Characterization of the 67 kDa laminin binding protein
by Terry Hinz Landowski

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology
Montana State University
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Abstract:
In order to successfully complete the process of metastasis, tumor cells must adhere to and invade the extracellular matrix basement membrane. As an essential step in the dissemination of metastatic disease, the adhesive event represents an opportunity for therapeutic intervention. Small synthetic peptides, which mimic the binding domain of an extracellular matrix ligand, have been shown to be effective in blocking the adhesion of tumor cells to basement membrane molecules, thereby terminating the metastatic process. However, before these agents can be effectively utilized in the treatment of human disease, the biological activities of their target proteins must be fully characterized.

Cell surface expression of the 67 kDa laminin binding protein has been shown to correlate with the metastatic potential of many solid tumors. Unique structural features of the cDNA deduced amino acid sequence of this protein include the lack of a signal sequence for plasma membrane localization, and the absence of a hydrophobic domain characteristic of transmembrane proteins. In addition, the isolated protein displays an apparent molecular weight of 67 kDa, while the cDNA is sufficient to encode only a 32 kDa protein. The goal of this study was to determine the mode of cell surface association of the 67 kDa laminin binding protein, and to identify the mechanisms responsible for the discrepancy between the predicted and the observed molecular weight.

The dhfr mutant CHO cell line, DG44CHO, was utilized as a homotypic overexpression. system in order to obtain sufficient protein for biochemical analyses. This system also provided an opportunity to assess the phenotypic affects of overexpression of the 67 kDa laminin binding protein. Surface expression of the 67 kDa laminin binding protein was found to be independent of mRNA levels in the overexpression system, indicating the possibility of a post translational regulation mechanism.

Treatment with endoglycosidases had no apparent affect on the molecular weight of affinity purified protein, indicating that post translational modification with carbohydrates is not likely to be responsible for the molecular weight shift. Transesterification and hexane extraction of the 67 kDa laminin binding protein demonstrated the presence of covalently bound fatty acids. While the quantity of lipids isolated is not likely to be directly responsible for the observed molecular weight shift, they may provide a mechanism for membrane localization.

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OF THE

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ABSTRACT

In order to successfully complete the process of metastasis, tumor cells must adhere to and invade the extracellular matrix basement membrane. As an essential step in the dissemination of metastatic disease, the adhesive event represents an opportunity for therapeutic intervention. Small synthetic peptides, which mimic the binding domain of an extracellular matrix ligand, have been shown to be effective in blocking the adhesion of tumor cells to basement membrane molecules, thereby terminating the metastatic process. However, before these agents can be effectively utilized in the treatment of human disease, the biological activities of their target proteins must be fully characterized.

Cell surface expression of the 67 kDa laminin binding protein has been shown to correlate with the metastatic potential of many solid tumors. Unique structural features of the cDNA deduced amino acid sequence of this protein include the lack of a signal sequence for plasma membrane localization, and the absence of a hydrophobic domain characteristic of transmembrane proteins. In addition, the isolated protein displays an apparent molecular weight of 67 kDa, while the cDNA is sufficient to encode only a 32 kDa protein. The goal of this study was to determine the mode of cell surface association of the 67 kDa laminin binding protein, and to identify the mechanisms responsible for the discrepancy between the predicted and the observed molecular weight.

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CHAPTER 1

INTRODUCTION

Although most tumors arise from the transformation of a single cell, by the time the tumor has become clinically detectable, it has undergone multiple genetic changes, resulting in a high degree of heterogeneity. This process is known as tumor progression, and is defined as the "independent, irreversible gain or loss of unit characters with time" (B. Pierce, Histopathobiology of Neoplasia, Keystone CO, 1991). Many of the phenotypic characteristics associated with this process confer a selective growth advantage to a subset of cells within the tumor (Lu and Kerbel, 1994). Antigen expression, growth factor and hormone responsiveness, drug resistance, and metastatic capability are but a few of the heterogeneities documented in human cancer. The most life threatening aspect of any malignant tumor is its ability to invade local tissues and metastasize to a distant site.

The Cascade Theory of Metastasis

Metastasis is defined as the outgrowth of a tumor at a site discontinuous with the primary growth. It can proceed via the hematogenous and/or the lymphatic routes. Hematogenous metastasis is easier to study, and so, better characterized. The process of hematogenous metastasis has been characterized as a series of sequential events, which can be summarized in the following steps: 1) initial transformation and outgrowth of the tumor;   2)

neovascularization; 3) invasion of the extracellular matrix and intravasation into the vasculature; 4) intravascular transport of tumor cells to a distant site; 5) adhesion to the endothelium and subendothelial basement membrane; 6) extravasation and migration into the parenchyma of the target organ; and 7) outgrowth of the secondary tumor. Each of these events requires specialized functions, and all must be successfully navigated by the tumor cells if they are to establish a distant, secondary growth. As such, metastasis is a highly inefficient process. Yet the majority of deaths attributed to cancer are due not to the primary tumor, but rather, to disseminated disease.

The lifespan of most solid tumors can generally be divided into two phases of growth. The first growth phase is avascular, and is typically quite slow. During this time, the metabolic requirements of the tumor cells are supplied through passive diffusion. Once the tumor reaches approximately 2 mm in diameter, it can often reach an equilibrium where the rate of cell death at the interior of the tumor is approximately equivalent to the growth at the periphery. This stage can last from a few months to several years, often without clinical detection. Metastases are rarely associated with this phase, an observation that has been considered to be indicative of the importance of the vascular system to tumor dissemination.

Further expansion of the malignant growth beyond approximately 2-4 mm requires a direct blood supply to the tumor. This occurs via a process known as tumor angiogenesis. Tumor angiogenesis takes place through the sprouting of

capillaries from existing vessels of the host tissue, and migration of endothelial cells towards the tumor. This response is initiated by the hypoxia-induced expression of genes for angiogenic factors, and is modulated by the angiogenic molecules expressed, some of which promote proliferation of the endothelium, and others of which enhance endothelial migration (reviewed in Folkman and Shing, 1992; Blood and Zetter, 1990). Tumor derived angiogenic factors which have been shown to be important in the proliferation of the endothelium include; angiogenin, basic fibroblast growth factor (bFGF), transforming growth factor α (TGFα), and vascular endothelial growth factor (v-EGF). Infiltrating inflammatory cells provide an additional source of angiogenic factors. Using the mast cell deficient W/W^v mouse model, it has been shown that tumor angiogenesis and spontaneous metastasis are reduced in the absence of matrix bound bFGF (Starkey et al., 1988).

The host tissue and extracellular matrix environment actively participate in vascularization of the tumor (reviewed in Liotta et al., 1991). One of the first observable responses to an angiogenic stimulus is the dissolution of the subendothelial basement membrane surrounding the existing microvessels. *In vitro* assays have shown that endothelial cells stimulated by angiogenic factors are induced to produce matrix degrading proteinases including type IV collagenases, serine proteinases, and other matrix metalloproteinases (Weinstat-Saslow and Steeg, 1994). Endothelial matrix degradation proceeds in a manner highly controlled by the presence of proteinase inhibitors in the

surrounding tissue stroma. This is a normal physiological phenomenon during tissue remodelling in development and wound healing, which is halted when the angiogenic stimulus is removed.

Once the tumor becomes vascularized and enters the second, more expansive phase of growth, tumor cells also secrete matrix degrading enzymes. As with angiogenic degradation of the extracellular matrix, the process is influenced by both tumor and host factors, and many of the same proteolytic enzymes and proteinase inhibitors are implicated. However, the invasive phenotype of a metastatic tumor is characterized by an imbalance favoring matrix proteolysis (Liotta et al., 1991).

Several classes of proteolytic enzymes have been associated with invasive carcinomas. The matrix metalloproteinase family are enzymes that can be classified based on their substrate preference: interstitial collagenases, which are highly specific for the degradation of type I collagen; stromelysins, which degrade proteoglycans, the non-collagenous domains of type IV collagen, and glycoproteins such as laminin and fibronectin; and gelatinases, which are highly specific type IV collagenases (reviewed in Kleiner and Stetler-Stevenson, 1993). Two forms of collagenases specific for type IV collagen have been identified, one with a molecular weight of 72 kDa, and the other of 92 kDa. All classes of matrix metalloproteinases are secreted as inactive zymogens, and must be activated by the disruption of a zinc-sulfhydryl interaction in the catalytic center. This results in a conformational rearrangement and the cleavage of the amino

terminal domain of the protein. The *in vivo* mechanism of this activation is unknown, but presumably involves another cell surface or matrix-associated proteinase. Elevated type IV collagenase activity has been implicated as particularly relevant to the metastatic phenotype of many solid tumors (Levy et al., 1991; Miyazaki et al., 1994). Normal and benign tissues express very low or negligible amounts of this enzyme. In contrast, highly metastatic carcinomas of the breast, colon, and gastric mucosa all have been shown to express high levels of 72 kDa type IV collagenase.

Secretion and activation of proteinases may not be sufficient to establish the metastatic phenotype, as the invasive properties of the tumor are balanced by the host expression of proteinase inhibitors. The tissue inhibitors of metalloproteinases (TIMPS) are a family of molecules that bind to both the latent and active forms of the type IV collagenases, inhibiting their proteolytic function (reviewed in Stetler-Stevenson et al., 1993). Recent studies have shown an inverse correlation between TIMP-2 expression and the invasive phenotype of several transformed cell lines (Ponton et al., 1991; Lotz and Guerne, 1991). Furthermore, non-invasive cells can be rendered invasive by the transfection of antisense mRNA which inhibits the expression of TIMP-2 (Khokha et al., 1989). Thus, the degradation of the extracellular matrix is characterized by an imbalance of the positive and negative regulatory activities of the tumor cells and the host tissues.

The metastatic potential of tumors invading the vasculature is modulated

by homotypic cell surface receptors. Tumors expressing high levels of cell-cell adhesion molecules are less likely to shed cells into the circulation, while decreased expression of homotypic adhesion molecules allow single cells or emboli of tumor cells to break away from the primary tumor. One class of molecules implicated in the metastatic process is the cadherins. E-cadherin is an epithelial cell adhesion receptor preferentially expressed at sites of cell-cell contacts. Loss of E-cadherin expression has been shown to correlate well with the metastatic phenotype, while over expression of E-cadherin in transformed cell lines reduces spontaneous metastasis in animal models (Behrens, 1993).

Intravascular survival of tumor cells *en route* to a metastatic site is the next obstacle in the metastatic cascade. Once the cells have gained entry into the vasculature, they must survive a hostile environment and evade the host immunological responses. Using radiolabelled tumor cells, Fidler *et al.* have shown that less than 1% of the cells are still viable 24 hours after entry into the circulation, and fewer than 0.1% eventually survive to establish a secondary tumor (Fidler, 1970). One mechanism that has been proposed to enhance the intravascular survival of tumor cells is the formation of emboli with platelets and other hematopoietic cells. This is based on the observation that patients with thrombocytopenia display fewer metastases than do patients with normal platelet counts. However, platelet-tumor emboli have not been directly demonstrated in clinical assessments.

The next step in the metastatic cascade is that of adhesion to the

endothelium and subendothelial basement membrane, a prerequisite to extravasation. Endothelial cells lining the vasculature have historically been considered as passive players in the metastatic cascade. More recent evidence has disproved this dogma, and multiple endothelial cell adhesion receptors have been identified as relevant to tumor extravasation. Many of these adhesion molecules, such as ICAM-1, ICAM-2, VCAM, and E-selectin are inducible receptors that also mediate leukocyte adhesion during inflammation. Activation of the endothelium by exposure to inflammatory cytokines such as interleukin-1, endotoxin, or tumor necrosis factor-α has been shown to markedly enhance the adhesion of tumor cells to an endothelial monolayer *in vitro* (Rice et al., 1988). Furthermore, pretreatment of mice with IL-1 has been shown to increase the metastasis of melanoma *in vivo*.

It has also been proposed that these endothelial adhesion molecules are responsible for the site-specific-metastasis of certain tumor types to a preferential target organ (reviewed in Zetter, 1993). For example, VCAM is preferentially expressed on lung endothelium, which is a frequent site of melanoma metastasis. A counter receptor for VCAM is the integrin $\alpha_4\beta_1$, whose expression on melanoma cells has been shown to correlate with a high metastatic potential. E-selectin is particularly abundant in the sinusoids of the liver, which is a common target organ for colon carcinomas expressing a carbohydrate ligand for E-selectin, Sialyl Lewis X. Although much of the evidence is circumstantial, it is becoming increasingly evident that adhesion

molecules are, at least, partially responsible for the predilection of tumors for specific organs.

The initial adhesion of tumor cells to the endothelium is generally followed by endothelial cell retraction, which exposes a portion of the subendothelial basement membrane. A second, more stable adhesive interaction then takes place between tumor cell surface receptors and extracellular matrix molecules. Since the basement membrane poses the most formidable barrier the cells will encounter, this adhesive event between circulating tumor cells and the subendothelial basement membrane is often cited as the rate limiting step. It is also considered to be the last opportunity for effective therapeutic intervention.

The principal components of basement membrane extracellular matrix are; collagens, types IV and V; laminins; and heparan sulfate proteoglycan. Of these, type IV collagen and laminin have been shown to be the primary ligands for tumor cell receptors in metastatic dissemination, and several receptors have been identified, including both integrin and non-integrin proteins. Once the cells have become adherent to the basement membrane, signal transduction is initiated, promoting the secretion of proteolytic enzymes and motility, allowing the tumor cell to traverse the extracellular matrix.

The final step in the metastatic cascade is the outgrowth of the secondary tumor, at which point the process may begin again and produce "metastases from metastases." The properties of a particular organ which allow the growth of a secondary tumor are only now being defined (Doerr et al., 1989; Tressler

et al., 1989), but it has been recognized for over a century that "congenial soil" is required for successful establishment of the metastatic "seed" (Paget, 1889).

Composition of the Extracellular Matrix Basement Membrane

Basement membranes are specialized extracellular matrices which form a structural barrier between epithelial cells and the underlying connective tissue. They also underlie endothelial cells and surround other cell types such as adipocytes and muscle cells. The typical morphology of basement membranes is that of a fibrous sheet-like structure, typically 20-30 nm thick, composed of collagens, primarily types IV and V, proteoglycans, and glycoproteins. The compositional ratios of these elements in a given basement membrane are reflective of both the developmental stage and the specific function of the epithelial tissue. Besides providing structural support, the extracellular basement membrane acts as a molecular sieve to allow the selective diffusion of small molecules in and out of the tissues it surrounds. It also participates in regulating the growth, differentiation, migration, and metabolic activity of adjacent cells via cell surface receptors for specific components.

Type IV collagen

Type IV collagen is produced by epithelial, myoepithelial, and endothelial cells, and is found exclusively in basement membranes (reviewed in Yurchenco and Schittny, 1990; Labat-Robert et al., 1990). In contrast to other types of collagen, type IV collagen is not proteolytically processed prior to chain

assembly, and forms a three dimensional lattice which provides the major structural network of basement membranes. Three polypeptide chains $[\alpha1(IV)_2\alpha2(IV)]$ have been shown to self assemble in a highly sequential manner to form collagen-like triple helices with extensive internal loops. Initial dimerization occurs between the carboxyl-terminal globular domains of two polypeptides. The third polypeptide is added in a parallel orientation, and fibril assembly proceeds from carboxyl to amino terminal, with lateral disulfide and non-reducible interactions between internal loops stabilizing an irregular polygonal structured matrix. The type IV collagen found in extracellular basement membranes is generally considered to be responsible for the flexibility and tensile strength of the structure. It also provides a structural scaffold with adhesive sites for the non-collagenous basement membrane components.

Proteoglycans

Proteoglycans are complex molecules containing anywhere from a few to several hundred glycosaminoglycan chains covalently attached to a protein core. Glycosaminoglycans are linear polymers of repeated disaccharides attached to the protein core by specific carbohydrate sequences. The best characterized of the proteoglycans are those of cartilage, in which several protein cores are frequently found attached to a single hyaluronate molecule by a smaller link protein, resulting in a heterogeneic macromolecular complex with molecular weight ranging from 50,000 to over 1,000,000 daltons. Proteoglycans are highly negatively charged, a feature that contributes to their proposed

function of molecular sieves. The most striking aspect of the proteoglycans is the high degree of molecular variability provided by the glycosaminoglycan side chains. Each proteoglycan molecule may contain one or two different types of glycosaminoglycans at varying densities, and each glycosaminoglycan may be of different lengths. Further heterogeneity is imparted by additional N-glycosidically linked polysaccharides attached to the core protein.

The majority of the proteoglycan found in most basement membranes is composed of heparin and heparan sulfate glycosaminoglycans. Heparin and heparan sulfates are the most complex of the glycosaminoglycans, with repeating subunits of β-D-glucuronic acid--α-D-N-acetylglucosamine and α-L-iduronic acid-α-D-N-acetylglucosamine. Heparin, which differs from heparan sulfate only in that it is more highly sulfated, and thus, more highly charged, is a secretory product of mast cells and often isolated from lung and intestine. Heparan sulfate is found on the surface of many cells as well as within basement membranes. While the physiological significance of cell surface heparan sulfate is not well understood, basement membrane heparan sulfate has been shown to function as a reservoir for soluble growth factors such as bFGF, interleukin-3 and granulocyte-macrophage colony stimulating factor (Ruoslahti and Yamaguchi, 1991). At least some of these growth factors can be released by competition with heparin.

Structural domains of laminin

The major glycoprotein components of basement membranes are

members of the rapidly expanding laminin family. Additional glycoproteins associated with the basement membrane include fibronectin, which exists primarily as a soluble serum factor, and entactin/nidogen, which is found in association with laminin. The laminins are large, highly glycosylated molecules, and are often referred to as the "glue" of the basement membrane because of their many adhesive domains for cells and other matrix molecules (Beck et al., 1990). Laminin-1, the best characterized of the laminin isoforms, is composed of three polypeptide chains which have been shown by rotary shadowing to form a cruciform structure with three short arms and one long arm (Fig. 1.1). Initially isolated from the Englebreth-Holm-Swarm (EHS) mouse tumor, the three chains of laminin-1 were designated as A (MW 440,000) (Nissinen et al., 1991), B1 (MW 210,000) (Sasaki et al., 1987; Pikkarainen et al., 1987), and B2 (MW 200,000) (Sasaki and Yamada, 1987). Recent studies have identified variant forms of the A and B chains expressed in a developmental and tissue specific manner (reviewed in Kleinman et al., 1993), and the nomenclature has been revised to reflect these isoforms (Burgeson et al., 1994). The A chain has been designated as α, the B1 chain has been designated as β, and the B2 chain is now designated γ. Classical laminin, produced by the EHS mouse tumor has been designated laminin-1, and the polypeptide chains are denoted as α1, β1, and γ1. Merosin, which has been renamed laminin-2, is the predominant form of laminin expressed in human placenta, striated muscle, and peripheral nerve tissues (Ehrig et al., 1990). It is composed of the EHS-type β1 and γ1 chains,
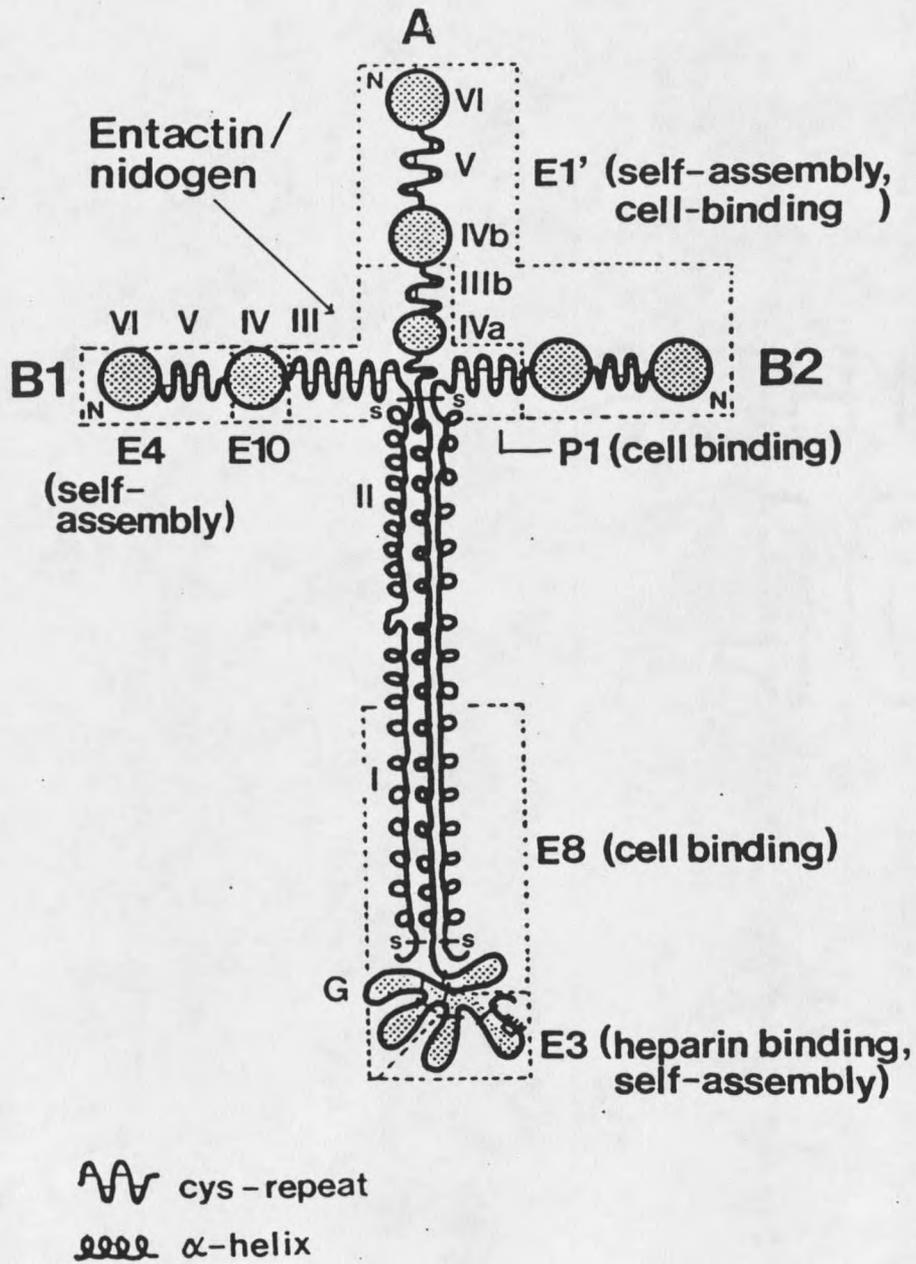
Figure 1.1. Schematic representation of the domain structure of laminin-1. The figure is taken from Yurchenco and Schittny, 1990.

and an A chain designated α2, which shows approximately 40% homology with the α1 of laminin-1. S-laminin, now designated laminin-3, has been found primarily in the synaptic cleft of neuromuscular junctions and in the glomerular basement membrane (Hunter et al., 1989b). It is composed of EHS-type α1 and γ1, however, the β1 polypeptide is replaced by a β2 isoform. Interestingly, the expression of laminin-3 (S-laminin) is concentrated in basement membranes with cell layers on both sides, as opposed to the classical basement membranes which contact cells on one side only, and connective tissue matrix on the other. The functional significance of this has not been elucidated, however, the β1 chain has been shown to contain several cell adhesive sites (Aumailley et al., 1987). Two of these adhesive sites, YIGSR and LGTIPG, are conserved to 60 and 67%, respectively in the β2 isoform (Hunter et al., 1989a). The activity of the homologous sequences in tumor cell adhesion has not been tested. The neuron adhesive site in laminin-3 has been identified as a tripeptide, LRE, located in the carboxyl-terminal half of the α1 chain.

Although all of the laminin chains identified to date are independent gene products, they demonstrate a striking degree of structural similarity with each other, and can be divided into domains based on their structural features. Domains I and II, located at the carboxyl-terminal ends of the β chains and in a related position of the α chains, are α-helical structures which interact to give a coiled-coil conformation. This rigid polymer, which is stabilized by disulfide bonds, forms the long arm of the laminin molecule. The α chain has an

additional cysteine-rich globular domain at the carboxyl terminal end, Domain G. Domain G, which has no β chain counterpart, has been shown to be one of the regions of the molecule that interacts with heparin in the basement membrane structure (Skubitz et al., 1988). It is also a region showing variations between different α chains. The next regions of the polypeptide chains diverge in spatial orientation; and Domains III-VI form the three short arms of the cross-like structure. Domains III and V are characterized by an amino acid sequence rich in glycine and cysteine, with homologous repeats of approximately 50 residues repeated 6-13 times. Each homologous unit has 6-8 cysteine residues spaced at regular intervals which are very similar to the motif found in epidermal growth factor (EGF) and EGF-related proteins. These homologous repeats are interrupted by the globular Domain IV, which contains mixtures of α-helix, β-sheet, and random coil structures. The larger α subunit contains two such globular domains, denoted IVa and IVb. Domain VI, at the amino terminal end of the polypeptides is the most highly conserved between the chains, and has been shown to be important in the adhesion of laminin to type IV collagen within the basement membrane (Kouzi-Koliakos et al., 1989).

Biological activities of laminin

Laminin evokes a variety of responses in cells of many different types. These responses, including cell growth, motility, proliferation, differentiation, and enzyme secretion, are all modulated by the interaction of laminin with specific cell surface receptors. Isolated proteolytic fragments have enabled the mapping

of some of these functions to discrete domains of the molecule (Barsky et al., 1984b). Further definition of a specific laminin binding sequence has, in some cases, been successful with the use of synthetic peptides corresponding to sequences within the laminin molecule, and function blocking antibodies raised to those peptides. Using these techniques, cell adhesion sites have been mapped to two primary sites: the P1 fragment, generated by pepsin digestion, which comprises the central cross region; and the E8 fragment, generated by elastin digestion, which is composed of the carboxyl terminal portions of all three chains (Aumailley et al., 1987).

The role of laminin in the adhesion and invasion of metastatic tumor cells was initially demonstrated by Terranova *et al.*, who co-injected either the intact laminin molecule, or proteolytic fragments of laminin with mouse melanoma cells (Barsky et al., 1984b; Terranova et al., 1984). The intact molecule enhanced the lung colony formation of the tumor cells in a dose dependent manner. A thrombin derived fragment, which lacked only the long arm of the molecule (Domains I and II) also increased lung colony formation, whereas a chymotrypsin produced fragment (C_1), lacking both the long arm and the globular domains of the short arms (Domains IV-VI), inhibited pulmonary metastasis. Intravenous injection of the C_1 fragment with ^{125}I-5-iodo-2-deoxyuridine (IUdR) labelled melanoma cells demonstrated that tumor cell retention in the lungs was reduced with the C_1 treatment. Since Domains IV and VI have been shown to contain a region of the laminin molecule important for assembly with type IV collagen in

basement membranes, it was postulated that intact laminin, or thrombin fragments containing Domains IV and VI, mediated cell adhesion to the basement membrane-resident type IV collagen. C_1 fragments, which do not include domains IV and VI, were unable to bridge cell adhesion to the basement membrane substrata, and specifically blocked interactions between the cell surface receptors and the basement membrane localized laminin.

In vitro experiments confirmed the presence of a cell adhesion site in Domain III of laminin-1 (Aumailley et al., 1987). When the C_1 fragment was immobilized on plastic and cells allowed to adhere, the affinity of cells for the proteolytic fragment was comparable to that seen with the intact laminin molecule. However, this adhesive site was not active in vitro with all cell types tested (Nurcombe et al., 1989). Proteolysis of laminin-1 with elastase allowed the isolation of a fragment (E8) containing Domains I and II, which represented the portion of the molecule removed by thrombin. This E8 fragment was also active in adhesion in vitro. The authors of this study hypothesized that the Domain III binding site was a latent binding site, and that the E8 fragment was the primary cell adhesion site in laminin. Subsequent studies have indicated that this interpretation may apply to some tissue types, but that differential expression of cell surface receptors is a more important determinant in binding site utilization, and the cellular response to laminin adhesion.

Cell Surface Receptors For Basement Membrane Glycoproteins

Given the multi-domain structure of laminin and the variety of cellular responses it can evoke, it is not surprising that multiple adhesive receptors have been identified. Cell surface proteins which interact with laminin can be loosely classified into two families, integrin and non-integrin.

Integrin receptors

Integrin receptors are heterodimers consisting of non-covalently associated α and β chains (Hynes, 1987; Hynes, 1992; Sonnenberg, 1993; Albelda, 1993). Both the α and β chains are glycosylated, integral membrane proteins with large extracellular domains and short cytoplasmic tails, with the exception of β_4, which has an extensive cytoplasmic region. The extracellular region of the α subunits contain three to four divalent cation binding domains, which have been shown to be essential to ligand binding function. The β chains are characterized by four repeated segments with a high cysteine content. To date, 14 α and 8 β subunits have been identified which combine to form 20 known receptor complexes. Alternative splicing of mRNA transcripts and post-translational modifications of the polypeptides add an additional level of complexity to the integrin family.

A wide variety of ligands for the integrins have been identified, including both extracellular matrix components and cell surface counter-receptors. Many integrins bind to more than one ligand, and have been termed "promiscuous receptors". Early work with the integrin receptors identified a tripeptide ligand

binding site, the amino acid sequence RGD, which is present in nearly all extracellular matrix molecules (Ruoslahti and Pierschbacher, 1987). Identification of the RGD binding domain proved to be instrumental in demonstrating the importance of integrin receptor-extracellular matrix interactions in tumor invasion and metastasis (Humphries et al., 1986). Intravenous coinjection of synthetic GRGDS peptide with syngeneic mouse melanoma cells results in a marked reduction of lung colony formation. The competitive inhibition of GRGDS with integrin-extracellular matrix binding is dose dependent, and is abolished with the conservative substitution of glutamic acid (E) for aspartic acid (D) (GRGES). *In vitro* crosslinking studies further demonstrated the specifity of the RGD peptides for the integrin receptors (Yamada et al., 1990).

The molecular context of the RGD tripeptide has been shown to be important in integrin adhesion. One level of control is the status of the adhesive molecule containing the RGD sequence. Werb *et al.* demonstrated that synthetic RGD, and fragments of fibronectin containing the RGD sequence are capable of inducing the expression of collagenase and stromelysin by fibroblasts, however, intact fibronectin is not (Werb et al., 1989). RGD peptide induction of the proteinases was mediated by an integrin receptor, presumably $\alpha_5\beta_1$, as shown by inhibition with monoclonal antibodies. Since proteolysis of basement membrane and interstitial matrix, and the adhesion of cells to the matrix molecules occur simultaneously during cell migration and tissue remodeling, it is reasonable to conclude that these events are coordinated by receptor

specificity for proteolytic fragments of matrix proteins.

An analogous situation may exist in the outgrowth of neurites on a laminin substrate. Laminin contains an RGD sequence in Domain IIIb of the α chain, between the second and third globular regions (Domains IVa and IVb) (Sasaki et al., 1988). Proteolytic fragments of laminin which contain this sequence are capable of supporting cell adhesion and neurite outgrowth *in vitro*, activities which can be inhibited by RGD peptides. However, synthetic RGD is not active in blocking the adhesion of neurons to intact laminin, nor does it elute integrin receptors from a laminin affinity column. Thus, the RGD sequence is likely to be sequestered in the intact laminin molecule, and only utilized by the cells when unmasked by limited proteolysis.

Receptor function has also been shown to be modulated by local molecular factors. Recent studies have identified a lipid mediator present in the cell membrane of neutrophils which enhances the binding affinity of β_2 integrins (Hermanowski-Vosatka et al., 1992). The identity of this neutral lipid moiety has not yet been discerned, nor has its mechanism of receptor activation. Although this is the first report of an integrin associated lipid cofactor, the phospholipid composition of the cell membrane has previously been shown to affect integrin function. Conforti *et al.* (1990) demonstrated that purified $\alpha_v\beta_3$ receptor inserted into liposomes consisting solely of phosphatidylcholine bound only vitronectin. Insertion into mixed micelles of phosphatidylcholine and phosphatidylethanolamine resulted in a broader specificity, and the receptor was

then able to bind vitronectin, fibronectin, and von Willebrand's factor. These studies would indicate a conformational requirement for specific integrin receptors in adhesion to their ligands.

Signal transduction by the integrin receptors

Signal transduction by the integrin receptors has been characterized as bidirectional, with integrin function influenced in both an "inside-out" and "outside-in" manner (reviewed in Hynes, 1992). Not only does the ligand binding event elicit a cellular response, the activation status of the cell also affects ligand binding. Signal transduction by the integrins is thought to be mediated by phosphorylation of residues in the cytoplasmic domains of the subunits, but downstream effectors remain largely uncharacterized.

Tyrosine phosphorylation of a 125 kDa cytoplasmic protein has been shown to be induced by the adhesion of β_1 integrins to fibronectin (Juliano, 1994). This cytoplasmic protein, which accumulates at focal contacts subsequent to cell adhesion, has been named pp125FAK (Focal Adhesion Kinase), and had been previously demonstrated to be a substrate for src kinase. The specific function of FAK and its substrates have not yet been identified, but are likely to involve the cytoskeleton. Ligand binding of the platelet gpIIb/IIIa receptor induces phosphorylation of a tyrosine residue in the cytoplasmic domain of the β chain (Elmore et al., 1990). Immunoprecipitation of the phosphorylated gpIIIa (β_3) subunit co-precipitates the tyrosine kinase, pp60$^{c\text{-src}}$, which is likely to be the effector kinase. Phosphorylation of the α_6 subunit in macrophages can

be induced by adhesion to laminin (Shaw et al., 1990). Differential extraction of the adherent cells demonstrated that the phosphorylated α_6 protein co-localized with the cytoskeleton.

The β_1 subunit has also been shown to associate with cytoskeletal proteins when activated either by phorbol esters or ligand binding (Tamkun et al., 1986). The cytoskeletal components talin (Otey et al., 1990) and α-actinin (Burn et al., 1988) have both been shown independently to physically interact with the cytoplasmic domain of β_1 integrins. Cytoskeletal organization is a prerequisite for cell motility, spreading, and formation of focal contacts, all of which are known cellular responses to basement membrane substrates.

"Inside-out" signalling is reflected by the activation status of the cell expressing the integrin receptor. The major integrin receptor expressed on platelets, gpIIb/IIIa ($\alpha_{IIb}\beta_3$), is not active as a receptor for soluble fibrinogen when the cells are in the resting state. Upon cell activation by thrombin, collagen, or other platelet agonists, the gpIIb/IIIa receptor undergoes a conformational change required for RGD binding (Parise et al., 1987; Frelinger et al., 1988). A similar activation-dependent ligand binding is seen in β_2 integrins, which are widely expressed on neutrophils, monocytes, and lymphocytes. Although these receptors do not interact with the RGD sequence, their activation by inflammatory mediators such as C5a, tumor necrosis factor α, or f-MLF are required for cell adhesion to the endothelium (Bohnsack et al., 1990).

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Laminin binding integrin receptors

Integrin receptors which recognize laminin include $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_3\beta_1$, $\alpha_6\beta_1$, $\alpha_7\beta_1$, and $\alpha_6\beta_4$. Of these, $\alpha_7\beta_1$, $\alpha_6\beta_1$, and $\alpha_6\beta_4$ appear to be the most relevant to cancer invasion and metastasis. The elastase derived fragment E8, which is comprised of most of the long arm (Domains I and II), has been shown to contain the binding site for $\alpha_6\beta_1$ (Hall et al., 1990; Sonnenberg et al., 1990) and $\alpha_7\beta_1$ (Kramer et al., 1991) receptors which are expressed on malignant cells. The α_7 subunit was initially thought to be a variant of α_6, because of its high degree of sequence similarity. It has since been shown to be a distinct gene product which is specifically induced in transformed melanocytes (Kramer et al., 1991). The $\alpha_6\beta_1$ integrin is expressed on many epithelial tissues in a developmentally regulated manner (Cooper et al., 1991). During embryogenesis, the appearance of $\alpha_6\beta_1$ coordinates with expression of the α subunit of laminin in the glomerular basement membrane of the kidney (Goodman, 1992). Similarly, immunohistochemical analysis of the expression and localization of α_6 in undifferentiated breast carcinoma has shown co-localization of this receptor with laminin on the cell surface (D'Ardenne et al., 1991).

The α_6 subunit of $\alpha_6\beta_1$ can also be found in a different heterodimeric complex, together with β_4, in a number of epithelial tissues (Sonnenberg et al., 1992). The specific ligand for this integrin receptor has recently been identified as also being within the E8 fragment of laminin (Lee et al., 1992). Previous reports conflicted with this finding (Sonnenberg et al., 1990), and may reflect

differences in the developmental stage or activation status of the cell. Immunoperoxidase staining of tissue sections probed with specific antibodies for the α_6 and β_4 subunits is concentrated near the basal surface of the epithelium in contact with the basement membrane (Sonnenberg et al., 1992).

<u>Non-integrin laminin binding proteins</u>

Since laminin is a highly glycosylated protein, it is not surprising that the non-integrin class of laminin receptors includes several lectins (Rao et al., 1983; Dennis et al., 1984). Macrophages express a 35 kDa protein, Mac-1, which can be eluted from a laminin-sepharose affinity column with galactose (Woo et al., 1990). Microsequencing of this protein showed it to be identical to a previously identified carbohydrate binding protein expressed on mouse fibroblasts. The biological function and significance of this protein remains to be elucidated. A related protein of M_r 14,000 has been identified on the surface of human melanoma cells (Castronovo et al., 1992). This lectin has been suggested as a modulator of interactions between laminin and other laminin binding proteins.

<u>The 67 kDa Non-Integrin Laminin Binding Protein</u>

<u>The relevance of the 67 kDa laminin binding protein in metastasis</u>

The non-integrin laminin binding protein, which is the focus of this study, is a cell surface molecule that migrates on SDS-PAGE with an apparent molecular weight of approximately 67 kDa. This laminin receptor was initially

identified in tissue sections of highly metastatic breast carcinoma (Terranova et al., 1983). A monoclonal antibody, LR1, raised to the laminin-affinity purified protein was used in immunohistochemical studies to assess the expression of the 67 kDa laminin binding protein in neoplastic vs. adjacent normal tissues (Hand et al., 1985). Antigen expression was found to be highest in invasive tumors of epithelial origin. This antibody was renamed 2H5, and used by Wewer et al. to identify the 67 kDa laminin binding protein cDNA in a λgt11 human umbilical vein endothelial cell expression library (Wewer et al., 1986). Although unable to isolate a full length clone, Wewer et al. obtained a partial sequence that was compatible with a cyanogen bromide fragment generated from laminin affinity purified protein, verifying the identity of the cDNA. They further demonstrated a correlation between elevated mRNA levels and the ability of several human epithelial cell lines to adhere to laminin. The full length sequence of the 67 kDa laminin binding protein cDNA was published as a serendipitous finding by an independent group of researchers (Yow et al., 1988). Their study was designed to identify tumor markers through the comparison of cDNA libraries prepared from colon carcinoma and adjacent normal human colonic epithelium of the same patient. A dominant 1.2 kb mRNA which hybridized to a probe from a well characterized colon carcinoma cell line was cloned and sequenced, and found to completely overlap the partial sequence reported by Wewer et al. The predicted amino acid sequence of the full length cDNA codes for a 295 residue polypeptide with a predicted molecular weight of 32-33 kDa.

This is in contrast to the 67 kDa native protein isolated from plasma membranes, and the discrepancy has generated considerable discussion in the literature. Features of the predicted amino acid sequence will be more completely addressed in a later section of this introduction.

cDNA probes derived from the published sequence have been used by many researchers to characterize the expression of the 67 kDa laminin binding protein in various tissues. Several lines of evidence exist to support the correlation of increased expression with high metastatic capability. Mafune *et al.* (1990) examined 67 kDa laminin binding protein mRNA from twenty-one surgical specimens of primary colon carcinoma and six liver metastases of colon carcinoma. Fifteen of the twenty-one primary tumors demonstrated mRNA levels of over 150% of adjacent normal tissue. In five of the remaining samples, expression was between 100% and 150%, and only one patient specimen showed mRNA levels to be decreased in the tumor tissue. Correlation of increased 67 kDa laminin binding protein mRNA with the patients Duke's classification of colon carcinoma was reported. In addition, expression of this mRNA was 3 to 10-fold higher in the liver metastases than in adjacent liver tissue for all six specimens. A similar study (Satoh et al., 1992) analyzed expression of the 67 kDa laminin binding protein mRNA in 25 lung cancer tissues and 11 lung cancer cell lines. Small cell and oat cell carcinomas, which are typically highly aggressive tumors, demonstrated the highest levels of mRNA expression, with up to 7-fold increases over normal adjacent lung tissue.

Interestingly, increased 67 kDa laminin binding protein mRNA levels correlated with a higher cell proliferation rate.

Immunological assessments of the surface expression of the 67 kDa laminin binding protein have been complicated by the existence of several laminin binding proteins of similar molecular weights, some of which appear to have cross-reactive epitopes. Lesot *et al.* (1983) isolated a 68 kDa laminin binding protein from the surface of mouse skeletal muscle cells, which displayed an affinity constant of K_D 1.5-2 x 10^9 on laminin immobilized to sepharose. This protein was subsequently shown to have AMPase activity (Dieckhoff et al., 1986), and likely represents a deglycosylated form of the ectoenzyme, 5'-nucleotidase. A direct comparison of 5'-nucleotidase, isolated from chicken gizzards, and 67 kDa laminin binding protein by two dimensional electrophoresis of tryptic digests demonstrated that, although these two proteins contained similar domains, they were, in fact, distinct from one another (Risse et al., 1989).

An elastin binding protein of M_r 67,000 which bound secondarily to laminin was reported, and suggested to be the laminin binding protein (Mecham et al., 1989). Direct amino acid sequencing of this protein later identified it as a splice variant of β-galactosidase with no regions in common with the 67 kDa laminin receptor (Hinek et al., 1993). This variant enzyme is inactive, and may have some relevance in tumor adhesion and invasion through aberrant glycosylation of cell surface components, or via its carbohydrate binding potential. However, it does not likely represent a laminin receptor of any significance.

A third laminin binding protein of similar molecular weight was identified on the surface of chicken and rat muscle cells (Hall et al., 1988). cDNA sequencing of this protein identified it as a distinct entity by its unique carboxyl terminus, containing a large number of aspartic acid residues (Clegg et al., 1989). This protein, named aspartactin, is suggested to be involved in the association of mesenchymal cells with basement membranes, but it has not been shown to be relevant to metastasis. Fibrosarcoma cells have also been shown to express a 68 kDa laminin binding protein which is capable of inducing the polymerization of actin *in vitro* (Malinoff and Wicha, 1983; Brown et al., 1983). No sequence information is available for this protein, so it is not clear whether it is a member of one of the families described here, or yet another tissue specific laminin binding protein.

The 67 kDa high affinity laminin binding protein initially isolated by Liotta's group at the NIH (Barsky et al., 1984a; Mafune et al., 1990), and sequenced by Wewer (1986) and Yow (1988), is highly conserved in evolution, and so, must play a critical role in the survival of the organism. It has been shown to be expressed quite early in development where it likely plays a role in the direction of cell migration on laminin substrates (Rabacchi et al., 1990; Laurie et al., 1991; Grant et al., 1991; Laurie et al., 1989). Homologous proteins have been identified in bacteria (Marques et al., 1994), yeast (Davis et al., 1992; Lopez-Ribot et al., 1994), Hydra (Keppel and Schaller, 1991), Drosophila (Melnick et al., 1993) and virtually all vertebrates thus far studied (Bignon et al., 1991). Given the highly

conserved nature of this protein and its apparent ubiquitous expression, attempts to raise antiserum to the isolated protein have typically been unsuccessful. However, immunization with whole cells has proven successful for two independent groups, Martignone *et al.* (1992), and Wang *et al.* (1992). The first group used a panel of highly metastatic human cell lines, both lung and breast carcinomas, to immunize mice. The second group immunized mice with hamster cells and hamster membrane extracts to obtain a monoclonal antibody that blocked the adhesion of Sindbis virus to hamster cells. Using this antibody to screen an expression library for the Sindbis virus receptor, they isolated a cDNA nearly identical to the human sequences previously published for the 67 kDa laminin binding protein.

Highly specific antibodies raised to synthetic peptides derived from the predicted amino acid sequence have also been used to demonstrate a strong correlation of surface expression with metastatic propensity. Castronovo *et al.* utilized an antibody raised to residues 2-9 of the predicted amino acid sequence of the 67 kDa laminin binding protein to demonstrate high levels of protein expression on the surface of invasive tissues of breast cancer (Castronovo et al., 1990), colon cancer (Cioce et al., 1991), and melanoma (Castronovo et al., 1991b). Poorly differentiated hepatocellular carcinomas, which were shown by Grigioni *et al.* to stain strongly with an antibody raised to a 20-mer peptide from the carboxyl half of the cDNA sequence showed a poor prognosis and high mortality rate (Grigioni et al., 1991). As highly specific probes, anti-synthetic

peptide antibodies are quite useful in identifying tumor markers. However they generally do not recognize the antigen with sufficient affinity to immunoprecipitate their protein target.

Features of the 67 kDa laminin binding protein
cDNA and deduced amino acid sequence

In spite of the wealth of data supporting the relevance of the 67 kDa laminin binding protein in tumor invasion and metastasis, characterization of the protein itself, its form of surface expression, and its mechanism of interaction with laminin has been substantially lacking. The amino acid sequence predicted from the cDNA sequence is sufficient to code for a polypeptide chain of approximately 32 kDa, while the isolated protein displays an apparent molecular weight of 67 kDa on reduced SDS-PAGE. Primer extension experiments by two separate groups (Yow et al., 1988; Rao et al., 1989) have verified that the cDNA is, in fact, the full length clone of the 67 kDa laminin binding protein. Analysis of the cDNA reveals several in frame stop codons in the 5' untranslated region, however, there is no typical translation initiation consensus sequence surrounding the methionine residue that marks the open reading frame (Yow et al., 1988; Rao et al., 1989; Wang et al., 1992). The deduced amino acid sequence of the 67 kDa laminin binding protein cDNA shows an open reading frame of 295 residues (Fig. 1.2). There is no leader signal sequence at the amino terminus for entry into endoplasmic reticulum, as has been reported for many cell surface proteins. There is no N-X-(S/T) consensus sequence for N-linked glycosylation which could account for the discrepancy between the
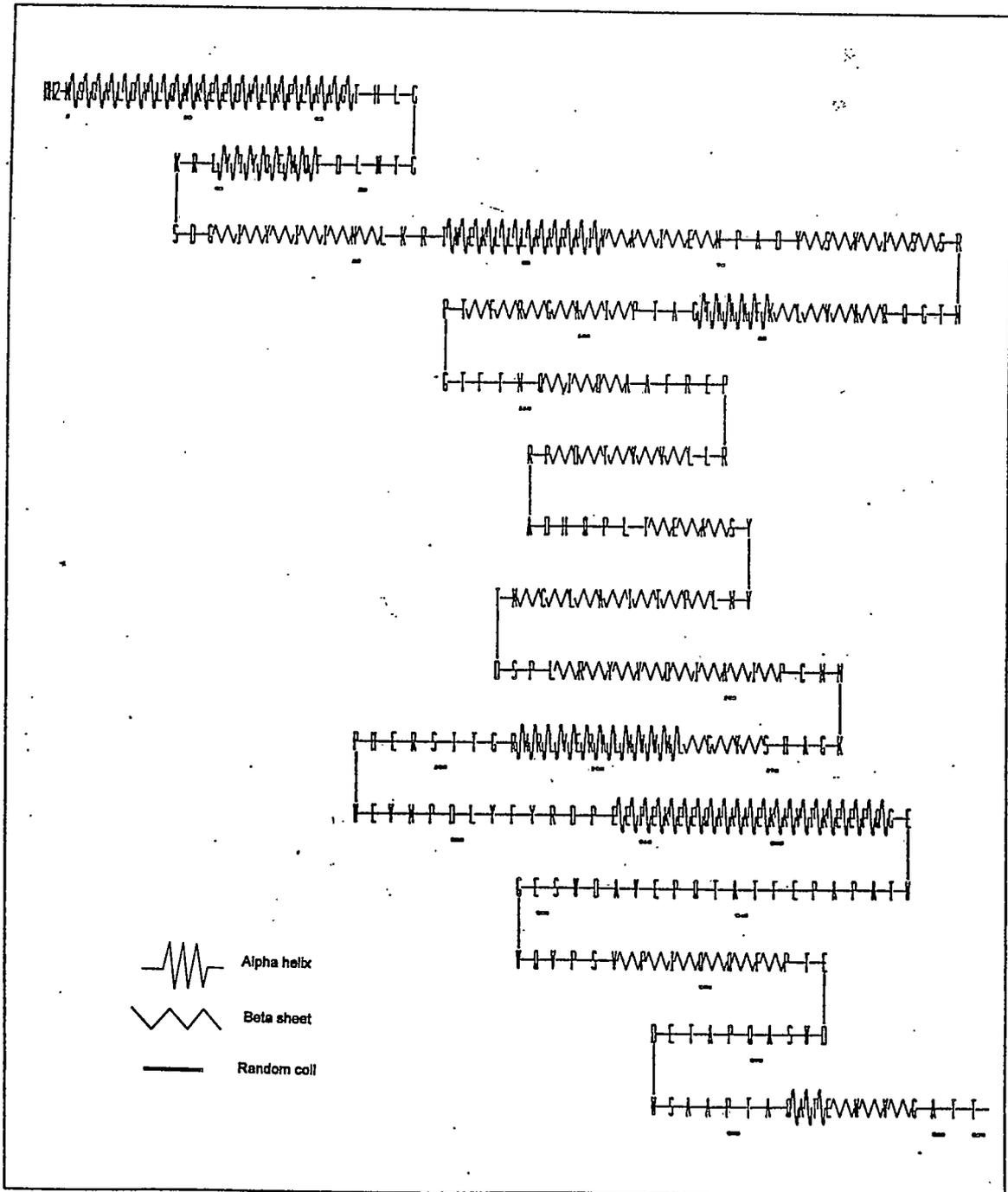
Figure 1.2. Schematic representation of the predicted secondary structure of the 67 kDa laminin binding protein. The structure was generated with the Stroud and MSEQ structure prediction programs using parameters suggested for a membrane associated protein (Finer-Moore and Stroud, 1984).

predicted molecular weight of 32 kDa and the observed molecular weight of 67 kDa. There are, however, 14 serine and 23 threonine residues which could be available for O-linked glycosylation. There are only two cysteine residues, at positions 148 and 163, which are believed to form an intrachain disulfide bond, based on the increased mobility of the isolated protein in non-reducing SDS-PAGE analysis (Wewer et al., 1986).

In vitro translation studies of the 67 kDa laminin binding protein cDNA have identified a native gene product of approximately 37 kDa (Rao et al., 1989). Total cellular mRNA from the human melanoma cell line A2058 and from mouse NIH-3T3 cells was translated in a rabbit reticulocyte cell-free translation system. The protein products from the translation reaction were immunoprecipitated with a polyclonal antiserum raised to a synthetic peptide derived from the carboxyl terminal region of the predicted amino acid sequence (residues 263-282). SDS-PAGE analysis of the immunoprecipitated products demonstrated a predominant protein of 37 kDa molecular weight. Pulse chase experiments, using a mouse-human chimeric cDNA in COS-7 cells under the control of a large T promoter, were used to demonstrate a precursor-product relationship. However, the biosynthetic mechanism responsible for the molecular weight shift from 37 kDa to 67 kDa was not determined (Castronovo et al., 1991a). In whole cell extracts, this 37 kDa putative precursor is immunoprecipitable by anti-synthetic peptide antibodies raised to sequences corresponding to residues 21-40, 64-83, or 104-123 (Castronovo et al., 1991b). It is not, however, immunoprecipitated by

antiserum specific for sequences between these regions. This could be due to the low affinity of the polyclonal antisera, as the authors surmise. Alternatively, it may reflect conformational requirements of the antibody that are not compatible with the secondary structure of the native protein.

Many studies have clearly identified the 67 kDa laminin binding protein as a cell surface protein. However, the predicted amino acid sequence contains no region with the hydrophobic characteristics of a simple transmembrane domain. With calculations based on an average membrane thickness of 30 nM, it is generally accepted that the minimum sequence required to span a eukaryotic plasma membrane in an alpha helical conformation is 18-20 residues (reviewed in Fasman and Gilbert, 1990; Jahnig, 1990). The amino acids present in a single transmembrane alpha helix must be entirely hydrophobic. β-sheet structures have been identified as transmembrane domains, however they are generally found in a closed structure of eight strands which are hydrogen bonded to form an amphiphilic channel. Orientation of two or more amphipathic alpha helices in such a manner as to impart a hydrophobic nature to the lipid-contacting portion of the structure is also a common mechanism for membrane imbedding of proteins. The 67 kDa laminin binding protein does contain a region predicted by computer analysis to form such an amphipathic helix (Starkey et al., 1990). Residues 53-70 of the amino acid sequence are predicted to form an alpha helical structure with the charged amino acids located on one side of the helix. These are spaced in such a manner that alignment of three helices could