1983). Developing limbs can be isolated, opened, stripped of mesoderm, and cultured as a flat explant (Lefcort & Bentley 1987). In these conditions, designed to eliminate the effects of electric fields, gradients of diffusible molecules, and tissues extrinsic to the limb, growth cones of pioneer neurons still follow normal trajectories. Thus the limb epithelium and its associated ECM appear to provide adequate guidance cues. Retraction of growth cones as a consequence of removal of the basal lamina by proteolysis implies that there are adhesive interactions between the growth cones and basal lamina (Condic & Bentley 1989a). In such preparations, the polarity of initial outgrowth and selective interactions with limb segment boundaries and neuronal cells still occur normally, thereby indicating that the intact basal lamina is not required in this system for normal guidance (Condic & Bentley 1989b). Oriented axon outgrowth has also been examined in explants of embryonic chick and quail retinae (cf. Halfter & Deiss 1984). In these cultures, also designed to eliminate guidance by diffusible molecules, already existing cues are followed by axons in developmentally older portions of the retina, but these cues cannot be established in the developing retinal rim. When tested on the isolated ECM, growth cones grow at normal rates but are not oriented normally, thus implying that necessary guidance cues are not contained in the retinal ECM (Halfter et al 1987). To summarize, both of these examples indicate that ECM molecules in vivo can provide permissive substrates for growth cone motility. Neither suggests the presence of information in the ECM that can steer them.

A mechanism by which ECM fibrils can guide cell migration is provided by recent studies on neural crest cells (Newgreen 1989). Fibrils of ECM are easily aligned by a variety of forces. On a major substrate for crest cell migration, the medial face of the somites, fibrils of matrix are aligned parallel to the direction of neural crest cell migration. In vitro, crest cells migrate preferentially along the axis of alignment of the matrix fibrils, thus suggesting that this directional cue is utilized in vivo. Thus, structural cues may promote preferential migration in one direction (Boocock 1989).

Substantial progress in identifying individual ECM glycoproteins implicated in cell migration has come from studies on the developing neural crest and cerebellum. FN, LN, tenascin, thrombospondin, and hyaluronic acid are each found in pathways followed by neural crest cells (cf. Perris & Bronner-Fraser 1989, Epperlein et al 1988). Several reagents that inhibit interactions of neural crest cells with ECM constituents in vitro dramatically impair the migration of chick cranial neural crest cells. These include RGDS-containing peptides, modeled as competitors for a cell-binding site in FN; the INO antibody, which prevents cell interactions with an isoform of LN; tenascin antibodies; and an antibody that blocks the functions of β_1 subunit-containing integrins (Boucaut et al 1984, Bronner-Fraser & Lalier 1988, Bronner-Fraser 1986, 1988). These results suggest that integrins are important receptors on neural crest cells and that an RGD-containing ECM glycoprotein (e.g. FN, thrombospondin, or vitronectin), a LN isoform, and tenascin are essential substrates for these cells. Since neural crest cells can migrate efficiently on substrates coated with individual ligands in vitro, such as FN or LN, it is not clear why interfering with the function of a single ligand in vivo has such dramatic effects. In analogous in vitro systems in which cells or growth cones move on substrates with multiple ligands, blocking the function of a single molecule usually has only a partial inhibitory effect (cf. Bixby et al 1988). One possibility is that some of the peptides and antibodies used in the in vivo studies do not inhibit migration directly, but instead inhibit steps in neural crest cell differentiation prior to migration. This can now be addressed more directly by visualizing individual cells during migration via confocal microscopy.

The ability to study cerebellar granule cell migration in tissue slices in vitro has provided an opportunity to assess the role of ECM proteins in the migration of central neurons. During postnatal development, cerebellar granule cells proliferate in the external granule cell layer and then migrate through the molecular layer to reach their final positions in the internal granule cell layer. Several ECM components and CAMs have been identified in the developing cerebellar cortex, including tenascin (cytotactin), thrombospondin, chondroitin sulfate proteoglycan, hyaluronic acid, and L1 (Hoffman et al 1988, Chuong et al 1987, O'Shea et al 1990, Ripellino et al 1989). Perturbation experiments implicate several of these molecules in granule cell development. Antibodies to the CAM, L1, strongly inhibit granule cell migration (Chuong et al 1987). Cells accumulate in the pre-migratory zone of the external granule cell layer. Since these cells may not have initiated migration, these antibodies may inhibit differentiation of premigratory granule cells instead of interfering directly with migration. Tenascin surrounds granule cells in the external granule layer and is also expressed in the molecular layer, primarily on the surfaces of Bergmann glia (Chuong et al 1987). In the presence of tenascin antibodies, granule cells accumulate in the molecular layer, thus indicating that they start, but do not complete, migration. Both observations suggest that tenascin functions as an important substrate for granule cell migration. Thrombospondin is associated with granule cells in the premigratory zone of the external granule cell layer and in the molecular layer (O'Shea et al 1990). In contrast to tenascin, thrombospondin is not associated with the surfaces of the Bergmann glia, but is instead concentrated at the leading edges of the migrating granule cells. Thrombospondin antibodies prevent the migration of granule cells into the molecular layer. Although thrombospondin may stimulate migration as a substrate, its ability to bind and activate plasminogen and tissue plasminogen activator (Silverstein et al 1986) suggests that it may function instead by localizing and activating proteases released by the granule cells (Verrall & Seeds 1989, O'Shea et al 1990). Consistent with this idea, inhibitors of the tissue plasminogen activator/plasminogen system can also inhibit cerebellar granule cell migration from the external granule cell layer into the molecular layer (Moonen et al 1982). To summarize, L1, tenascin, and thrombospondin have been shown to promote cell attachment in vitro and may therefore also act as substrates in vivo. Differences in their distributions, activities, and effects of antibody injection suggest, though, that each also has a very specific, nonredundant function.

The characterization of genes that guide circumferential migrations of mesodermal cells and pioneer axons in *C. elegans* has provided convincing evidence for a role for an ECM constituent in providing directional information (Hedgecock et al 1990). In *C. elegans*, pioneer axons migrate between the epidermis and basal lamina. Mesodermal cells migrate on the opposite side of the basal lamina. Three genes—*unc-5*, *unc-6*, and *unc-40*—are required for guiding both classes of cells along the dorsoventral but not anterior-posterior axis. *Unc-6*, which affects migrations in both dorsal and ventral directions, has been shown to be a homologue of the LN B2 chain (cited in Hedgecock et al 1990). Considering the importance of LN in regulating epithelial cell development (cf. Klein et al 1988), it seems likely that some isoforms of LN are still present in *unc-6* mutants. The genetic analyses of migration-deficient mutants in *C. elegans* has provided the most specific role for an ECM molecule in cell guidance that has been identified thus far. That guidance and motility are not affected along the anterior-posterior axis is particularly striking. There is persuasive genetic evidence for interaction of two other gene products—*unc-5* and *unc-40*—with *unc-6* (Hedgecock et al 1990). *Unc-5* has been cloned and has properties consistent with its being a receptor, including a predicted external Ig domain and a putative transmembrane domain (cited in Hedgecock et al 1990). Existence of opposing adhesive gradients have been proposed as a model to explain guidance in *C. elegans*. One gradient could potentially be formed by a LN isoform containing the *unc-6* product. In vertebrate systems, though, neurons in vitro do not seem able to follow gradients of LN (McKenna & Raper 1988).

Many ECM molecules stimulate neuronal process outgrowth in vitro. These include LN, FN, several collagens, thrombospondin, and tenascin (cf. Manthorpe et al 1983, Rogers et al 1983, O'Shea et al 1990, Chiquet 1990). Some of these glycoproteins are also capable of guiding axons in vitro (cf. Gunderson 1987). Essentially all of these molecules are localized in vertebrate embryos where they could potentially serve as permissive substrates or provide guidance cues for growth cones (reviewed in Sanes 1989). LN isoforms, for example, have been detected in both the vertebrate peripheral and central nervous systems in locations that would suggest a role in axonal growth: in developing sensory and autonomic ganglia and spinal nerve roots (Rogers et al 1986), in the pathway of trigeminal nerve fibers (Riggott & Moody 1987), in developing muscle prior to innervation (Sanes & Chiu 1983), in the spinal ventral longitudinal pathway (Letourneau et al 1988), and in the optic nerve (cf. Cohen et al 1987). In almost all cases, LN expression appears to be transient, occurring at high levels during periods of axonal growth and, at least, in the central nervous system, diminishing later in development. Two notable exceptions are the continued expression of LN isoforms in the basal lamina of peripheral nerve (Chiu et al 1986) and the optic nerves of goldfish (Hopkins et al 1985, Liesi 1985), two favorable sites in the adult for either continued axonal growth or nerve regeneration. In contrast to studies on cell migration, surprisingly few attempts have been made to use antibodies or other reagents to interfere with axonal growth. In one of these, the INO antibody, which prevents cell interactions with an isoform of LN, was shown to slow, but not prevent reinnervation of the iris by sympathetic neurons (Sandrock & Matthew 1987).

Although experimental evidence is largely missing, it seems likely that ECM constituents function as permissive substrates for axonal growth in many areas of the developing nervous system. The ability of many ECM constituents to guide growth cones in vitro has suggested that they might also guide growth cone movements in vivo (cf. Gunderson 1987). In principle, observed heterogeneities in distribution of ECM proteins or changes in their concentrations could direct axonal growth. Recent results indicate that heterogeneity in both the number and function of ECM glycoproteins has been underestimated. Most strikingly, there are many more genes for ECM glycoproteins than was previously appreciated. The collagens, for example, have proliferated in both number of new collagens and number of genes encoding new isoforms of existing collagens (Burgeson 1988). Some of these have quite restricted distributions (cf. Hostikka et al 1990). The recently described heterogeneity in LN is of particular interest to

neurobiologists, because LN has many dramatic effects on neuronal differentiation. In addition to its effects on neurite outgrowth, LN also modulates the activities of neurotrophic factors, regulates expression of transmitter enzymes, and has many other striking effects on the differentiation of neurons, not mimicked by other ECM glycoproteins (cf. Edgar et al 1984, Kalcheim et al 1987, Acheson et al 1986). Although it has been appreciated for several years that 1aminins from different sources have different structures and antigenic properties (cf. Lander et al 1985, Edgar et al 1988), only in the past year, with the discovery of new genes encoding LN homologues, has a definite advance been made in understanding the causes of this heterogeneity.

Merosin is an A-chain homologue that associates with the B1 and B2 chains to form a novel isoform of LN (Ehrig et al 1990). Merosin-containing LN generally appears late in development and is the major LN in adult skeletal muscle, cardiac muscle, and peripheral nerve (Leivo & Engvall 1988, Sanes et al 1990). Specific functions relevant for cell differentiation appear to be differentially encoded by different A chain genes. For example, expression of the classic A chain appears necessary for normal development of kidney epithelial cells in organ cultures of embryonic kidney anlage (Klein et al 1989, Sorokin et al 1990). Consistent with the possibility that A chain genes differentially encode sites relevant for axon growth, recent work has demonstrated that sensory neurons utilize different combinations of integrins to extend neurites on merosin-containing and A-chain containing isoforms of LN (K. Tomaselli et al 1991, unpublished).

S-laminin was originally identified as a synapse-specific component of muscle basal lamina (cf. Sanes & Chiu 1983). Recently shown by sequencing to be a homologue of the LN B1 chain (Hunter et al 1989a), it has not yet been shown to associate with other LN subunits. S-laminin has a much more restricted distribution than LN in vivo (Hunter et al 1989a). Only a subset of neurons, at this point only motoneurons from the ciliary ganglion, appear to interact with it (Hunter et al 1989b). It is associated with sites at which axons cease elongation, suggesting that it effects on neuronal differentiation are quite different from those of other LN isoforms (Covault et al 1987). Recent work has implicated a peptide sequence LRE as the attachment site for motoneurons in S-laminin fusion proteins (Hunter et al 1989b). Since S-laminin is likely to associate with A and B2 chain homologues, the function of this sequence needs to be confirmed by using native isoforms of LN containing this subunit.

The discoveries of merosin and S-laminin make it possible in theory to assemble at least four different LN isoforms. In addition, there is evidence suggesting additional diversity in LN isoforms, not attributable to different genes. The INO antibody was isolated as an inhibitor of the activity of LN-containing neurite outgrowth factors (Matthew & Patterson 1983). It clearly binds an epitope associated with LN-heparan sulfate complexes (Chiu et al 1986). It also inhibits axonal extension on sections of the peripheral nerve where merosin, B1, and B2 are the major visible LN subunits (Sandrock & Matthew 1987b, Sanes et al 1990). Even though this suggests that INO recognizes LN isoforms containing merosin, its distribution in vivo is quite distinct from that of merosin or any other identified LN subunit (Chiu et al 1986, Sanes et al 1990). This suggests that the structure and possibly biological activities of LN can be modified by associating with other molecules, such as proteoglycans.

FN, which promotes axonal outgrowth by peripheral neurons, illustrates how differential splicing may contribute to diversification of the ECM (Hynes 1990). Embryonic chick sensory and sympathetic neurons extend neurites on two non-overlapping fragments of FN, a 75 kDa fragment containing the RGDS sequence and a 33 kDa fragment containing an alternatively spliced FN type III repeat (cf. Humphries et al 1988, Rogers et al 1987). Spinal cord neurons interact poorly with the RGDS-containing fragment, but interact efficiently with the 33 kDa fragment (Rogers et al 1987). These differences in behavior almost certainly reflect differences

in integrin subunit expression by the different populations of neurons. The integrin $\alpha_4\beta_1$ interacts with the alternatively spliced CS-1 domain in the 37 kDa fragment (cf. Guan & Hynes 1990) and is thus probably present on both central and peripheral neurons. The integrins $\alpha_5\beta_1$ and $\alpha_3\beta_1$ both interact with the RGDS-containing fragment of FN (cf. Hemler 1990) and are consequently not likely to be expressed by spinal cord neurons. As mentioned previously, expression of specific domains in other ECM glycoproteins, including tenascin, is also regulated by differential splicing. In principle, this could generate tremendous diversity in the temporal and spatial patterns of ECM protein expression during development of the nervous system.

Tenascin is a particularly intriguing ECM glycoprotein because it is expressed at high levels in the nervous system, has already been implicated by perturbation experiments as a mediator of neural crest and granule cell migration, and has an anti-adhesive as well as adhesive domain (Spring et al 1989; for review, see Erickson & Bourdon 1989). The anti-adhesive domain actually prevents responsive cells from attaching strongly to permissive substrates such as FN (cf. Lotz et al 1989). Tenascin's anti-adhesive activity in vitro suggests that it might function in vivo to inhibit the growth of responsive axons, similar to intrinsic membrane proteins isolated recently from myelin and posterior somite, two tissues avoided by axons in vivo (Caroni & Schwab 1988, Davies et al 1990). In particular, since tenascin is found in boundaries of vibrissae-related barrel fields in the somatosensory cortex of mice (Steindler et al 1989), it has been suggested that its anti-adhesive activity may function in vivo to separate the fields of different afferent inputs. Recent work indicates that there is a family of tenascins and tenascin-like glycoproteins. First, there are several isoforms of tenascin derived from one gene by differential splicing (cf. Spring et al 1989). These differ in the number of FN type III repeats included in the translation product. Still more diversity is provided by a second gene encoding an isoform of tenascin that was discovered recently by virtue of its proximity to the gene encoding complement factor IV (Morel et al 1989). Further heterogeneity in tenascin-like proteins has been discovered by characterization of the J1 family of proteins (cf. Pesheva et al 1989). ECM proteins recognized by J1 antibodies include the major isoforms of tenascin and, in addition, two smaller proteins, J1-160 and J1-180. When examined in the EM, these smaller forms are dimeric and trimeric kinked arm rods, respectively, whereas tenascin is a hexamer of kinked arm rods (Pesheva et al 1989). J1-160 and J1-180 thus have striking structural similarities with tenascin. In contrast to tenascin, which is primarily synthesized by astroglia and fibroblasts (ffrench-Constant et al 1986, Gatchalian et al 1989), the J1-160 and J1-180 are synthesized by oligodendrocytes (Pesheva et al 1989). Like tenascin, J1-160 and J1-180 are nonpermissive substrates for attachment of some cells, including cerebellar neurons and astroglia. Similar to tenascin, J1 isoforms also have anti-adhesive activities on mixed substrates (cf. Pesheva et al 1989, Lotz et al 1989). To summarize, tenascin and its relatives are expressed at high levels in the nervous system. In different parts of the nervous system, evidence suggests that tenascin may have quite different functions, e.g. promoting migration of cerebellar granule cells in the cerebellum but limiting the extent of afferent branching in the barrel fields of the somatosensory cortex (Chuong et al 1987, Steindler et al 1989). The recent discoveries of new genes, differential splicing, and related glycoproteins increase its potential functions and the interest in identifying TN receptors.

To summarize this section, there is comparatively strong evidence that ECM constituents function as permissive substrates for cell and growth cone migration. Except for studies in *C. elegans*, there is little evidence that ECM constituents provide directional cues. Recent discoveries of new genes encoding ECM glycoprotein homologues and differential splicing of transcripts encoding these molecules indicate that the ECM in the developing nervous system may well be much more heterogeneous spatially than was previously appreciated. Thus there is at least the potential for the ECM to provide some of the guidance cues required for establishing migratory routes and axon tracts in the nervous system. At this point, though, ECM

proteins have not yet been shown to distinguish between individual fiber pathways, as has been shown for some cell adhesion molecules, e.g. fasciclins I, II, and III in the insect nervous system (Harrelson & Goodman 1988). On balance, these results suggest that much of the information specifying direction and pathway choice is likely to be provided by other molecules, such as cell adhesion molecules and chemotropic factors (cf. Harrelson & Goodman 1988, Dodd & Jessell 1988).

ECM REGULATION OF SYNAPSE FORMATION

Studies on regeneration of amphibian neuromuscular junctions have demonstrated a critical role for the basal lamina in inducing formation of both pre- and postsynaptic specializations (see Nitkin et al 1983, Sanes & Chiu 1983). The basal lamina at the neuromuscular synapse has been shown to be molecularly distinguishable from non-synaptic basal lamina. ECM glycoproteins present only in synaptic basal lamina include two LN subunits (A and S) and two collagen IV chains [α_3(IV) and α_4(IV)] (Sanes et al 1990); agrin, an apparently novel ECM glycoprotein (Nitkin et al 1987); and, in *Torpedo*, a large proteoglycan named TAP-1 (Carlson & Wight 1987). Studies of these molecules imply that synaptic basal lamina acquires its unique molecular constituents from both neurons and myotubes. Thus, S-laminin is a biosynthetic product of myotubes (Sanes & Chiu 1983). Although its function in the synapse is unknown, its effects on peripheral motoneurons described above suggest that it may function to induce one or more steps in differentiation of the presynaptic terminals of motoneurons. The glycoprotein agrin was identified initially as a factor that induces clustering of acetylcholine receptors on skeletal myotubes (cf. Nitkin et al 1983). Purified agrin has been shown to induce concentrations of both ECM-associated and membrane-associated synaptic molecules on skeletal myotubes. Specifically it clusters acetylcholine receptors, acetylcholine esterase, and butyrylcholine esterase (Nitkin et al 1987, Wallace 1986). Agrin antigen is specifically localized at neuromuscular junctions in vivo (cf. Reist et al 1987), making it a strong candidate to induce clustering of the same molecules in vivo. Agrin is transported anterogradely by motoneurons (Magill-Solc & McMahan 1988). Agrin can also be detected in uninnervated muscle masses (Fallon & Gelfman 1989). Thus, it seems likely that it is synthesized by both neurons and skeletal myotubes. TAP-1 is a large synaptic vesicle–associated proteoglycan that appears to be synthesized by motoneurons in *Torpedo* (Carlson & Wight 1987). An isoform of this proteoglycan is associated with the synaptic basal lamina in the *Torpedo* electric organ. Since it also has properties of an integral membrane protein, it is a good candidate to function as a receptor that mediates binding of the nerve terminal plasma membrane to the synaptic basal lamina.

Although synapse-specific constituents of the ECM have been identified, very little is known about the intracellular signaling mechanisms involved in induction of pre- and postsynaptic differentiation. Extracellular Ca^{2+} is required for the formation of agrin-induced aggregates of acetylcholine receptor (Wallace 1988). Inhibitors of energy metabolism and activators of kinase C both inhibit clustering of this receptor. Future studies extending these promising initial results should be informative.

At this point, it is possible to propose how molecular differences between synaptic and nonsynaptic basal lamina are established. In part, this must reflect the contributions to the synaptic basal lamina of constituents synthesized by motoneurons, such as TAP-1 and agrin and, in turn, must reflect localization in the synaptic basal lamina of muscle-specific products, such as S-laminin. Mechanisms by which ECM constituents synthesized by skeletal myotubes become localized to synaptic basal lamina are not well understood. Preferential transcription of genes encoding synaptic molecules in myotube nuclei located near the synapse is one potential mechanism. The genes encoding the acetylcholine receptor appear to be regulated in this fashion (cf. Goldman & Staple 1989).

ECM REGULATION OF CELL PROLIFERATION AND DIFFERENTIATION

At early stages of development of the CNS, shortly after neural tube closure, the neuroepithelium is a single cell layer in thickness. At this time, germinal neuroepithelial cells in many areas of the nervous system contact at their basal surfaces an ECM that is rich in fibronectin, laminin, thrombospondin, and entactin (e.g. Tuckett & Morris-Kay 1986, O'Shea & Dixit 1988). At later stages, in some parts of the CNS that have undergone considerable cell proliferation (e.g. the retina) contact between germinal cells and overlying basement membrane is maintained. Recent studies on retinal neurogenesis in frogs provide evidence for a role of ECM molecules in controlling the extent of proliferation of retinal germinal neuroepithelial cells. When slices of tadpole retinae are maintained in culture with the surrounding basement membrane intact, neuroepithelial cells in the proliferative zone continue to divide for up to 3 weeks. Removal of the basement membrane by collagenase treatment, however, leads to a significant decline in germinal cell proliferation, and this deficit can be partially ameliorated with a basement membrane extract that is rich in laminin, collagen, and heparan sulfate proteoglycan (Reh & Radke 1988). Therefore, at least in some regions of the CNS, cell proliferation apparently may be stimulated by the presence of proteins within a basal lamina. In regions of the CNS where proliferating cells are not in contact with the basement membrane (e.g. the cerebral cortex), other ECM molecules, including FN, thrombospondin, and tenascin, are often in the proliferative zone (cf. Chun & Schatz 1988, O'Shea & Dixit 1988).

Although the mechanisms by which ECM constituents regulate proliferation of neuroepithelial cells are not well understood, studies on non-neural systems provide evidence for two roles of the ECM. First, the ECM localizes growth factors. FGF, for example, binds heparan sulfate on proteoglycans in the ECM (cf. Saksela et al 1988). This has been shown to protect FGF from proteolysis. As a result, the ECM serves as a reservoir of FGF (cf. Rifkin & Moscatelli 1989). Since FGF is both a potent mitogen and a differentiation factor for embryonic neuroepithelial cells (cf. Bartlett et al 1988), these observations are very relevant for understanding early development of the nervous system. Other growth factors, including a mitogen for Schwann cells, also bind to heparin (e.g. Ratner et al 1988). The activity of TGF-β is regulated in two distinct ways by proteoglycans. First, heparin activates it by dissociating it from an inactive complex with another protein (McCaffrey et al 1989). Second, a cell surface proteoglycan named decorin seems to function as a low-affinity receptor, binding and concentrating TGF-β on the cell surface, where it can then be bound by the high-affinity TGF-β receptor (Boyd & Massague 1989, Ruoslahti 1989). In addition to indirect effects mediated by growth factors, the ECM contains major constituents, such as LN, tenascin, and thrombospondin, that can directly promote cell proliferation. In vitro, LN has been shown to promote proliferation of several cell types, including fibroblasts and Schwann cells (cf. Panayatou et al 1989, McGarvey et al 1984). Thrombospondin and tenascin also promote the proliferation of specific cell types, including smooth muscle cells and fibroblasts, respectively (see Engel 1989). All three proteins contain domains with homology to EGF. In the case of soluble LN, mitogenic activity has been localized within a fragment containing multiple EGF repeats that does not contain the major cell attachment or neurite outgrowth-promoting sites (Panayatou et al 1989). A cell line lacking EOF receptors does not respond to soluble LN, thus making it attractive to imagine that the EOF receptor mediates the mitogenic response. Soluble LN does not compete with EGF for its receptor, however, so the mechanisms of LN's action are unclear. Whatever the mechanisms, the effectiveness of soluble LN suggests that the mitogenic action of LN is due to direct modulation of intracellular second messengers rather than effects on cell adhesion.

ECM REGULATION OF CELL SURVIVAL AND DIFFERENTIATION

The effects of the ECM on neuronal survival and differentiation also seem likely to reflect modulations of cytoplasmic second messenger systems. In particular, LN has striking effects on the survival of early embryonic neurons and on the responsiveness of older neurons to neurotrophic factors. Early embryonic sympathetic neurons require an LN substrate but not a trophic factor to survive in vitro (Ernsberger et al 1989). For initial survival in vitro, embryonic sensory neuron precursors have a similar requirement for an ECM-coated substrate, but not a trophic factor (Ernsberger & Rohrer 1988). At later stages, both sympathetic and sensory neurons become dependent on trophic factors. Some sympathetic neurons remain dependent on a LN substrate (cf. Edgar et al 1984). Those capable of surviving without LN can be maintained on LN by much lower concentrations of NGF than would otherwise be effective. As LN is a prominent constituent of embryonic sympathetic and sensory ganglia at these stages of development, the ECM is likely to be critical in regulating proliferation and differentiation of these cells in vivo. Consistent with this view, embryonic sensory neuron precursors, when separated from the neural tube in vivo, can be rescued from cell death by the trophic factor BDNF only in combination with LN (Kalcheim et al 1987).

The effects in the nervous system of ECM molecules are not limited to neurons. Studies on Schwann cells, the myelin-forming cells of the peripheral nervous system, indicate that constituents of the basal lamina are required for myelination. Agents that disrupt Schwann cell basal lamina production in vitro—e.g. inhibitors of collagen or proteoglycan biosynthesis—prevent myelination of axons in vitro (Carey et al 1987, Eldridge et al 1987). Basal lamina–deficient Schwann cells can be induced to mye1inate axons by the addition of LN. Eldridge et al (1989) have proposed that LN promotes assembly of a normal basal lamina that in turn enables Schwann cells to myelinate the available axons. Schwann cells thus provide an interesting example of an autocrine role for the ECM in inducing expression of a highly differentiated phenotype. The mechanisms by which the ECM promotes Schwann cell differentiation are not understood. Both adhesive interactions that polarize the Schwann cells and modulation of second messenger systems may be important.

ECM components have been shown to regulate differentiation of neural precursor cells into neurons and to regulate the expression of transmitter enzymes in neural cells. Thus, LN has been shown to induce amphibian retinal pigment epithelial cells to express a neuronal phenotype (Reh et al 1987). A substrate-attached factor from conditioned medium, which is probably an isoform of LN, dramatically enhances the ability of NGF to convert neonatal adrenal chromaffin cells to sympathetic neurons (Doupe et al 1985). The same factor is absolutely required for NGF-induced conversion of adult adrenal chromaffin cells. ECM components have also been shown to induce expression of an adrenergic phenotype by neural crest cells and adrenal chromaffin cells (Maxwell & Forbes 1987, Acheson et al 1986). When used as a substrate for adrenal chromaffin cell growth, LN increases the amount of tyrosine hydroxylase over a period of days, but is also able to increase the activity of tyrosine hydroxylase within a few hours (Acheson et al 1986). The latter result suggests that LN acts on tyrosine hydroxylase by regulating the activity of one of the many protein kinases known to modulate its activity (cf. McTigue et al 1985).

MECHANISMS OF ACTION OF ECM CONSTITUENTS AND THEIR RECEPTORS

ECM constituents obviously influence many aspects of cell behavior during development of the nervous system. Knowledge of the mechanisms by which ECM components exert their effects is still sketchy; however, in the case of integrin-mediated interactions with the ECM, a picture has begun to emerge. Upon binding of ligand, integrins appear to transduce signals

across the plasma membrane in two ways: by interacting directly with cytoskeletal proteins, and by regulating the levels of second messengers. Some integrin-mediated actions may, in principle, reflect simply effects of interactions with the cytoskeleton. The functions of macromolecules such as FN in modulating migration of neural crest celis, for example, seem potentially understandable in thisconceptual framework. Other effects of ECM constituents, though, such as effects upon cell survival, mitosis, or trans-determination, are difficult to explain as simple consequences of adhesion. Instead, they seem much more easily explicable if ECM macromolecules also influence the intracellular second messenger systems of the cells. Studies on integrins, particularly in lymphocytes and platelets, provide evidence that they can indeed transfer signals across the membrane that influence both the cytoskeleton and intracellular second messenger systems. There is also convincing evidence that intracellular metabolism can alter the ligand-binding functions of several integrin receptors.

An important effector of integrin-mediated signaling is likely to be a change in integrin structure, mediated by proteins inside and outside the cell. With regard to extracellular mediators, both binding of ligands and of divalent cations have been shown to alter integrin conformation (Parise et al 1987). Regulated conformation-dependent epitopes have been identified on the platelet integrin α_{IIb}/β_3 and on the lymphocyte integrins by using monoclonal antibodies (Frelinger et al 1988, Dransfield & Hogg 1989). The epitope on glycoprotein α_{IIb}/β_3 is located in the α_{IIb} subunit sequence at a site that is well separated in the linear sequence from the ligand and cation-binding sites, a position suggesting that it is a reporter for a significant structural change (Frelinger et al 1988).

Binding of antibodies and ligands to external domains of integrin receptors has been shown to regulate several cytoplasmic events. Among these, binding to an ECM protein-coated substrate promotes the formation of focal contacts in which integrins are associated with the termini of F-actin filaments and several other proteins, most notably talin, vinculin, and α-actinin (cf. Burridge et al 1988, Fath et al 1989). Each of these proteins has been detected in growth cones (cf. Letourneau & Shattuck 1989). Purified integrins have been shown to bind talin, which can in turn bind vinculin (Horwitz et al 1986, Tapley et al 1989). Based on observed interactions of these proteins in vitro, it has been proposed that talin, vinculin, and α-actinin link the integrins to the cytoskeleton (reviewed in Burridge et al 1988). Analysis of early events in cell attachment has demonstrated that binding to ligand induces integrin-talin coaggregation at sites of membrane-ECM contact, which is followed by association of other cytoskeletal proteins (Mueller et al 1989). Agents that disrupt adhesion, such as RGD-containing peptides, have been shown to dissociate these cytoskeletal proteins from integrins. In addition to regulation by adhesion, colocalization can also be regulated by intracellular messengers and protein phosphorylation (cf. Tapley et al 1989).

The cytoplasmic domain of the integrin β_1 subunit has been shown to be important for its colocalization with the cytoskeleton and for its function in cell adhesion (Solowska et al 1989, Hayashi et al 1990). When expressed in heterologous cells, integrin β_1 subunits lacking the cytoplasmic domain associate with α subunits to form receptors that can still bind ligands in biochemical assays. Within the cell, though, they do not localize to focal contacts and are not effective at promoting cell adhesion. This indicates that integrins must interact with the cytoskeleton to promote cell adhesion (cf. Lotz et al 1989). The cytoplasmic domains of several integrin subunits are substrates in vivo for tyrosine or serine-threonine protein kinases (cf. Tapley et al 1989). Some evidence suggests that phosphorylation of these domains regulates associations of integrins with the cytoskeleton.

Regulation of integrin interactions with the cytoskeleton is important for controlling the motility of cells and growth cones. In stationary cells, ligand binding by integrins leads to their accumulation in stable focal adhesions. Integrin associations with adhesive zones are

comparatively stable (Duband et al 1988). In motile cells, however, integrins have becn shown to diffuse rapidly in the membrane, thereby indicating that associations with the ECM and cytoskeleton are transient. The importance of actin dynamics in growth cone motility and guidance is well established (cf. Smith 1988, Mitchison & Kirschner 1988). Growth cone motility is driven in part by the behavior of the actin-based cytoskeleton. The flow of actin depends on actin polymerization at the leading edge of cells, energy-dependent retrograde movement of actin filaments, and depolymerization of these filaments in the cell interior. Integrin-mediated interactions with F-actin can in principle regulate any of these steps. Lateral interactions have the potential to slow retrograde movements, generating traction against the substrate. Interactions at filament ends may regulate capping, polymerization, or depolymerization. Receptors interacting with moving filaments of actin will generate tension inside the growth cone, which can potentially regulate the shape and dynamics of all cytoskeletal elements in the growth cone. In addition, stretch-activated and stretch-inactivated K^+ channels have recently been discovered in growth cones (cf. Sigurdson & Morris 1989). By regulating membrane potential, these channels may modulate voltage-dependent Ca^{2+} channels and cytoplasmic Ca^{2+} levels, which in turn may affect several other second messengers. As Ca^{2+} has been shown to be an important modulator of growth cone motility (cf. McCobb et al 1988), critical examination of the functions of these channels should prove interesting.

Binding to integrin receptors of antibodies and ligands has been shown to regulate other cytoplasmic events, at least some of which are not related to cell shape or adhesion. Some anti-integrin monoclonal antibodies can initiate signaling cascades; for example, binding of some monoclonal antibodies to the platelet integrin $\alpha_{IIb}\beta_3$ induces platelet activation (Jennings et al 1985). During platelet activation, tyrosine protein kinases phosphorylate proteins in three waves. The function of the platelet integrin glycoprotein $\alpha_{IIb}\beta_3$ is required for the third wave of phosphorylation, which does not occur in platelets lacking this integrin or in platelets activated in the presence of RGDS peptides (Ferrell & Martin 1989). Similarly, in fibroblasts, interactions of the fibronectin receptor with fragments of fibronectin or with antibodies capable of crosslinking this receptor induce expression of genes encoding ECM-degrading proteases (Werb et al 1989). These effects are not related to visible changes in cell shape or cytoskeletal organization. Consistent with this evidence implicating integrins as modulators of intracellular signaling, the functions of integrins have been shown to be required for differentiation of several cell types (e.g. myoblasts) on substrates for which they are not essential for cell adhesion (cf. Menko & Boettiger 1987).

Mechanisms by which integrins regulate signal cascades are not understood. In platelets, there is evidence that the integrin $\alpha_{IIb}\beta_3$ can associate with an additional membrane-associated protein, CD9, which itself is involved in platelet activation (Slupsky et al 1989). Similar associations of other proteins with integrins may potentially be involved in transducing signals in other cells. Intriguingly, the cytoplasmic domain of an individual integrin subunit has typically proven to be highly conserved among species (cf. Marcantonio & Hynes 1988), but α and β subunits within a species have quite different cytoplasmic domains (cf. Takada & Hemler 1989). It seems possible, therefore, that these domains interact with additional, unknown proteins, and that different subunits have significant differences in their interactions and signaling capabilities. This may provide at least a partial explanation for the existence of multiple integrin receptors that interact with a single ECM glycoprotein (Table 1).

Intracellular metabolism can alter both the structure and function of the extracellular domains of the integrins. The ligand-binding functions of several integrins, most strikingly the platelet integrin ($\alpha_{IIb}\beta_3$ and the lymphocyte integrins $\alpha_{LFA\text{-}1}\beta_2$ and $\alpha_{Mac\text{-}1}\beta_2$, have been shown-to be regulated by ligand binding to other receptors, by physiological stimuli, and by agonists or activators of second messenger systems (cf. Frelinger et al 1988, Dustin & Springer 1989).

Appearance of a conformation-dependent epitope shared by each of the lymphocyte integrins ($\alpha_{LFA-1}\beta_2$, $\alpha_{Mac-1}\beta_2$,and $\alpha_{gp150}\beta_2$) has been shown to require not only Mg^{2+} binding but also cellular metabolism (Dransfield & Hogg 1989). Phosphorylation of cytoplasmic domains of integrin subunits seems likely to be one mechanism by which integrin-mediated cell adhesion is regulated (Tapley et al 1989, Danilov & Juliano 1989). This may be one mechanism by which trophic factors and chemotropic factors influence neuronal growth cone interactions with the ECM.

Additional mechanisms for rapid regulation of integrin function have been described in lymphoid cells and may be useful paradigms for understanding effects of substrates, growth factors, and chemotropic agents on growth cone behavior. Large fractions of the integrins ($\alpha_{Mac-1}\beta_2$ and $\alpha_{gpl50}\beta_2$ are sequestered in intracellular vesicles in neutrophils (Bainton et al 1987). Physiological stimuli induce exocytosis of these granules, transferring them to the cell surface. Factors that induce aggregation of these receptors or their ligands have also been shown to enhance their activity (cf. Hermanowski-Vosatka et al 1988). In a series of ingenious experiments in which a membrane-impermeant, cleavable crosslinking agent was used, integrins have been shown to cycle rapidly between intracellular and surface compartments in fibroblasts, being inserted preferentially at leading edges of motile cells (Bretscher 1989). By similar mechanisms, chemotropic agents and trophic factors could potentially regulate growth cone behavior by regulating the cycling or by targeting the insertions of integrins and other adhesion molecules.

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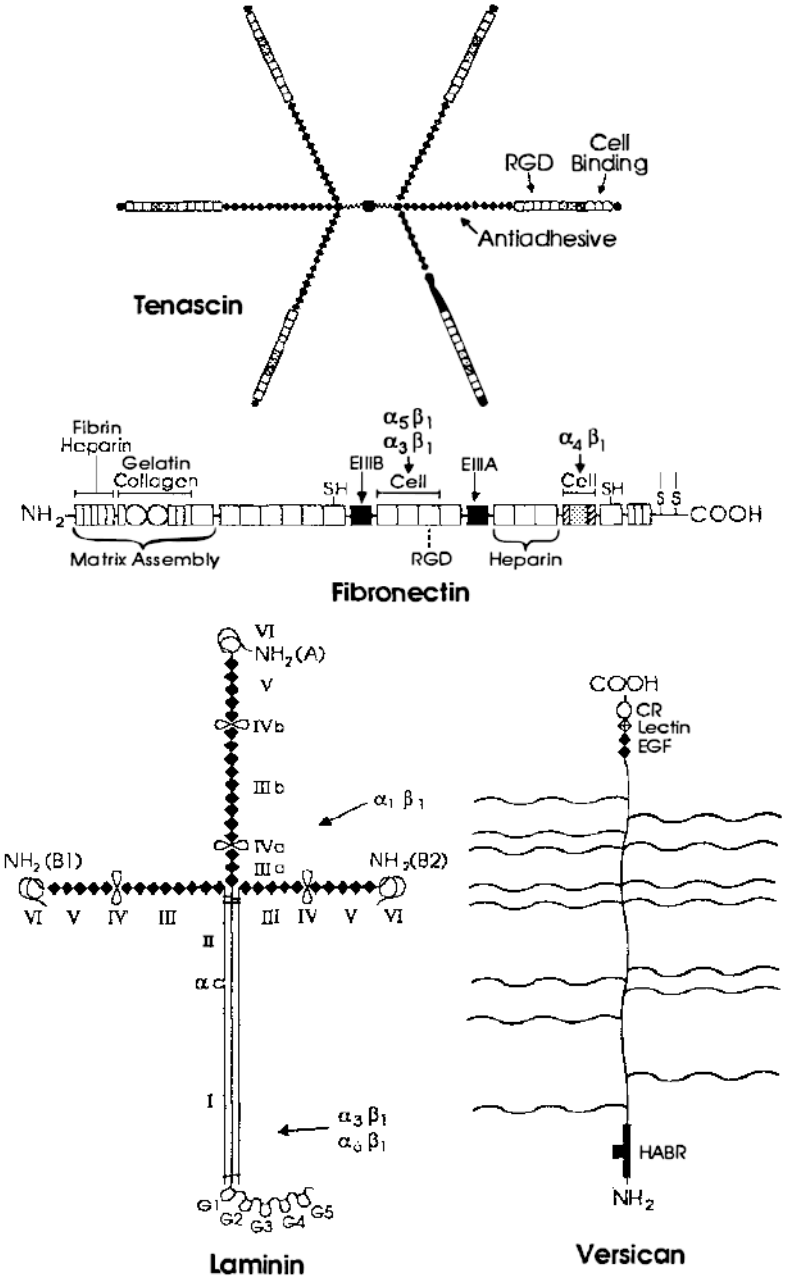

Figure 1.

Examples of extracellular matrix glycoproteins and proteoglycans. Schematic structures for the adhesive glycoproteins tenascin, fibronectin, and laminin and for the proteoglycan versican arc presented. Fibroncctin is a dimer, but only one subunit is depicted. The sulfhydryl residues near its carboxyl terminus mediate its dimerization. The positions of FN type I [▯] FN type II [○], and FN type III [□] repeats are indicated in fibronectin. FN type III repeats are also found in tenascin, where their positions are marked by the same symbol [□]. Shaded FN type III repeats in fibronectin and tenascin are encoded by differentially spliced exons and are thus missing from some forms of these glycoproteins. The positions of domains homologous to those in the EOF-precursor, the EOF repeats [◆], are indicated in tenascin, laminin, and versican. The positions of domains homologous to functional domains in lectins [⌖],

complement regulatory proteins [○], and hyaluronic acid-binding proteins [▬] are indicated in versican. Note that many of these domains are also found in receptors, some of which are illustrated in Figure 2. In tenascin, the positions of the ROD sequence, the major cell-binding domain, and the region implicated in the anti-adhesive activity of this protein are shown. Similarly, in fibronectin, the positions of the RGD sequence and domains mediating interactions with fibrin, heparin, gelatin, collagen, and cells are indicated. Both the N-terminal region and the major cell attachment site containing the RGD sequence are required for assembly of fibronectin into the extracellular matrix. The regions of fibronectin recognized by the integrins $\alpha_5\beta_1$, $\alpha_3\beta_1$ and $\alpha_4\beta_1$ are indicated by arrows. Note that the integrin $\alpha_4\beta_1$ recognizes a sequence located in a differentially spliced exon that is not present in all forms of fibronectin. In laminin, homologous domains present in the A, B1, and B2 chains are indicated by roman numerals. Loops indicate the locations of putative globular domains, many of which have been visualized in the electron microscope. Domains I and II in the long arm of laminin's cruciform structure represent regions in which the three subunits associate in a triple coiled rod. The major proteolytic fragments of laminin E3, E8, E1, and E1-4 described in the text contain approximately the following domains: E3 (G4 and G5), E8 (G1, G2, G3, and most of I), E1 (part of II, III, IV, IIIa, IVa, IIIb, IVb), EI-4 (part of II, III, IIIa, IIIb, IV, IV', IVa, IVb, V, and VI). As indicated, the binding domains of the integrins $\alpha_1\beta_1$, $\alpha_3\beta_1$ and $\alpha_6\beta_1$ have been roughly mapped by using these fragments. The site utilized by the integrin $\alpha_2\beta_1$ has not yet been defined. The wavy lines in versican indicate the positions of several chondroitin sulfate side chains. All of these glycoproteins contain N-linked carbohydrate, but this is not shown.

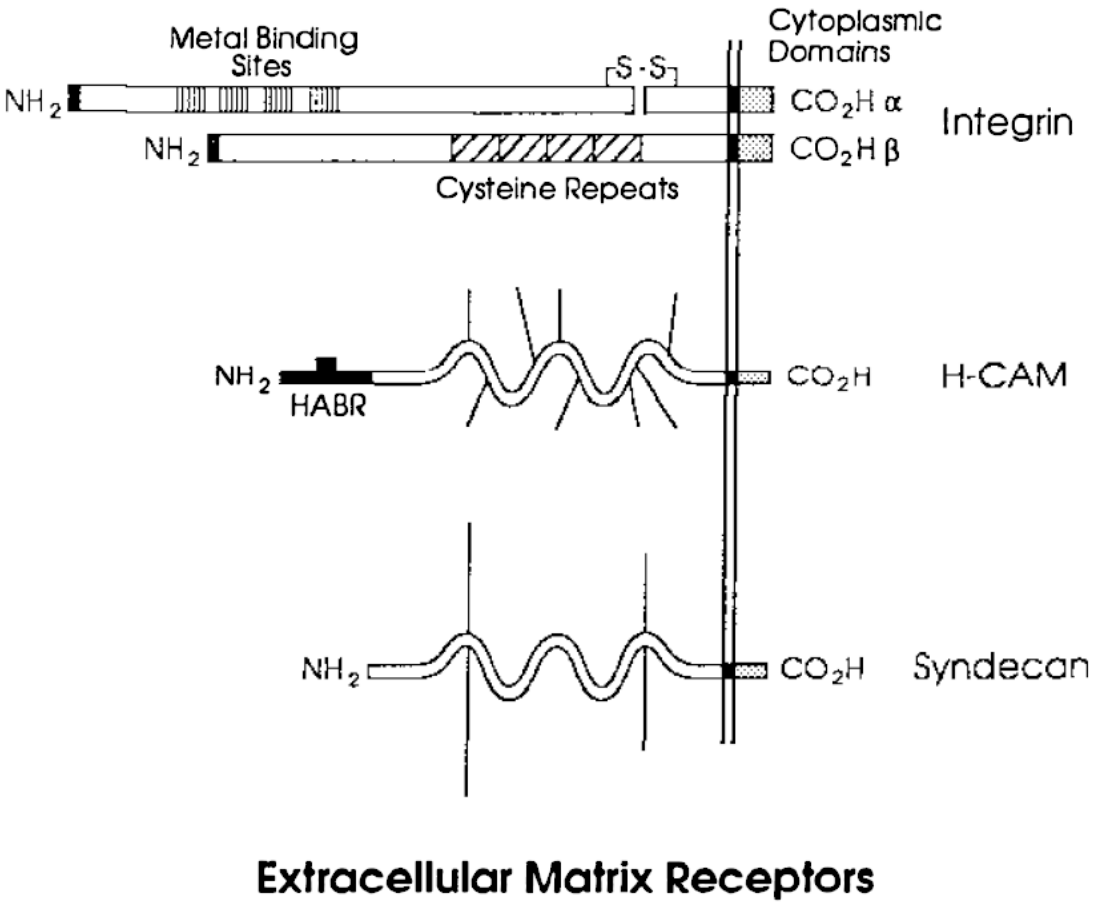

Figure 2.

Extracellular matrix receptors. Examples of three classes of integral membrane glycoprotein receptors are illustrated in this figure. An integrin similar to the fibronectin receptor $\alpha_5\beta_1$ is shown *above*. The α subunit contains four putative metal-binding sites, a site at which it is cleaved into large and small fragments during maturation, a transmembrane domain, and a cytoplasmic domain (Argraves et al 1987). Some integrin α subunits contain I domains inserted at approximately the position of the first metal-binding site, and lack protease cleavage sites. The β subunit contains four repeats of a cysteine-rich motif, a transmembrane, and a cytoplasmic domain. H-CAM, also named the Hermes antigen or CD44, is illustrated in the *middle* with positions of a hyaluronic acid–binding domain [▬]. The positions of several carbohydrate chains are indicated by *thin lines.* The structure of syndecan, a proteoglycan that mediates binding of several cell types to several collagens and fibronectin is illustrated at the *bottom*. Positions of glycosaminoglycan chains are indicated by *thin lines*.

Table 1

Summary of integrin heterodimers and their ligands

Class	M_r	α Subunit	M_r	I domain	Ca/Mg sites	Cleaved	Ligands	References
β_1	115K	α_1	200K	+	3	−	col, LN (E1)	*a*
		α_2	150K	+	3	−	col, LN[1]	*b, c*
		α_3	105K		3[2]	+	LN (E8), FN[4], col[1]	*c, d*
		α_4	140K	−	3	+/−[3]	FN (CS-1), VCAM-1	*e*
		α_5	150K	−	4	+	FN (RGD)	*f*
		α_6	150K	−	3	+	LN (E8)	*g*
		α_7	130K	?[2]	?[2]	+	LN	*h*
		α_{VN}	150K	−	4	+	FN[4], VN[1, 4]	*i*
β_2	90K	α_{LFA-1}	l70K	+	3	−	I-CAM-1, I-CAM-2	*j*
		α_{Mac-1}	180K	+	3	−	C3bi[4], FG	*k*
		α_{p150}	l50K	+	3	−	?	
β_3	95K	α_{IIb}	136K		4	+	FN[4], VN[4], vWF[4], FG[4]	*l*
		α_{VN}	150K		4	+	VN[4], TS[4], vWF[4], FG[4]	*m, n*
β_4	205K	α_6	150K		3	+	?	*o*
β_5	90K	α_{VN}	150K		4	+	VN[4], FN[4]	*n*
β_p	95K	α_4	l40K	−	3	+/−[3]	?	*p*

Six integrin classes, distinguished by distinct but homologous β subunits, are shown, Each β subunit can associate noncovalently with a subgroup of homologous α suhunits. Tntegrin nomenclature is described in Hemler (1990). The numerical designations of $\alpha_{1\text{-}6}$ correspond to the human T cell VLA antigen α subunit nomenclature. The indicated subunit M_rs were determined by SDS-PAGE in nonredueing conditions. Note, some a/β heterodimers recognize more than one ligand. In some cases a ligand is recognized only by a subset of the cells expressing a heterodimer. Additional ligands may yet be discovered for those receptor heterodimers listed, Additional α and β subunits and additional associations between listed α and β subunits are also being discovered. The reference list below is selective and does not always list original reports because of limited space.

Abbreviations: col, collagen; FG, fibrinogen; FN, fibronectin; ICAM, intercellular adhesion molecule: LN, laminin; TN, tenascin; TS, thrombospondin; VCAM, vascular cell adhesion molecule; VN, vitronectin; vWF, von Willebrand factor; (RGD), RGDS site in FN; (CS-1), CS-1 site in FN; (E1) and (E8), elastase fragments of LN.

[1] Indicates ligands recognized in some, but not all cells.

[2] The sequence was not completed at the time of this review.

[3] Cleavage of α_4 is variable.

[4] The RGD sequence is on this ligand and appears to be used by this receptor.

[a] Turner el al (1989), Hall et al (1990), Ignatius et al (1990), Tamkun et al (1986).

[b] Takada & Hemler (1989), Elices & Hemler (1989), Languino et al (1989).

[c] Wayner & Carter (1987), Staatz et al (1989).

[d] Gehlsen et al (1989), Tsuji et al (1990).

[e] Guan & Hynes (1990), Holzmann et al (1989), Elices et al (1990), Takada et al (1989).

[f] Pytela et al (l985a).

[g] Hall et al (1990), Tamura et al (1990).

[h] Kramer et al (1989).

[i] Vogel et al (1990), Suzuki et al (1985).

[j] Marlin & Springer (1987), Staunton et al (1989).

[k] Wright et al (1987).

[l] Pytela et al (1986), Fitzgerald et al (1987).

[m] Pytela et al (1985b), Lawler et al (1988).

[n] Cheresh et al 1989), Suzuki et al (1990).

[o] Kajiji et al (1989), Suzuki & Naitoh (1990), Tamura et al (1990).

[p] Holzmann & Weissman (1989).