



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 effects 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 effect 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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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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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.

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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 (TGF α), 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 [$\alpha 1(IV)_2\alpha 2(IV)$] 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 heterogeneous 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 $\alpha 1$, $\beta 1$, and $\gamma 1$. 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 $\beta 1$ and $\gamma 1$ chains,

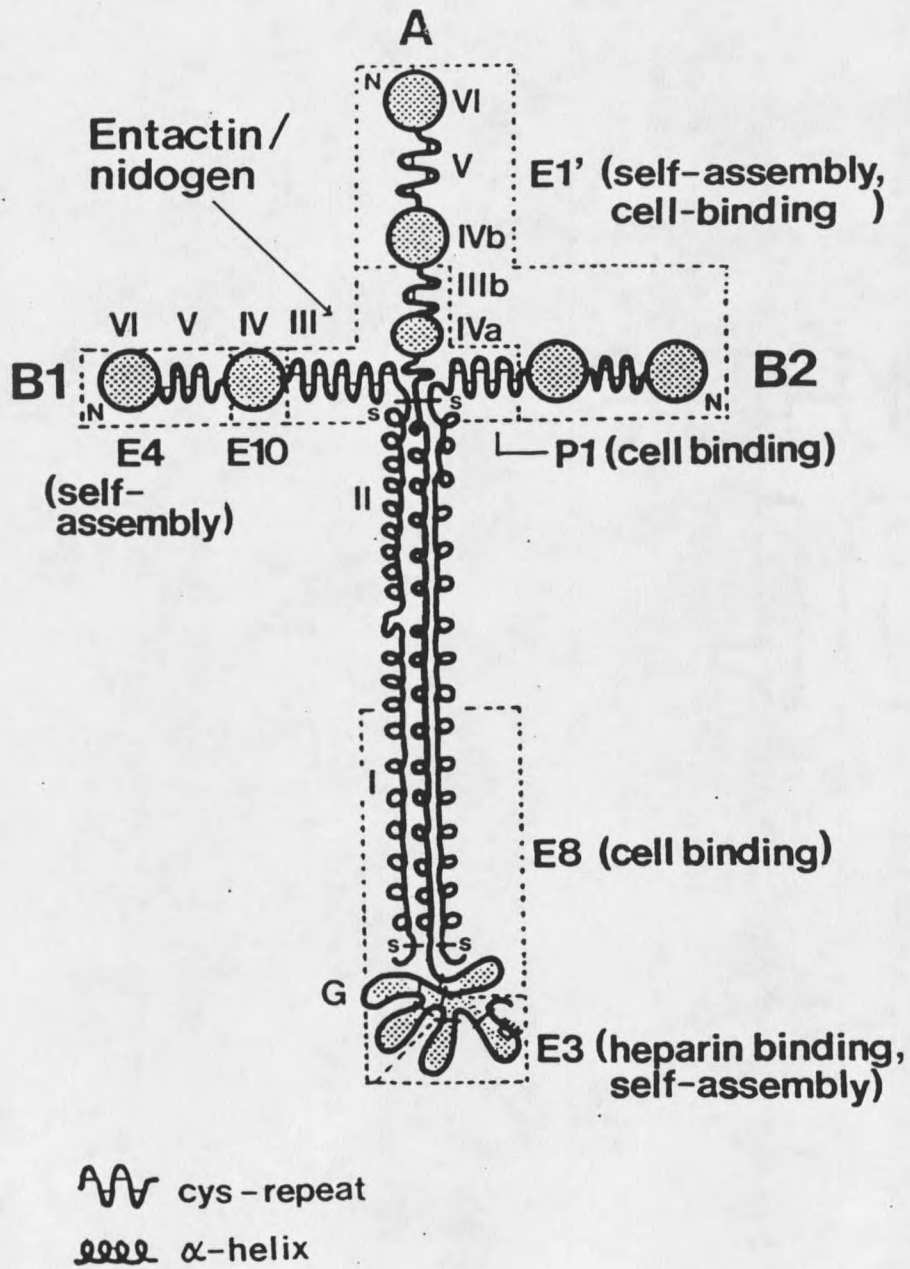


Figure 1.1. Schematic representation of the domain structure of laminin-1. The figure is taken from Yurchenco and Schittny, 1990.

and an A chain designated $\alpha 2$, which shows approximately 40% homology with the $\alpha 1$ 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 $\alpha 1$ and $\gamma 1$, however, the $\beta 1$ polypeptide is replaced by a $\beta 2$ 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 $\beta 1$ 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 $\beta 2$ 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 $\alpha 1$ 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 (C₁), lacking both the long arm and the globular domains of the short arms (Domains IV-VI), inhibited pulmonary metastasis. Intravenous injection of the C₁ fragment with ¹²⁵I-5-iodo-2-deoxyuridine (IUdR) labelled melanoma cells demonstrated that tumor cell retention in the lungs was reduced with the C₁ 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. C₁ 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 C₁ 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 specificity 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-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).

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).

Non-integrin laminin binding proteins

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.

The 67 kDa Non-Integrin Laminin Binding Protein

The relevance of the 67 kDa laminin binding protein in metastasis

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 λ gt11 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 (Sato *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 $1.5-2 \times 10^9$ 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

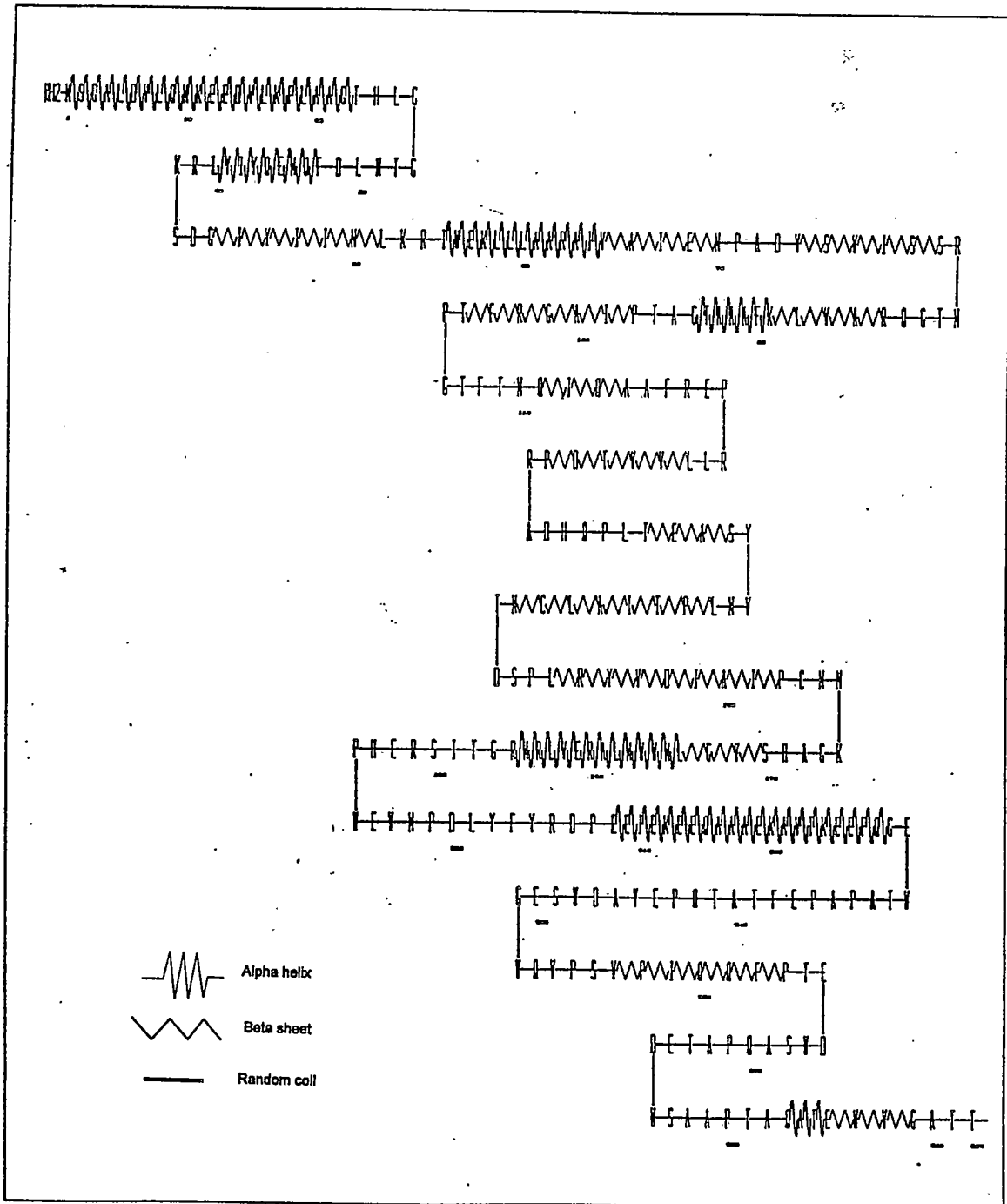


Figure 1.2. Schematic representation of the predicted secondary structure of the 67 kDa laminin binding protein. The structure was generated with the Stroud and MSEQ structure prediction programs using parameters suggested for a membrane associated protein (Finer-Moore and Stroud, 1984).

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; Jahning, 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

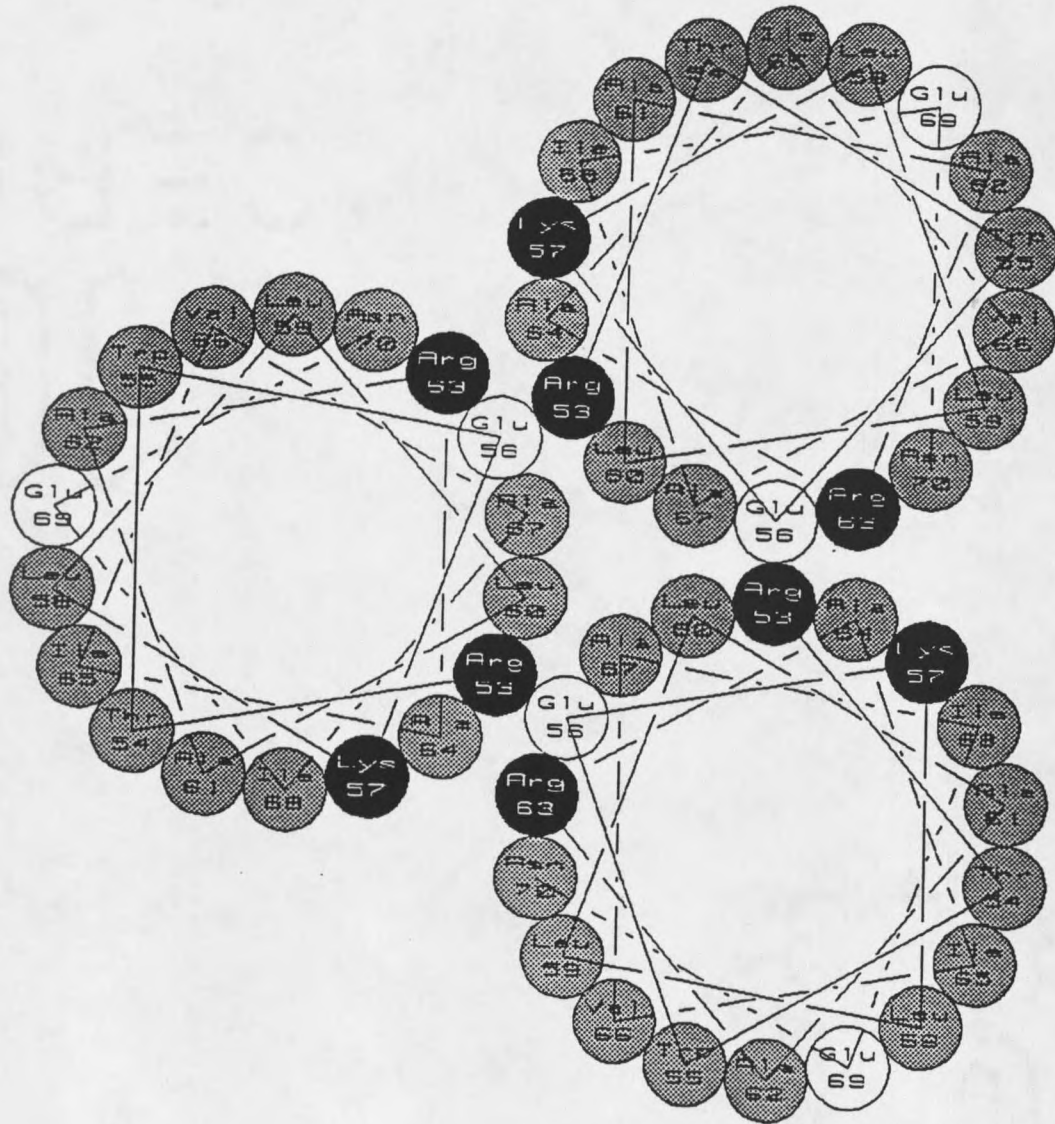


Figure 1.3. Axial wheel plot of residues 53-70 of the 67 kDa laminin binding protein demonstrating possible charge interactions within the lipid bilayer in a putative transmembrane association. Positively charged residues are represented by dark circles, negatively charged residues are represented by white circles, and all other hydrophobic and neutral amino acids are gray. The figure was generated using a program developed by the MSU Computer Science GRAIL Laboratory, courtesy of Dr. J.D. Starkey.

place the hydrophobic residues on the exterior of the structure, with the charged residues sequestered from the lipid environment of the membrane (Fig.1.3).

While this model has not been entirely disproven, subsequent data would support the association of only two transmembrane helices, rather than three (Landowski *et al.*, this study, manuscript submitted). Castronovo *et al.* (1991c) have proposed residues 86-101 as a potential transmembrane domain based on immunofluorescent staining of unpermeabilized cells with a panel of anti-synthetic peptide antibodies. Polyclonal antisera raised to peptides amino to this sequence did not stain unpermeabilized cells, while those antibodies raised to peptides on the carboxyl side of residues 86-101 did. While this study does not fully define the transmembrane domain, it does clearly demonstrate that the carboxyl terminal region of the protein is extracellular. The authors interpreted the inability of the anti-sera raised to amino terminal sequences as an indication of the intracellular location of these sequences. However, it is quite possible that the secondary structure of the 67 kDa protein in its membrane associated form was not consistently recognized by the antisera, and further study is required to define the exact nature of the membrane association of the 67 kDa laminin binding protein.

The carboxyl terminal half of the predicted amino acid sequence of the 67 kDa laminin binding protein includes a large number of negatively charged residues, a characteristic which is consistent with an extracellular location of this region (Nilsson and von Heijne, 1990; Beltzer *et al.*, 1991). Antibody mapping

has shown the laminin binding domain to reside within the carboxyl terminal half of the molecule (Wewer et al., 1987). This region also contains some interesting repeated sequences. The sequence K-E-E appears three times in the coding sequence of the protein, with two of these occurrences in the carboxyl terminal region. Two repeats of T-E-D-W-S-A-X-P are found at residues 264-271 and 273-280, and one palindromic sequence of L-M-W-W-M-L is noted at residues 173-178. Although these repeated sequences are reported to be unique to the 67 kDa laminin binding protein, their significance is not known (Yow et al., 1988).

Identification of a bioactive peptide

Residues 161-180, which include the palindrome, L-M-W-W-M-L, have been proposed by Castronovo *et al.* to be the specific domain of the protein involved with adhesion to laminin (Castronovo et al., 1991c). A synthetic peptide corresponding to this sequence, Peptide G, has been shown to adhere to laminin-1, which was immobilized on nitrocellulose, in a dose dependent manner. Additionally, the peptide was demonstrated to elute the 67 kDa laminin binding protein from a laminin affinity column. The adhesion of the human melanoma cell line A2058 to cultured bovine aortic endothelial cells (BAEC) could also be blocked with Peptide G (Castronovo et al., 1991b). However, polyclonal anti-serum raised against the peptide had no inhibitory effect on cell adhesion. Since BAEC were also shown to express 67 kDa laminin binding protein on their surface, the mechanism of cell adhesion, and inhibition by Peptide G was postulated to be mediated by a laminin "bridge". In this model, the laminin

binding proteins expressed on the BAEC surface would interact with laminin which was secreted by, and coating the surface of, the melanoma cells. Support for this model was garnered by the observation that melanoma-endothelial cell adhesion could be inhibited by either soluble laminin-1, or anti-laminin antiserum. However, no direct evidence of the laminin "bridge" was presented. Ensuing studies by an independent group of researchers demonstrated that the interaction of Peptide G with laminin was mediated by heparin (Guo et al., 1992). This study was initiated because of the similarity between Peptide G, and a heparin binding peptide derived from the adhesive protein, thrombospondin. Both Peptide G, and the thrombospondin derived peptide contain at least two tryptophan residues, and several basic amino acids, which are postulated to interact with sulfated glycoconjugates. In this study, the adhesion of A2058 cells to immobilized Peptide G was almost completely inhibited by the presence of heparin, or by the thrombospondin peptide. When the A2058 cell line was grown in the presence of chlorate to inhibit sulfation, the attachment and spreading of the melanoma cells on Peptide G was significantly reduced, leading to the conclusion that the 67 kDa laminin binding protein derived peptide does not interact with laminin directly, but rather is an adhesive site for proteoglycans. These proteoglycans, which could be present either on the cell surface, or resident in the extracellular matrix, might then adhere to laminin through one of its several heparin binding domains.

Although not noted by the authors, this model would appear to be supported by the *in vivo* effects of Peptide G in a lung colony assay (Taraboletti et al., 1993). B16BL6 mouse melanoma, or A2058 human melanoma cells were pretreated with the peptide for ten minutes prior to intravenous injection into C57BL/6 or nude mice, respectively. This treatment resulted in a 2 to 10 fold increase in the number of experimental lung metastases. Intravenous administration of the peptide alone at various times before or after the injection of tumor cells did not enhance the lung colony formation, indicating that the mechanism of enhancement was most likely a tumor cell specific effect. The effects of coinjection of the peptide without pretreatment of the tumor cells were not presented. To determine if Peptide G was affecting the retention of the tumor cells in the lungs, B16BL6 mouse melanoma cells were metabolically labelled with $^{125}\text{IUdR}$, pretreated for 15 minutes with Peptide G, and intravenously injected. The experimental mice were sacrificed at various time periods following injection, and assayed for radioactivity in lung tissues. Peptide G treatment was shown to modulate the retention of the tumor cells in the lungs in a bivalent fashion. At up to eight hours post injection, tumor cell retention in the lungs was decreased relative to untreated controls. At times greater than eight hours, lung retention of radiolabelled tumor cells was increased over untreated controls. These data were interpreted as an allosteric modification of the subendothelial laminin that increases its affinity for other laminin receptors on the surface of tumor cells. An alternative explanation may be a coordination

of multiple adhesive sites on the 67 kDa laminin binding protein for laminin or other surface structure, possibly including proteoglycans.

Peptide 11 and YIGSR

A nine amino acid sequence, CDPGYIGSR, located in the P1 fragment of laminin-1, has been identified as a primary binding site for the 67 kDa laminin binding protein (Graf et al., 1987a). This sequence, named peptide 11, represents residues 925-933 of the β 1 chain of laminin-1, and is located within the EGF-like repeats of Domain III. Peptide 11 has been shown to inhibit the invasion of B16F10 or B16BL6 mouse melanoma cells through a reconstituted basement membrane matrix *in vitro*, and to inhibit experimental metastases in a lung colonization assay *in vivo* (Iwamoto et al., 1987; Ostheimer et al., 1992). In these assays, the amide form of the peptide consistently demonstrated greater inhibitory activity than did a peptide with a free carboxyl terminus, indicating the importance of the positive charge on the side chain on the terminal arginine residue. Anti-angiogenic activity of peptide 11 has also been demonstrated *in vivo*. In a study by Sakamoto *et al.* (1991), Peptide 11 was shown to suppress embryonic chorioallantoic membrane angiogenesis in a dose dependent manner. Intravenous administration of Peptide 11 in sarcoma bearing mice resulted in a significant reduction in the growth of the tumor. Clearly, this peptide is capable of specifically interfering with the interactions of tumor cells with basement membrane molecules, and represents a potential therapeutic lead.

The minimum sequence required for inhibition of tumor cell adhesion to laminin-1 has been shown to reside in the carboxyl terminal 5 residues, YIGSR. This peptide also demonstrates activity in the inhibition of tumor cell invasion of a basement membrane matrix *in vitro* (Ostheimer et al., 1992), and inhibition of experimental metastasis *in vivo* (Iwamoto et al., 1987). However, the pentapeptide is not as efficient as the full nine residue sequence in eluting the 67 kDa laminin binding protein from a laminin affinity column (Graf et al., 1987b), presumably due to an inability of the short peptide sequence to maintain the appropriate conformation. Conservative substitutions and glycine-to-alanine substitution studies have demonstrated that the activity of Peptide 11 is highly dependent on the secondary structure of the peptide (Graf et al., 1987b; Ostheimer et al., 1992). The NMR-derived preferred solution structure of Peptide 11 demonstrates an "S" shaped conformation, which appears to be stabilized by a carbonyl interaction between the terminal arginine residue, and the backbone of the tyrosine residue. The substitution of d-alanine for gly-7 (CDGPYI(dA)SR) accommodates this conformation, and the substituted peptide has biological activity equal to that of Peptide 11 (Ostheimer et al., 1992). However, the substitution of l-alanine at the same position (CDPGYI(lA)SR) reduces the biological activity of the peptide to 50% of Peptide 11 activity, and the truncated sequence, IGSR is entirely inactive (Graf et al., 1987b). Likewise, the amino terminal cysteine appears to be required for optimal activity of Peptide 11, as the conservative substitution of serine for cysteine (SDPGYIGSR) is

inactive (J.R. Starkey, unpublished observations). The cysteine residue may not be a structural requirement, but rather, the cysteine residue may simply physically contact the receptor.

Other laminin peptides with bioactivity

Other laminin derived bioactive peptides that have been identified include PDSGR, also in the P1 fragment (Kleinman et al., 1989), F-9, located in the globular Domain IV of the β 1 chain (Charonis et al., 1988), and IKVAV, located in the carboxyl terminal region of fragment E8 (Kanemoto et al., 1990; Tashiro et al., 1989). PDSGR has been shown to inhibit lung colonization, although the activity is approximately 20% less than that demonstrated for Peptide 11 (Kleinman et al., 1989, J.R. Starkey, unpublished observations). No specific cell surface receptor has been identified for this sequence. The F-9 peptide, characterized by Charonis *et al.*, was identified as a heparin binding sequence. This peptide was shown to inhibit the adhesion of cells to laminin, presumably by blocking an interaction between cell surface proteoglycan, and laminin.

One of the most interesting bioactive peptides derived from the sequence of laminin, which perhaps has a great deal of relevance for tumor metastasis, is the sequence IKVAV. This peptide is located in the carboxyl region of the α chain within the E8 fragment. IKVAV has been shown to initiate a signal transduction cascade in various cell types including melanoma (Kanemoto et al., 1990; Royce et al., 1992), and neural cells (Tashiro et al., 1989; Kubota et al., 1992). Cellular responses induced by this peptide include the secretion of

collagenolytic enzymes by invasive carcinomas and neurite outgrowth, however, the specific receptors involved in the signal transduction by this peptide have not yet been fully characterized. Several proteins have been identified with adhesive activity for the E8 fragment of laminin, including the integrins $\alpha_6\beta_1$, and $\alpha_6\beta_4$ (Sonnenberg et al., 1990; Lee et al., 1992), and other proteins with molecular weights in the range of 90-110 kDa which may also be members of the integrin family (Tashiro et al., 1989). However, the IKVAV peptide is but a small portion of the E8 fragment, and may not be the specific adhesive site for these receptors, which require the coiled coil structure formed by all three laminin chains (Lissitzky et al., 1992). The 67 kDa laminin binding protein has not been shown to directly attach to this domain. Differential responses to the IKVAV peptide are seen in cells of different origins (Tashiro et al., 1989), indicating a highly regulated interaction between cells and this region of the laminin molecule. As such, it represents a valuable tool for the further investigation of laminin mediated signal transduction, but does not appear to involve the 67 kDa laminin binding protein.

Preview to the Experiments

General project goals

The general goal of the project which includes this study is the development and assessment of specific anti-metastatic therapies. Since the adhesion of tumor cells to the extracellular basement membrane is considered

to be critical to successful completion of the metastatic cascade, interference with this step represents an opportunity for therapeutic intervention. Small synthetic peptides which mimic the binding domain of a receptor-ligand interaction can specifically inhibit such an adhesive event. As previously described, peptides of this design have been shown to be effective in reducing tumor growth and lung colony formation in experimental animals. These molecules have the added advantage of being small enough to evade immune detection, and so, do not elicit an antigenic response in the host. The major challenge in the design of synthetic peptides as therapeutic agents is their susceptibility to serum proteases, which results in a very short half life *in vivo*. Additionally, as small fractions of a much larger molecule, the synthetic peptide may experience minimal structural constraints, and may not stably represent the conformation of the amino acid sequence in its native context. If the peptide is to effectively mimic the binding domain of a ligand, and so, block the receptor-ligand interaction, tertiary structure is likely to be of key importance. The determination of the three dimensional structure when it is associated with its target receptor will allow the synthesis of organic compounds locked into the appropriate conformation. This approach has the potential to alleviate the problem of instability, as well as extend the *in vivo* half life of the molecule. However, care must be taken to ensure that the interaction of the synthetic peptide with its target receptor does not have unexpected adverse effects.

Since cell surface receptors are typically used by cells to communicate

with their environment, any receptor-ligand interaction may have the potential to initiate a signal transduction cascade, evoking a cellular response. Many cellular activities are governed by the highly controlled interactions of molecules on the cell surface with those of the extracellular matrix. If the ligand-derived peptides physically interact with the receptor molecule in such a manner as to activate the cell, rather than simply blocking the adhesion, the results could be disastrous. Thus, it is imperative that the activities and mechanisms of the target molecule be well characterized before these agents can become fully useful in the treatment of human disease.

As detailed in earlier sections of this paper, expression of the 67 kDa laminin binding protein has been shown to directly correlate with the metastatic potential of human tumors. Peptide 11, a specific laminin derived binding sequence for this protein, represents a potential anti-metastatic agent through its ability to interfere with the adhesion of tumor cells to a laminin substrate. In a collaborative project with investigators in the biochemistry department, studies are progressing to determine the secondary structure of peptide 11 in association with the 67 kDa laminin binding protein. This section of the study is intended to address the structural features of the 67 kDa laminin binding protein, and to begin to understand its biological activities.

Unpublished results

Since the metastatic character of the B16BL6 mouse melanoma cell line is well documented, this cell line was initially selected as an experimental model.

Isolation of the 67 kDa laminin binding protein was carried out by the method of Wewer *et al.* (1986), and resulted in a pure preparation of high affinity laminin binding protein of approximately 65-67 kDa as determined by silver stained SDS-PAGE. Yield of this protein was approximately 10 $\mu\text{g/ml}$ packed cell volume, and was increased to approximately 30 $\mu\text{g/ml}$ when isolated from cells which had been previously selected for their ability to traverse an EHS basement membrane barrier (B16BL6/EHSx3). Amino acid analysis of the affinity purified 67 kDa laminin binding protein by HPLC of PITC labelled hydrolysates by the method of Heinrickson and Meredith (1984), indicates a composition compatible with the sequence published by Yow *et al.* (Table 1.1). This protein is clearly not the same as that identified by Mecham *et al.* (1989), nor is it consistent with the amino acid composition of the laminin binding protein isolated from muscle cells by Lesot *et al.* (1983).

Replicate HPLC analysis of protein hydrolyzed for 24, 48, or 72 hours demonstrated complete hydrolysis at 48 hours. All samples were noted to contain several large peaks that eluted at a high concentration of organic solvent, and most probably represent non-protein material. At least one of these peaks is likely to represent excess PITC. Others may represent non-protein molecules associated with the 67 kDa laminin binding protein.

Computer comparison of the cDNA predicted amino acid sequence with other proteins having well characterized structural domains, was carried out to identify domains of the 67 kDa laminin binding protein which might indicate

Table 1.1:

Amino Acid Ratios for Various Laminin Binding Proteins

	Aspartactin Hall <i>et al.</i>	Muscle LB-68 Lesot <i>et al.</i>	Elastin binding Mecham <i>et al.</i>	Colon cDNA Yow <i>et al.</i>	B16BL6 67 kDa receptor
Ser/Gly	0.69	1.05	1.03	0.93	0.97
Ala/His	3.0	2.6	2.2	9.25	ND*
Ala/Asp	0.26	0.84	1.09	2.45	2.45
Val/Glu	0.54	0.184	0.16	0.76	0.98

*Histidine not separated

biological activities of the protein. The ALIMAT program, developed by Argos (1987), identifies regions within compared protein sequences with similar specific structural characteristics. These characteristics have been shown, on an empirical basis, to indicate similar functional domains. Using this program, several regions were identified which may be of functional significance. When combined with the MOTIF program comparing the laminin binding protein sequence with functional motifs in the Prosite database, the ALIMAT homologies gained additional significance in some cases.

Previously published reports have indicated that the 67 kDa laminin binding protein may interact with the cytoskeleton, specifically with actin (Brown et al., 1983; Cody and Wicha, 1986). The amino acid sequence was therefore compared to several known actin binding proteins. Most of these proteins showed no homologies with the laminin binding protein sequence. However, the sequence did show regions of strong structural homology with both chicken alpha-actinin and plasma gelsolin. The 75 amino-terminal residues of the laminin binding protein sequence align with residues 669-743 of alpha-actinin, with 14 identical and 13 conserved residues. This region of alpha-actinin is known to be required for dimerization of the molecule during the polymerization of F-actin (Simonidze et al., 1988), and is also the domain known to contain the region which interacts with the cytoplasmic domain of the β_1 integrin chain (Otey et al., 1990).

Gelsolin is an actin modulating protein. It also demonstrated an 85

residue region with a high degree of similarity to the laminin binding protein. Gelsolin has been shown to contain two actin-binding domains, one which is calcium dependent and one which is not (Kwiatkowski et al., 1986). The non-calcium dependent actin-binding region has been identified as residues 421-738. This domain encompasses the region of homology with the laminin binding protein, residues 470-540, suggesting the possibility that the laminin binding protein may also be capable of interacting with actin through a similar structural domain.

A strong structural homology was found with the β_1 integrin chain, aligning residues 203-251 of the laminin binding protein sequence with residues 195-243 of the chicken β_1 sequence. This domain lies some 15 residues to the carboxyl side of a conserved site in the β integrins which has been shown to cross-link the RGD motif of the ligand by β_3 (gp111a) (D'Souza et al., 1988). The β_1 integrin chain and the laminin binding protein are oppositely oriented, resulting in the homologous regions of both proteins being located extracellularly. In the laminin binding protein sequence, the homologous region encompasses a highly charged domain predicted to form an alpha helix, which is demonstrated in this study to interact with laminin.

Phorbol esters have been shown to enhance the laminin binding activity of mast cells (Thompson et al., 1990), NIH 3T3 cells (Kato et al., 1988), and neutrophils (Yoon et al., 1987), as well as to stimulate the metastatic capacity of tumorigenic cells (Gopalakrishna and Barsky, 1988; Nishizuka, 1984).

Furthermore, one of the well characterized responses to phorbol esters is that of protein phosphorylation via activation of nucleotide binding proteins (Parker et al., 1986). We therefore compared the sequence of the 67 kDa laminin binding protein to several kinases, including some known to be stimulated by phorbol esters. These included protein kinase C, lactic dehydrogenase, alcohol dehydrogenase, and adenylate kinase. The degree of homology seen with each of these proteins was not particularly striking. However, what was significant was the observation that residues 1-50 of the laminin binding protein consistently aligned with the specific region known to contact the nucleotide in each of these proteins. In an analysis of the critical residues required for orientation of the nucleotide, all consensus residues are satisfied.

Several potential phosphorylation motifs are identified by comparison with the Prosite database. Threonine²⁸ in the laminin binding protein sequence is a potential phosphorylation site in a casein kinase phosphorylation motif (Pinna, 1990), serine⁴³ is predicted as a potential c-AMP dependent phosphorylation site, and tyrosine⁴⁷ was predicted to be a tyrosine kinase phosphorylation site. Comparison of the laminin binding protein sequence to the β_3 integrin subunit of platelets (gpIIIa) by the ALIMAT program demonstrated a strong structural homology of residues 1-50 with the cytoplasmic tail of β_3 , which has been shown to be phosphorylated on tyrosine in response to ligand binding (Elmore et al., 1990).

No significant similarities were detected with proteins chosen to represent several other classes of cell surface proteins, including some with well characterized transmembrane domains, members of the EGF-like and CAM families of receptors, and mammalian lectins (Table 1.2).

Table 1.2:

Representative proteins compared to the deduced amino acid sequence of the 67 kd high affinity laminin binding protein which demonstrated no significant structural homology

Transmembrane	Receptors	Lectins
M13 Coat protein (J02461) Bacteriorhodopsin (M11720) Porin (E.coli) (M74489) Cytochrome b (X12783) Calcitonin (J00109) Glucagon (X05388)	ELAM-1 (M24736) ICAM-1 (M31585) ICAM-2 (X15606) PDGF receptor (M34480) gpIIb (M34480) gpIIIa (M35999)	Macrophage lectin (M35368) Rat hepatic lectin (K02817) Galactoside binding (X16074)

Numbers in () are Genbank/EMBL accession numbers (releases 69/27)

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CHAPTER 2FATTY ACYLATION AND EVIDENCE FOR MOLECULAR ASSOCIATIONS INVOLVED IN THE ADHESIVE FUNCTION OF THE 67 kDa LAMININ BINDING PROTEINIntroduction

To be successful in metastasis, cancer cells must adhere to and invade extracellular basement membranes. This adhesion and invasion is accomplished by the use of cell surface receptors specific for basement membrane components (for reviews, see Aznavoorian et al., 1993; Stetler-Stevenson et al., 1993). Several laminin binding receptors have been identified, one of which is a high affinity laminin binding protein that migrates with an apparent molecular weight of 67 kDa on SDS-PAGE. This 67 kDa high affinity laminin binding protein was initially isolated from a highly metastatic breast carcinoma by immunoprecipitation with a function blocking antibody (Barsky et al., 1984). Using this antibody as a probe, a partial cDNA sequence was identified in a human endothelial cell expression library. The sequence was found to completely overlap that of a mRNA which was preferentially over expressed in highly metastatic colon carcinoma as compared to adjacent normal colonic epithelium (Yow et al., 1988).

This 67 kDa laminin binding protein has since been shown to be expressed at high levels on lung carcinoma (Satoh et al., 1992), melanoma (Vacca et al., 1993), breast carcinoma (Martignone et al., 1992), and several other invasive solid tumors. The 67 kDa laminin binding protein is expressed at

relatively low levels in benign and normal tissues, and its level of expression correlates very well with the metastatic phenotype of the tumor (Mafune et al., 1990). Since adhesion to the basement membrane is considered to be a critical step in the metastatic cascade, the laminin binding function of this protein makes it a potential target for therapeutic intervention in metastatic disease as well as a useful prognostic indicator.

The full length nucleotide sequence of the high affinity laminin binding protein is sufficient to encode a protein with predicted molecular weight of approximately 32 kDa, while the laminin affinity isolated protein migrates with an apparent molecular weight of 67 kDa on reduced SDS-PAGE. The mechanism of this apparent molecular weight shift is not readily apparent, and is somewhat controversial. Although the original data have not been published, one group has reported that an *E. coli* expression product of a full length clone fails to bind to a laminin affinity column (discussed in Mecham, 1991). The 67 kDa laminin binding protein, when isolated from tumor cell membrane extracts, initially binds with high affinity to a laminin-Sepharose column. However, following laminin affinity purification, the protein does not re-bind to laminin with the same high affinity (M. Sobel and H. Kleinman, personal communication, this manuscript). More recently, another group has reported that a fusion protein of the 67 kDa laminin binding protein cDNA with TrpE which was expressed in *E. coli* did bind to a laminin-sepharose affinity column (Siyanova, 1992). However, it is not clear whether the TrpE secretory signal sequence was present or not, or what

molecular weight the affinity isolated product displayed. Taken together, these data would imply that there are unidentified structural characteristics of the laminin binding protein which play a critical role in its adhesion to laminin.

Rao *et al.* (1989) have reported the existence of a 37 kDa precursor in mouse NIH3T3 cells which can be chased into a 67 kDa product. These proteins were immunoprecipitable with an antibody raised to a synthetic peptide corresponding to the amino terminal region of the cDNA sequence. However, they were unable to identify the mechanisms underlying the molecular weight shift. Although there are 14 serine and 23 threonine residues in the putative amino acid sequence of the cDNA clone which might be modified with O-linked glycosylation, there are no consensus sites for N-linked carbohydrates. Analysis of the predicted amino acid sequence of the protein reveals no simple hydrophobic domain characteristic of a transmembrane region, leading to some speculation that the protein may not actually be a cell surface receptor (Grosso *et al.*, 1991). Several groups, in independent, and often unrelated studies, have identified proteins in yeast (Davis *et al.*, 1992), hydra (Keppel and Schaller, 1991), and *Drosophila* (Melnick *et al.*, 1993), with extensive sequence similarity to the 67 kDa laminin binding protein. These proteins are not cell surface proteins, but are apparently components of the translational machinery. They have not been shown to possess post translational modifications, and are associated with ribosomes. While the cDNA sequence homology of these proteins with the mammalian laminin binding protein is very high in the amino

terminal half of the molecule, the carboxyl terminal domain, which has previously been identified to contain the region important for association with laminin, is much less highly conserved. In the present paper, we hypothesize that the 67 kDa laminin binding protein has two functions in mammalian cells. One function may be intracellular, perhaps similar to that seen in lower species. The second function, which may be dependent on post translational modifications, is responsible for its surface localization and laminin binding characteristics.

Recently, many proteins have been identified with post translational modifications in which lipid moieties are covalently attached to the protein (for review see Schmidt, 1989; McIlhinney, 1990). These acylproteins have varied functions in the cell, including playing key roles in adhesion and signalling. Several mechanistic roles for the lipid modifications have been proposed, including localization of the protein to a lipid bilayer membrane. Lipid modifications have also been shown to modify protein:protein interactions. Finally, acyl modifying groups may be active in generating a second messenger in signal transduction cascades (reviewed in Magee, 1990; Chow et al., 1992).

We report here that the 67 kDa high affinity laminin binding protein is acylated by the fatty acids palmitate, oleate and stearate. These fatty acids appear to be covalently associated via an ester or thioester linkage and, in the absence of other known mechanisms, are likely to be responsible for targeting the protein to the cell surface, where it can participate in the adhesion of cells

to the extracellular matrix. From our results, we also postulate that associations with other molecule(s) are required to mediate high affinity ligand binding.

Materials and Methods

Expression of the laminin binding protein

A cDNA clone of the laminin binding protein, isolated from a hamster expression library, was kindly provided by Dr. James Strauss of the California Technical Institute in the pcDNA1/neo vector (Invitrogen) (pcLR). This expression vector utilizes a CMV promoter and G418 selectable marker (Wang et al., 1992). An expression vector for the dihydrofolate reductase (dhfr) gene, under the control of an SV40 promoter, was obtained from ATCC (Rockville, MD). Both plasmids were simultaneously transfected by calcium phosphate precipitation (Chen and Okayama, 1988) into DG44CHO cells which were obtained from Dr. Lawrence Chasin of Columbia University. These cells are double negative mutants for the dhfr gene, and rely on the presence of exogenous hypoxanthine and thymidine (HT) in the culture media for proliferation (Urlaub and Chasin, 1980). Cotransfection of the pcLR and dhfr plasmids was carried out at 1:1, 5:1, 10:1, and 20:1 ratios of pcLR:dhfr with a total of 20 μ g of DNA per 10^6 cells. Untransfected cells were grown in α MEM (Sigma) containing 10% FBS (Intergen) and supplemented with HT, 5 μ g/ml bovine insulin, 10 mM L-glutamate, and 100 U/L each of penicillin and streptomycin. Selection of transfectants was carried

out in the same medium without HT, containing 10% dialyzed FBS, and 400 $\mu\text{g/ml}$ G418 (Gibco). Methotrexate treatment of dhfr/pcLR transfected cells was initiated at a concentration of 0.03 μm , and increased by 0.02-0.05 increments approximately every 5th cell passage. Amplification of pcLR expression was monitored by Northern analysis and FACScan (Landowski *et al.*, manuscript submitted).

For metabolic labelling of glycosyl phosphatidylinositol (GPI) modified proteins, ^{14}C -ethanolamine (NEN) was added to the culture medium at a concentration of 0.1 $\mu\text{Ci/ml}$. Labelling of cells was carried out for 16 hours at 37°C.

Laminin binding protein isolation

The laminin binding protein was extracted by the method of Wewer *et al.* (Wewer *et al.*, 1986) with minor modifications. DG44CHO cells, selected for expression of the pcLR and dhfr plasmids, were harvested by rinsing twice with Puck's CMF Saline G solution, approximately 10 minutes each at 37°C, and a third time with the same solution containing 0.01 mM EGTA until the cells detached. This harvesting technique was used to avoid the use of trypsin and preserve membrane proteins. Cells were centrifuged for 10 minutes at 500 x g, the pellet resuspended in 10 volumes of CMF-Dulbecco's PBS (v/v), and centrifuged again under the same conditions. Included in this, and all subsequent buffers, were the protease inhibitors 50 $\mu\text{g/ml}$ PMSF, 1mM N-ethylmaleamide, and 5 mM benzamidine. Cells were then suspended in 2

volumes of ice cold 25mM Tris/0.3 M sucrose, pH 7.4 (v/v), and sonicated with 4-5 x 5 second bursts on ice using a Fisher Sonic Dismembrator, Model 50 at 50% power. Nuclei, cytoskeletal proteins and unbroken cells were pelleted by centrifugation at 500 x g for 10 minutes at 4°C, the supernatant collected, and the pellet resuspended in 5 volumes of Tris/sucrose buffer. Sonication was repeated, and the supernatant again collected and added to the first supernatant. These steps were repeated three times, or until the vast majority of the cells appeared lysed under microscopic examination. The membrane fraction was then collected on a sucrose cushion by centrifugation at 143,000 x g for 90 minutes at 4°C, and adjusted to a protein concentration of approximately 1 mg/ml with 25 mM Tris/150 mM NaCl/1 mM CaCl₂/3 mM MgCl₂, pH 7.4 buffer. An equal volume of the same buffer containing 1% NP-40 detergent was added, and the solution was rotated end over end for 12-16 hours at 4°C. Insoluble material was pelleted by centrifugation at 200,000 x g for 60 minutes. The supernatant was combined with a laminin-sepharose column which had been pre-equilibrated with 10 column volumes of 25 mM Tris/150 mM NaCl/1 mM CaCl₂/3 mM MgCl₂ + 0.05% NP-40 followed by the same buffer containing 400 mM NaCl. Laminin-sepharose affinity columns were prepared by coupling EHS laminin (Gibco) to CnBr-activated Sepharose 4B at a concentration of 0.5 mg/ml according to the procedure recommended by the manufacturer (Pharmacia). After application of the detergent extract, the column was washed with 50 mM Tris/0.1 M NaCl, pH 7.4 and the UV absorbance was monitored at 214 nm until it returned to

baseline. Approximately one column volume of 50 mM Tris/1.0 M NaCl, pH 7.4, was added and the column allowed to stand 15-20 minutes prior to elution of the high affinity binding protein with the latter elution buffer.

Isolated laminin binding protein was labelled with ^{125}I by the lactoperoxidase method using Enzymobeads (Bio-Rad), and labelled protein was separated from free iodine by chromatography on a BioGel P6 column with 1 M NaH_2PO_4 , pH 7.4. Specific activity of the labelled protein was 1.5×10^6 cpm/mg.

Antibody production and western blotting

A peptide corresponding to residues 205-229 of the amino acid sequence of the 67 kDa laminin binding protein (RDPEEIEKEEQAAAEKAVTKKEEFQG) was synthesized using standard Fmoc chemistry on a Milligen 9050 automated peptide synthesizer. Following purification by reverse phase HPLC and assessment of purity with electrospray mass spectrometry, the peptide was conjugated to KLH (Sigma) by glutaraldehyde crosslinking with 0.2% glutaraldehyde for 2 hours at room temperature. The solution was dialyzed against Dulbecco's PBS for 48 hours with 4 changes of dialysate to remove glutaraldehyde and free peptide prior to immunization. New Zealand white rabbits were injected with 1 mg of peptide-KLH and Freund's complete adjuvant at multiple subcutaneous sites, and boosted every two weeks with peptide-KLH and incomplete Freund's. Thirty days later a test bleed was obtained and the anti-peptide activity titered by ELISA using KLH, peptide-KLH, and peptide conjugated to ovalbumin as target antigens. The polyclonal antiserum was

purified on a Protein A column (Pierce) and the majority of the anti-KLH activity was removed with a KLH-Sepharose column.

Affinity isolated laminin binding protein was electrophoresed on a 10% SDS-PAGE gel and transferred to PVDF membrane (Bio-Rad) using a semi-dry blotter (Ellard Instrumentation). We found that the 67 kDa protein did not adhere well to PVDF membrane, therefore, the filter was fixed in Dulbecco's PBS containing 0.02% glutaraldehyde for 2 hours at room temperature prior to washing and blocking with 5% non-fat dry milk and 0.1% Tween-20 in PBS. To ensure that the glutaraldehyde fixation was not introducing false positive results, fixation in 10% acetic acid/20% methanol was also used, and found to give identical results. Antibody detection was performed using alkaline phosphatase conjugated goat anti-rabbit antibody (Bio-Rad) and BCIP/NBT chromogenic substrate (Kirkegaard Perry Inc.).

Determination of molecular weight

Determination of the molecular weight of the affinity isolated laminin binding protein was performed by Matrix Assisted Laser Desorption Time of Flight Mass Spectrometry (MALDI TOF-MS) on a Vestec instrument. One μl of 0.5 mg/ml laminin binding protein solution in 50 mM Tris/1.0 M NaCl was applied to a sample well, along with 1 μl of a saturated water solution of the UV sensitizer, sinapinic acid (Aldrich). Samples were air dried and subjected to 5 ns flashes from a 366 nm nitrogen laser. External mass calibration was carried out with cytochrome c, bacteriorhodopsin, and ovalbumin.

Lipid Characterization

Affinity purified 67 kDa laminin binding protein was frozen and lyophilized to dryness. Fifty μg of dry weight protein was then dispersed in 200 μl of Methyl Prep II (Alltech) and transesterified at 65°C for 60 or 90 minutes. The reaction was quenched by the addition of 200 μl of double distilled H₂O, and 100 μl of methanol containing 20 $\mu\text{g}/\text{ml}$ butylated hydroxytoluene, and the fatty acid methyl esters were extracted with hexane. The Methyl Prep II reagent converts esterified fatty acids to methyl esters, and any non-esterified fatty acids that may be present partition into the aqueous phase as (*m*-trifluoromethylphenyl) trimethylammonium salts (McCreary et al., 1978). The hexane extracted fatty acid methyl esters were then dried down under a stream of argon, and redissolved in 20 μl of iso-octane. Gas chromatographic analyses were performed with a Hewlett-Packard Model 5890 Series II gas chromatograph equipped with a split injector and flame ionization detector, using a DB-8 column. Temperatures used were: injector, 250°C; column, 100°C to 300°C at 5°C/min. The affinity isolation buffer plus NP-40 was similarly treated as a control for external lipid contamination. In separate assays, lyophilized laminin binding protein samples were first washed with methylene chloride, and the wash was treated with Methyl Prep II and analyzed by gas chromatography as described above. The methylene chloride extraction was carried out to test for the presence of non-covalently associated lipids.

Glycosidase treatments

Affinity purified 67 kDa laminin binding protein was treated with Endo-F (Boehringer-Mannheim), Neuraminidase (Boehringer-Mannheim) and O-Glycanase (Genzyme) under conditions recommended by the manufacturers. Endo-F treatment was carried out at 37°C in 10 mM NaHPO₄ with 1 U enzyme per mg protein. For O-Glycanase treatment, ¹²⁵I labeled 67 kDa laminin binding protein was dialyzed into 10 mM calcium acetate/20 mM sodium cacodylate buffer. Duplicate samples of 50 µl each (0.5 mg/ml) were denatured with 0.1% SDS at 80°C for 5 minutes, and a 6 fold excess of NP-40 was added to reduce the concentration of SDS prior to treatment with 0.35 U Neuraminidase at 37°C for 2 hours. O-glycanase was added to a concentration of 0.1 U/µg protein, and the incubation carried out for an additional 2 hours or overnight.

Triton X-114 solubility

To assess the potential membrane association of the protein, the method of Bordier (Bordier, 1981) was used to determine Triton X-114 solubility. Affinity purified, ¹²⁵I-labelled laminin binding protein was dialyzed to 10 mM Tris/150 mM NaCl, pH 7.4, and dissolved in precondensed 1.0% Triton X-114 (Sigma) in the same buffer at 0°C. The samples were then warmed to 30°C for 5 minutes and the phases separated by centrifugation at 10,000 x g on a 6% sucrose cushion at room temperature. The upper aqueous phase was removed and brought to 0.5% Triton X-114, cleared on ice, and again brought to 30°C for five minutes. This sample was reapplied to the 6% sucrose cushion, and centrifuged to

separate the phases. Aqueous and detergent-rich phases were then analyzed by electrophoresis on 10% SDS-PAGE and the radioactive components were visualized on a Molecular Dynamics™ phosphorimager using ImageQuant™ software.

Reconstitution of laminin binding

Affinity purified ^{125}I labelled laminin binding protein was dialyzed back to the laminin affinity column binding buffer, 25 mM Tris/150 mM NaCl/ 3 mM MgCl_2 /1 mM CaCl_2 , pH 7.4. In one experiment, this solution was brought to 0.05% NP-40 and reapplied directly to a laminin-sepharose column. In a second experiment, the solution was reconstituted with fractions 1 and 4 of the 50 mM Tris/0.1 M NaCl wash from a previous laminin binding protein extraction. These fractions were chosen because fraction 1 contains the majority of the NP-40 soluble cellular proteins that do not bind to laminin directly, while fraction 4 includes a "shoulder" which was consistently noted on the low ionic strength peak, and may include components which bind to laminin with intermediate affinity. The reconstituted solution was brought to 0.05% NP-40, and reapplied to a laminin-sepharose affinity column equilibrated as for the initial purification. Column elution was carried out exactly as for the *de novo* isolation procedure for the high affinity laminin binding protein.

Results

Expression and identification of the laminin binding protein

Since other labs have reported difficulties in obtaining a high affinity laminin binding product in artificial expression systems, we postulated that post translational modification may be required to obtain a fully functional protein. Post translational modifications may be species specific, or may be determined by the non-coding sequences in the transfected plasmid. We therefore chose to transfect CHO cells with the hamster cDNA clone for the laminin binding protein. The hamster protein was identified by Wang *et al.* as a cell surface receptor for the Sindbis virus (Wang *et al.*, 1992). The cDNA shows 99% identity with the mouse cDNA (Rao *et al.*, 1989) and 96% identity with the human cDNA (Yow *et al.*, 1988). Only two amino acids differ with each species.

Northern analysis of transfected cell populations demonstrated two mRNA products, one an endogenous product which was also found in untransfected controls, and a second at a slightly higher molecular weight consistent with the expected product of the transfected plasmid (Landowski *et al.*, manuscript submitted). Although the mechanism is not well understood, it has been shown that methotrexate causes reduplication of transfected dhfr plasmids and simultaneous amplification of any cotransfected plasmid (Kaufman and Sharp, 1982). Using this protocol, we were able to achieve amplification of the transfected gene and to increase expression of the transfected plasmid by approximately 10 fold as measured by quantitative analysis of densitometry. The

protein product isolated by laminin affinity from these transfected cell lines was also increased by approximately 10 fold, resulting in a yield of approximately 300 μg of purified laminin binding protein per 8 ml of packed cell volume (Fig. 2.1a). The identity of the laminin binding protein was confirmed by Western blot using a sequence specific polyclonal antiserum (Fig. 2.1b). This antibody stained the 67 kDa laminin affinity isolated protein from the transfected cell lines, as well as laminin affinity isolated proteins from the EHS tumor and B16BL6 mouse melanoma cells. Antibody binding on Western blots could be completely inhibited by the presence of 1 mg/ml of the immunizing peptide. The antiserum failed to react with bovine serum albumin, ovalbumin, or any other protein tested.

The MALDI-TOF MS measurement of molecular weight is compatible with the apparent MW observed by SDS-PAGE

Since SDS-PAGE provides apparent molecular weights, and mobility artifacts are common with membrane proteins, the true molecular weight of the affinity isolated material was determined by MALDI-TOF mass spectrometry. Mass spectra of the protein indicated the major molecular species at a mass of 66.7 kDa. Minor peaks were identified at 33 kDa, 133 kDa, and 201 kDa (Fig. 2.2). The calibration of the mass spectrometer was with external standards, and the accuracy of the molecular weight determination is expected to be in the range of $\pm 0.2\%$ (Aitken, 1992). To determine if disulfide bonding is involved in the shift in molecular weight from the cDNA predicted 32 kDa to the 67 kDa apparent MW on SDS-PAGE, ^{125}I labelled laminin binding protein was subjected

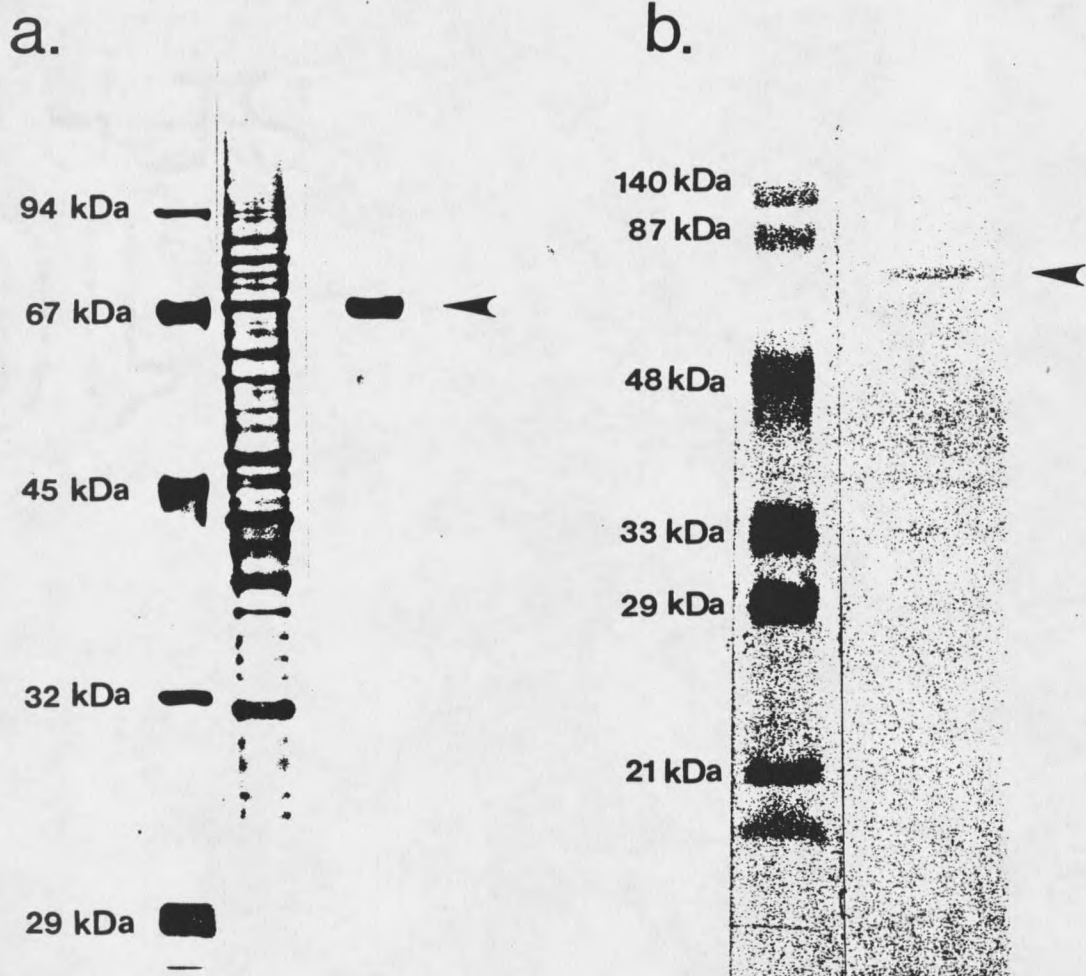


Figure 2.1. a) Silver stained SDS-PAGE demonstrates that the 67 kDa laminin binding protein is isolated in pure form by elution from a laminin-Sepharose column by high ionic strength solution. Lane 1, molecular weight standards (Pharmacia); Lane 2, starting membrane detergent extracts applied to the laminin-Sepharose column; Lane 3, high salt eluate of DG44CHO pcLR/dhfr detergent extracts from laminin-Sepharose column.

b) Polyclonal antiserum raised to a synthetic peptide derived from the deduced amino acid sequence of the 67 kDa laminin binding protein recognizes the affinity isolated product on a Western blot. Lane 1, prestained molecular weight standards (Bio-Rad); Lane 2, 67 kDa laminin binding protein probed with the sequence specific anti-peptide 205-229 antibody.

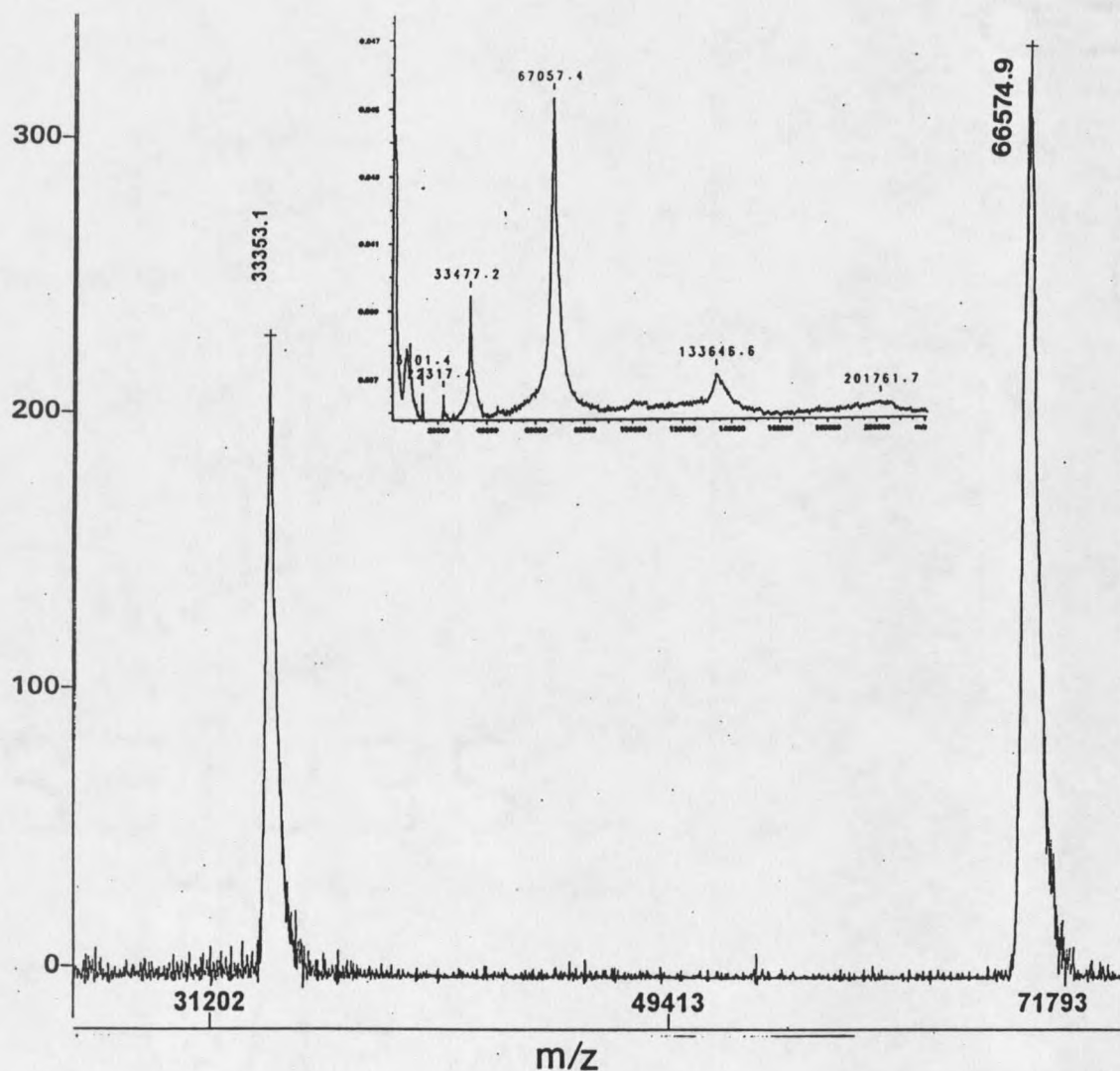


Figure 2.2. Affinity purified 67 kDa laminin binding protein was subjected to MALDI-TOF mass spectrometry for accurate molecular weight determination, and found to be 66.7 kDa. The accuracy of this method is $\pm 0.2\%$, with external calibration carried out using cytochrome c, bacteriorhodopsin, and ovalbumin. The insert demonstrates the relative quantities of the minor molecular weight species seen at 33 kDa, 133 kDa, and 201 kDa.

to reduction with 1 M DTT at 80°C for 1 hour. This treatment did not affect the apparent molecular weight of the protein on SDS-PAGE (Fig. 2.3). This finding is consistent with the report by Rao *et al.* (1989). Treatment of the material with 10 mM DTT followed by MALDI-TOF MS also did not significantly change the relative quantities of the four peaks obtained (data not shown).

The 67 kDa laminin binding protein is covalently associated with lipids, but does not appear to be modified by carbohydrates

The treatment of affinity isolated 67 kDa laminin binding protein with O-Glycanase, or O-Glycanase and Neuraminidase had no effect on the apparent molecular weight of ¹²⁵I-labelled protein (Fig. 2.4). Consistent with the absence of consensus sites for N-linked glycosylation, no molecular weight shift was seen when the affinity isolated protein was treated with Endo-F (data not shown). Affinity isolated laminin binding protein was dialyzed against double distilled H₂O to remove buffer salts and reduce detergent remaining from the extraction procedure prior to lyophilization. The lyophilized material was analyzed on SDS-PAGE and showed the same apparent molecular weight as the starting material (data not shown). Samples of 50 µg of protein (dry weight) were transesterified with the alkaline methanolic reagent Methyl Prep II. The resulting fatty acid methyl esters were hexane extracted and subjected to GC and GC-MS. Comparison of peaks observed in electron ionization GC-MS with a library of mass spectrometry data enabled us to identify three lipid moieties: palmitate, stearate, and oleate (Figs. 2.5 and 2.6). Semi-quantitative comparison of the lipids extracted from the 67 kDa laminin binding protein with external standards

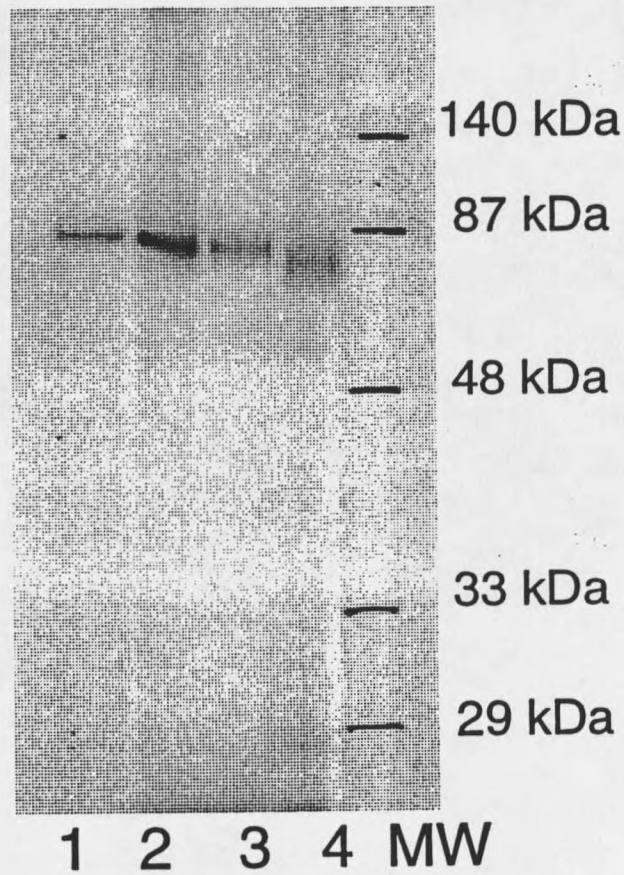


Figure 2.3. Reduction of 67 kDa laminin binding protein with β -mercaptoethanol or dithiothreitol has no effect on the apparent molecular weight of the protein on 10% SDS-PAGE. ^{125}I -labelled laminin binding protein isolated by laminin-Sepharose affinity chromatography is used in all cases. Lane 1, β -ME treatment for 2 hours at 80°C. Lanes 2 and 3, DTT treatment for 2 hours at room temperature and 80°C. Lane 4, starting material.

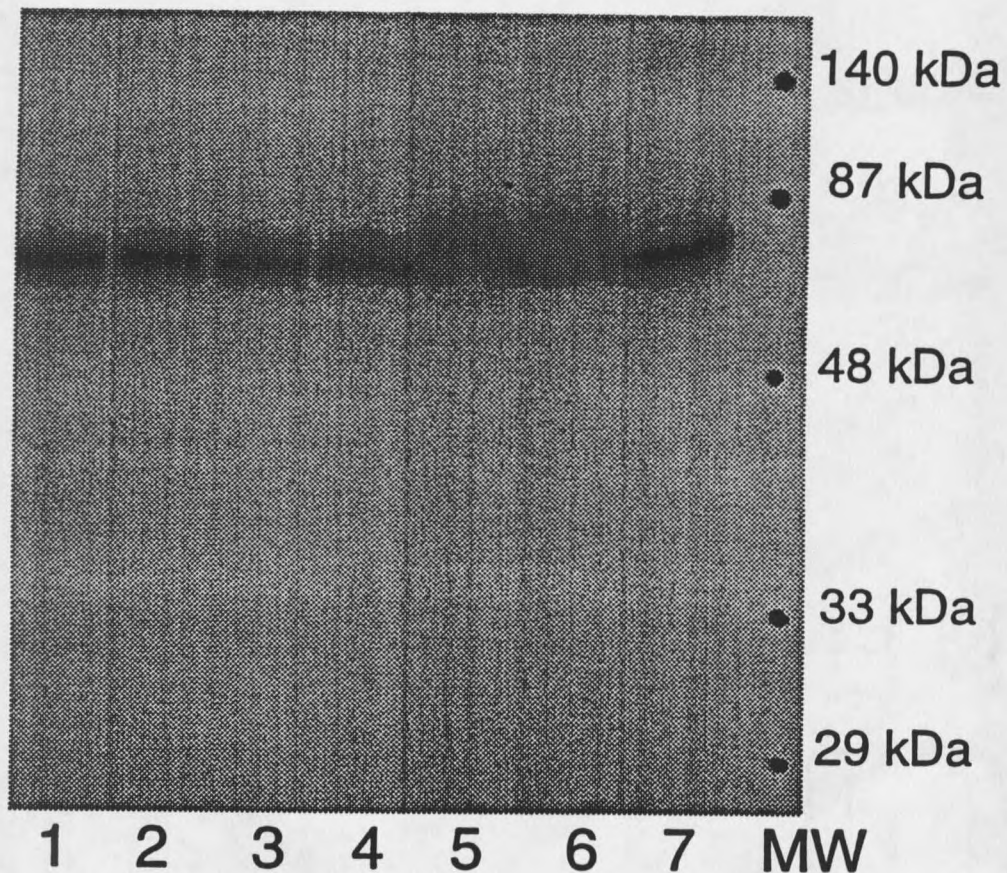


Figure 2.4. The molecular weight shift from a cDNA predicted polypeptide of 32 kDa to the observed 67 kDa of affinity isolated laminin binding protein does not appear to be mediated by glycosylation. Treatment with Neuraminidase, or Neuraminidase and O-Glycanase has no effect on the apparent molecular weight of affinity purified 67 kDa laminin binding protein. ^{125}I -labelled laminin binding protein is used in all cases, and experiments are analyzed by SDS-PAGE. Lanes 1 and 2, Neuraminidase treatment for 60 minutes (1) or 2 hours (2); Lanes 3 and 4, Neuraminidase followed by O-Glycanase treatment for 2 hours (3) or for 16 hours (4); Lanes 5 and 6, 67 kDa laminin binding protein denatured at 80°C with 0.1% SDS to control for detergent effects; Lane 7, untreated starting material. The positions of prestained molecular standards were marked with ^{14}C ink prior to exposure of the phosphorimaging screen.

indicated approximately 0.05 μg of palmitate, 0.04 μg of stearate, and 0.02 μg of oleate per 50 μg protein sample. Transesterification at room temperature resulted in a significantly lower yield of palmitate, and the stearate and oleate were not detected in all experiments under these conditions. Organic solvent extraction and esterification of the extracts of the isolated protein yielded no detectable fatty acid methyl esters. Analyses of lyophilized laminin affinity elution buffer containing NP-40 were entirely negative for fatty acids, as were methylene chloride wash extracts. While our data are in fairly good agreement with a 1:1 molar ratio for each lipid species identified, this acylation is not likely to be sufficient to account for the entire molecular weight shift from the predicted 32 kDa to the observed 67 kDa.

Lipids identified with the 67 kDa laminin binding protein are not associated via a glycosyl-phosphatidylinositol (GPI) structure

Lipid modifications of plasma membrane bound proteins are frequently found to be attached by a GPI moiety. Two separate assays were used to determine whether the lipids we identified were present as part of a glycosyl-phosphatidylinositol structure. Temperature-induced phase separation in Triton X-114 showed that the ^{125}I labelled laminin receptor partitioned primarily into the aqueous phase (Fig. 2.7). This finding is compatible with the predicted hydrophobicity index of the cDNA deduced amino acid sequence (Rao et al., 1989), but unlikely for a GPI-tailed protein. Metabolic labelling of the 67 kDa laminin binding protein with ^{14}C -Ethanolamine in the DG44CHO pcLR/dhfr overexpression system did not lead to incorporation of detectable levels of

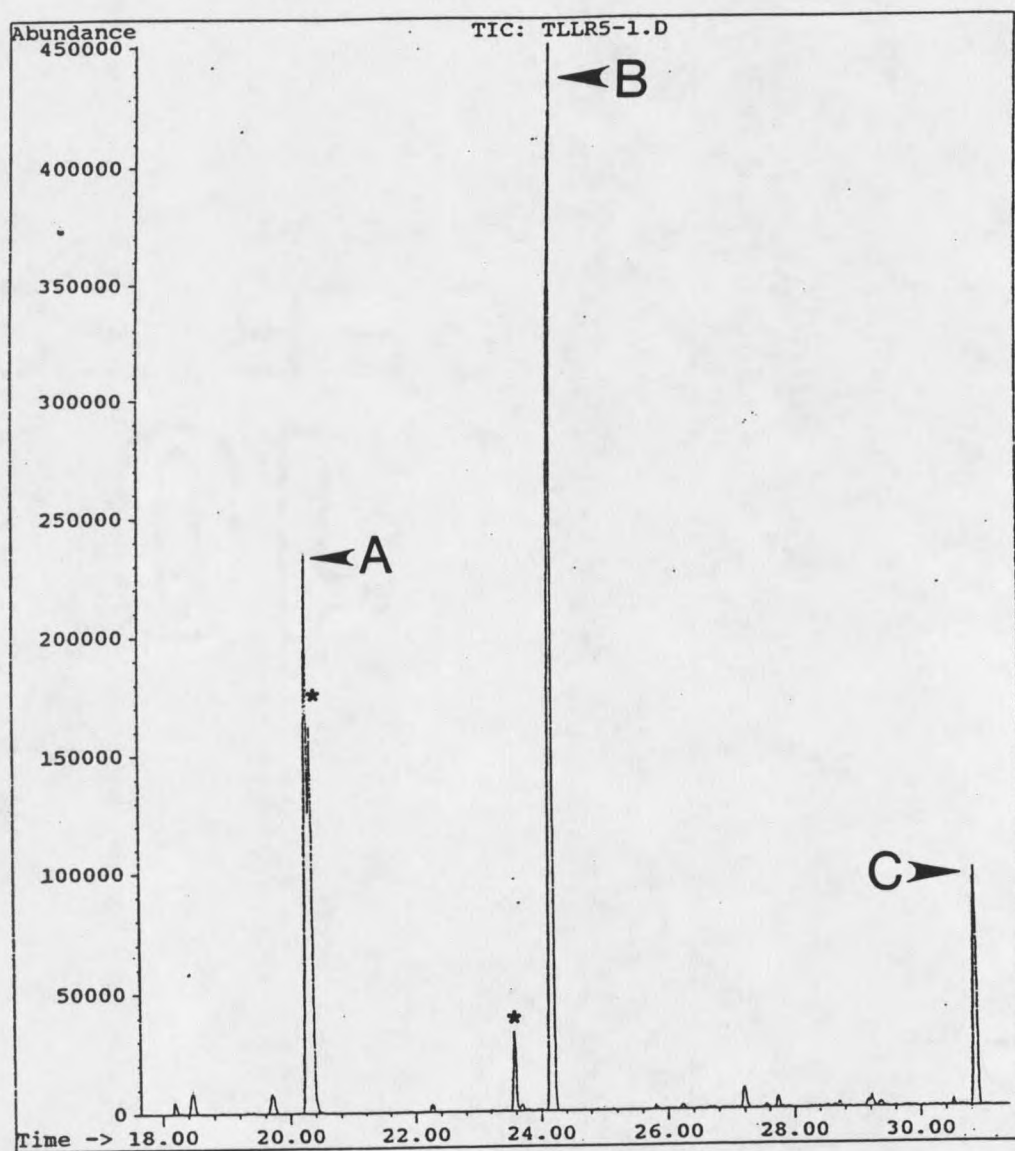
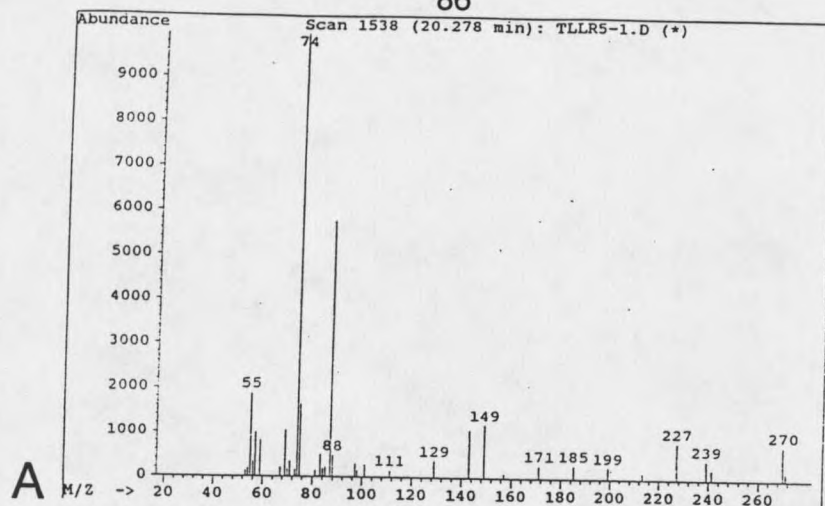
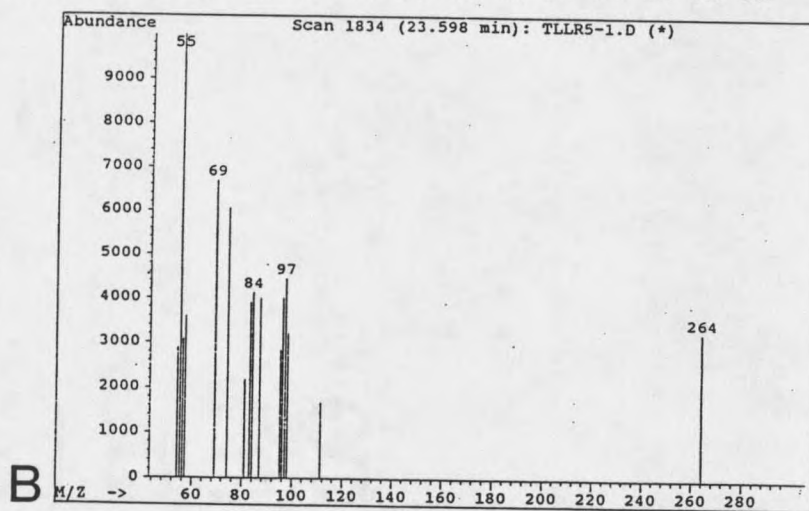


Figure 2.5. Affinity isolated protein was transesterified by alkaline methanolysis, the fatty acid methyl esters extracted with hexane, dried down under argon, and redissolved in iso-octane for GC analysis. Peak A was identified as palmitate (Figure 2.6, panel A), Peak B was identified as oleate (Figure 2.6, panel B) and Peak C was shown to be stearate (Figure 2.6, panel C) The two small peaks indicated by * were identified as plasticizers.

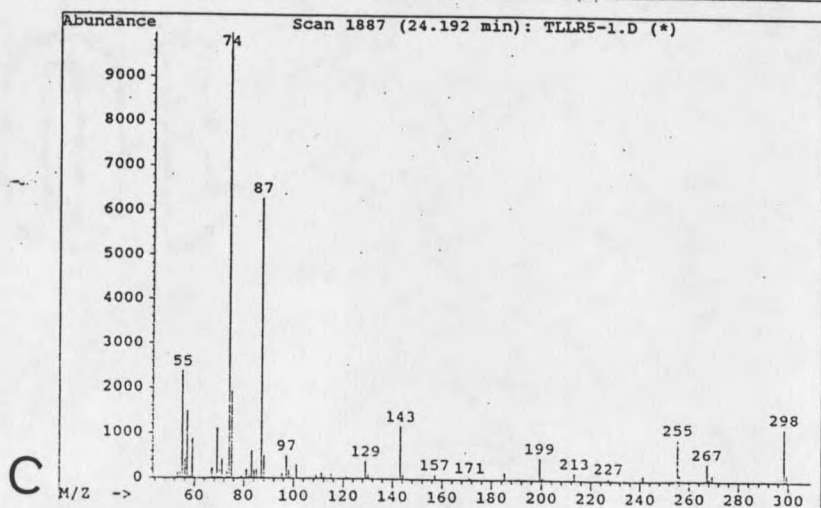
86



A



B



C

Figure 2.6. Mass spectrometry identification of fatty acid methyl esters. Peak A, palmitate; Peak B, oleate; Peak C, Stearate.

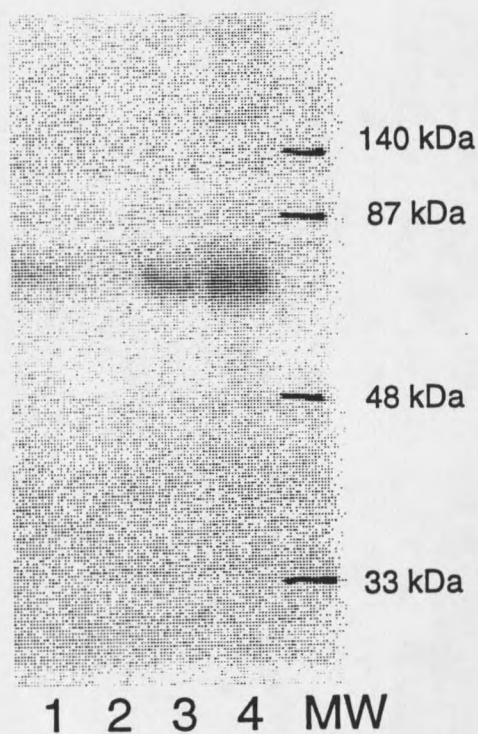


Figure 2.7. Partitioning of affinity purified laminin binding protein into the aqueous phase of a Triton X-114 solubility assay indicates that it is not likely to be modified by a glycosyl-phosphatidylinositol tail. ^{125}I -labelled protein was subjected to temperature induced phase separation at 0° and 30°C , and separated on a sucrose cushion. Lane 1, 1st detergent phase; Lane 2, 2nd detergent phase; Lane 3, aqueous phase; Lane 4, untreated protein; M, prestained molecular weight markers (Bio-Rad) marked with ^{14}C ink.

radioactive label into the affinity isolated product. Extended exposure of the phosphorimage screen indicated that, while specific activity of the labelling was relatively low, it was sufficient for detection. Non-laminin binding fractions of the affinity purification protocol showed a low level of radioactivity derived from the ^{14}C -ethanolamine, some of which was clearly associated with a protein of approximately 40-45 kDa on SDS-PAGE (data not shown).

High affinity laminin binding is modulated by accessory factors

When ^{125}I -labelled laminin binding protein was reapplied to a laminin-Sepharose column under conditions identical to its initial purification, approximately one half of the applied activity was recovered in the unbound column effluent (Fig. 2.8). The remaining bound activity was entirely eluted from the column with a low ionic strength buffer. During the initial purification of the 67 kDa laminin binding protein, this low ionic strength buffer is used to remove all loosely associated material, and is found to contain many proteins with a wide range of molecular weights. The high affinity products are then eluted with a high ionic strength buffer. Upon repurification, no detectable level of ^{125}I -labelled 67 kDa protein was recovered in the high ionic strength elution. The addition of fractions 1 and 4 of the low affinity eluate to the purified 67 kDa laminin binding protein solution resulted in an elution profile very similar to that seen on the initial isolation. SDS-PAGE analysis of the eluates demonstrated a significantly higher proportion of the 67 kDa laminin binding protein in the high affinity fractions of the reconstituted rebinding experiments (data not shown).

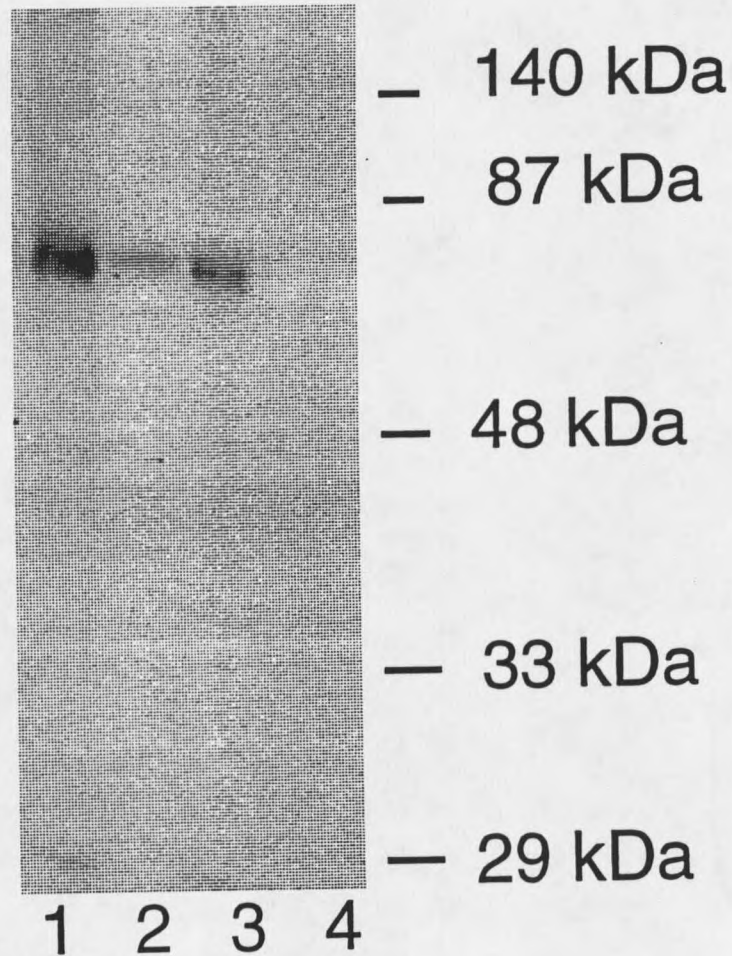


Figure 2.8 Fractions collected from a laminin-Sepharose affinity column following application of purified, ^{125}I -labelled 67 kDa laminin binding protein indicated that the majority of the applied material did not rebind, but was present in the column effluent. All samples were analyzed by SDS-PAGE. Lane 1, unbound fraction; Lane 2, first fraction of low ionic strength column wash; Lane 3, fourth fraction of low ionic strength column wash; Lane 4, high ionic strength column eluate. Molecular weight standards are as marked.

Discussion

The 67 kDa laminin binding protein has been shown to play an important role in the adhesion and extravasation of tumor cells during the metastatic dissemination of cancer (reviewed in Sobel, 1994). However, neither the structure, nor the ligand binding mechanism of this protein is understood in any detail. Considerable confusion in the literature has been generated by the fact that a number of laminin binding proteins of similar apparent molecular weight have been described, and frequently assumed to be the same molecule (reviewed in Mecham, 1991). Subsequent sequencing has identified some of these proteins as 5'-nucleotidase (Misumi et al., 1990), aspartactin (Clegg et al., 1989), and a splice variant of β -galactosidase (Hinek et al., 1993). Antibodies to these various laminin binding proteins have been shown to cross react with each other, possibly due to common epitope structures (Risse et al., 1989).

The isolation of the cDNA clone for the laminin binding protein failed to clarify the issue, as the cDNA clone was sufficient to encode only a 32 kDa protein. Furthermore, the deduced protein sequence contained no consensus sites for N-linked glycosylation which could account for the molecular weight discrepancy, and no obvious transmembrane region is apparent from the sequence (Yow et al., 1988). Classical biochemical analyses such as peptide mapping and direct amino acid sequencing have been limited by insufficient material for standard assays. Reticulocyte lysate *in vitro* translation studies (Castronovo et al., 1991; Rao et al., 1989) were reported to result in a 37 kDa

protein. In those studies, anti-synthetic peptide antibodies which immunoprecipitated the 67 kDa product did not always recognize the putative 37 kDa precursor. In the current study, we have utilized a homotypic eukaryotic over-expression system to produce the 67 kDa laminin binding protein in its physiologically functional form.

Using the technique of MALDI-TOF mass spectrometry, we were able to demonstrate that the molecular weight of the protein is consistent with its SDS-PAGE migration. It is attractive to speculate that the 33 kDa species seen on the MALDI TOF-MS could represent the native gene product with the 66 kDa species representing a dimer, and the 133 and 201 kDa representing 4x and 6x subunits, respectively. Treatment with disulfide reducing agents did not affect the molecular weight, either on SDS-PAGE or in the mass spectra, indicating that, if this protein does exist as a dimer or higher multimer, the association is not dependent on disulfide bonds. If the high molecular weight peaks identified on the TOF MS represent nonspecific aggregates of a 33 kDa monomer, one would also anticipate the presence of 3x and 5x multimers, which were not detected. However, it should be emphasized that with the methods employed here, it is not possible to definitively distinguish between a singly protonated monomer (MH^{+1}) of 33 kDa, and a doubly protonated monomer (MH_2^{+2}) of 66 kDa which would also appear at M/Z 33 kDa. Therefore, the cDNA predicted product of 32 kDa has not been unambiguously rationalized with the principal 67 kDa observed species. Further studies, such as mass spectrometry of tryptic

digest, or cyanogen bromide, cleavage products will be required to definitively determine the monomeric unit of this macromolecule, and these studies are underway in our laboratory.

Castronovo *et al.* reported that N-glycosidases had no effect on the molecular weight of isolated 67 kDa laminin binding protein (Castronovo *et al.*, 1991), and there are no consensus sites for N-linked carbohydrates. They were also unable to metabolically label a transfected construct in COS-7 cells, with ^3H -glucosamine or galactosamine. Our results, using endoglycosidase digestion of affinity purified laminin binding protein, support the conclusion that neither N-, nor O-linked glycosylation is responsible for the molecular weight shift from the cDNA predicted product of 32 kDa to the affinity isolated 67 kDa product. It is conceivable that there are carbohydrates associated with the molecule in a manner not recognized by commonly used endoglycosidases. However, glycoproteins typically show some degree of heterogeneity in glycosylation. The MALDI-TOF spectra show sharp peaks, with no evidence of glycosylation induced heterogeneity. The lack of heterogeneity in molecular weight is strong evidence against the 67 kDa laminin binding protein being a glycoprotein. Therefore, modifications with non-carbohydrate moieties are a more likely explanation for the discrepancy between the predicted and observed molecular weights.

Acylation of proteins has recently been shown to be relevant to the structure and function of numerous mammalian proteins (reviewed in Chow *et*

al., 1992). Three primary mechanisms of fatty acid attachment have thus far been described: 1) ester or thioester linkages to an internal amino acid; 2) amide linkages to an amino-terminal glycine residue; 3) a phosphodiester linkage to a glycan moiety, forming a glycosyl phosphatidylinositol (GPI) tail on the carboxyl terminus of the protein. The fatty acid most commonly identified with an ester linkage is palmitate, whereas cysteine residues in a CAAX consensus sequence are frequently modified with isoprenoids through a thioester linkage.

We have identified the covalent association of palmitate, stearate, and oleate with the affinity isolated 67 kDa laminin binding protein. These lipids may provide a mechanism for membrane association of the molecule on the cell surface, as there is no standard transmembrane domain apparent within the predicted amino acid coding sequence of the cDNA. Lipid modified proteins are known to be associated with both the extracellular and cytoplasmic surfaces of plasma membranes. The majority of the known acylproteins on the extracellular surface are associated with the membrane by lipids covalently bound to the protein through a glycosyl phosphatidylinositol linkage. Using two separate assay methods, we did not find any evidence for the presence of a standard GPI linkage. Triton X-114 solubility is a technique commonly used to separate amphipathic proteins, and to identify transmembrane proteins. This technique is based on the ability of the non-ionic detergent Triton X-114 to partition into two distinct phases at 30°C; a detergent rich phase and an aqueous phase.

Amphipathic proteins with a transmembrane domain tend to partition predominantly (>80%) into the detergent rich phase, while hydrophilic proteins generally partition into the aqueous phase. Proteins which are anchored in the membrane with GPI tails have been shown to partition into the detergent phase even when associated with a very hydrophilic protein (Hooper, 1992). However, the 67 kDa laminin binding protein remained predominantly in the aqueous phase. In a second assay for GPI association of the identified lipids, we attempted to metabolically label the 67 kDa laminin binding protein with ^{14}C -Ethanolamine. We failed to detect any ^{14}C labelling in isolated 67 kDa laminin binding protein. Ethanolamine is a specific label for GPI moieties, but would result in only a 1:1 molar ratio under optimum biosynthetic conditions. It is possible that the specific activity used was below the limits of detection in this system, however, radioactivity was detected in a non-laminin binding fraction of the cell extract, so this is considered unlikely.

The conditions required to release the covalently bound lipid from the protein were rather stringent, relative to standard fatty acid analyses (McCreary et al., 1978). These conditions also precluded the recovery of the intact protein from the reaction mix, so the effects of deacylation could not be evaluated. Transesterification under milder conditions resulted in significantly lesser quantities of methyl esters, and, in fact, the oleate and stearate were not always detectable under the milder experimental conditions. Acid hydrolysis also failed to release fatty acids, indicating the linkage mechanism is not likely to be an

amide bond (unpublished observations). The most common mechanism for palmitate attachment to proteins has been shown to be an ester bond with the hydroxy group on serine or threonine (McIlhinney, 1990). Our data are compatible with this chemistry. Hydroxyester linkage of stearate and oleate are also a possibility. As these two lipids are common components of the phospholipid bilayer, great care was taken to ensure their presence was not due to contamination of the protein preparations. Given the harsh conditions required for isolation of these fatty acids, we are confident they are, in fact, covalently associated with the protein. However, we were unable to definitively identify the linkage chemistry.

On a molecular basis, we did not find the quantity of lipid to be sufficient to account for the total molecular weight shift from the cDNA predicted 32 kDa to the observed 67 kDa. However, as discussed earlier, given the experimental conditions required to release the lipid, complete transesterification may not have been achieved. One possible mechanism for the shift from 32 kDa to 67 kDa would be a dimerization of the native gene product which may be stabilized by lipid:lipid association. With an error range of $\pm 0.2\%$, the MALDI-TOF molecular weight measurement compared to the cDNA predicted molecular weight could accommodate up to 2 long chain fatty acids per 32 kDa molecule, or 4 per 67 kDa molecule. Lipid modification of the protein may be responsible for plasma membrane association of the 67 kDa laminin binding protein in the absence of a hydrophobic membrane anchor polypeptide domain. Alternatively,

the lipids may promote an association with a second protein. Castronovo *et al.* (1991) have proposed a covalent linkage with a second protein molecule, and our results do not eliminate that as a possibility. Indeed, we show evidence in this paper, that the high affinity ligand binding function is modulated by an, as yet, unidentified factor in detergent extracts of tumor cells.

The ligand binding activity of many cell surface receptors is known to be modulated by local environmental factors. The ligand binding activity of the integrin receptor $\alpha_M\beta_2$ has been shown to be enhanced by an, as yet, unidentified lipid factor, which is suggested to function as an allosteric activator (Hermanowski-Vosatka *et al.*, 1992). Neutrophil activation is required for the localization of this factor, which appears to be a single, low molecular weight anionic species. Ligand specificity of the $\alpha_V\beta_3$ integrin is modulated by the lipid composition of the surrounding membrane (Conforti *et al.*, 1990). Isolated $\alpha_V\beta_3$ receptors incorporated into liposomes composed solely of phosphatidylcholine bound specifically to vitronectin, while those incorporated into a mixed vesicle of phosphatidylcholine and phosphatidylethanolamine also bound fibronectin and von Willebrand factor.

We show evidence in this paper that reconstitution of the laminin binding protein with cell detergent extracts restores the original avid laminin binding ability of the affinity isolated 67 kDa laminin binding protein. It is possible that the plasma membrane components in the extracts provide the proper hydrophobic environment for a conformation dependent binding. However,

detergent alone was not sufficient to restore the high affinity binding of the isolated protein, and it seems more likely that a second protein or lipid factor is required for proper conformation or orientation.

Expression of the 67 kDa laminin binding protein has been shown to correlate with expression of the $\alpha_6\beta_1$ integrin receptor on small cell lung cancer cell lines (Pellegrini et al., 1994). These authors speculated that the 67 kDa laminin binding protein did not bind laminin directly, but functioned as an accessory molecule for $\alpha_6\beta_1$. This integrin receptor is thought to bind to the E8 fragment of laminin-1, which is located in the distal region of the long arm (Sonnenberg et al., 1990). The 67 kDa laminin binding protein has been shown to interact with a five amino acid sequence, YIGSR, located in the proximal region of the short arm (Graf et al., 1987). This finding, however, was not supported by rotary shadowing experiments, which showed isolated 67 kDa laminin binding protein associated with the long arm of laminin-1 (Cioce et al., 1993). Coordinated binding by the two receptors, $\alpha_6\beta_1$, and the 67 kDa laminin binding protein, could reconcile these discrepant observations. Since the laminins are large, multi-domain proteins with multiple functions, it is also possible that two or more adhesive sites exist within the laminin molecule, whose activities are modulated by environmental or conformational factors. We suggest a working model where, in the presence of accessory factors, possibly molecules such as $\alpha_6\beta_1$, or other non-proteinaceous factors, a high affinity binding site is accessible and used. In the absence of such factors, perhaps the 67 kDa

laminin binding protein utilizes a lower affinity binding site. Additionally, the expression of both laminin binding proteins and laminin isoforms are known to be developmentally regulated (reviewed in Kleinman et al., 1993). It would be reasonable to expect that the determination of binding sites available for specific laminin binding proteins is also influenced by the developmental stage of the tissue. Since neoplasia is, by definition, an unregulated growth, one would anticipate a loss of coordination of cell surface receptor functions with their extracellular matrix ligands. The present work has implicated cofactors in the high affinity adhesion of the 67 kDa laminin binding protein with laminin. Further definition of the surface form and adhesive characteristics of laminin binding proteins should clarify our understanding of their mechanisms of action and so, facilitate the design of highly specific therapeutics for the treatment of metastatic disease.

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CHAPTER 3CONTROL PATHWAYS OF THE 67 kDa LAMININ BINDING PROTEIN:
SURFACE EXPRESSION AND ACTIVITY OF A NEW LIGAND BINDING
DOMAINIntroduction

The greatest challenge to clinicians in the treatment of neoplasia is the threat of distant metastases. Metastasis is described as a multi-step process which is modulated by both the tumor cells and the host tissues (reviewed in Liotta et al., 1991; Stetler-Stevenson et al., 1993). Since the extracellular basement membrane represents the most formidable barrier to metastatic dissemination, tumor cell interaction with basement membrane molecules is considered a critical determinant of the tumor's ability to invade adjacent tissues and spread to distant sites. A major class of basement membrane glycoproteins is the laminins. Large, heterotrimeric molecules with multiple structural domains, the laminins have been shown to contain multiple adhesive sites for other matrix molecules and cellular receptors (reviewed in Kleinman et al., 1993). Invasion of the extracellular basement membrane is mediated in part by the adhesion of tumor cells to laminins via specific cell surface receptors. Understanding the biology of these receptors promotes the ability of the clinician to predict the course of metastatic disease in the patient. Furthermore, definition of their ligand binding sites and associated signal transduction pathways should enable the design of highly effective anti-metastatic therapies.

One of the laminin binding proteins whose expression has been shown to

correlate with the metastatic potential of solid tumors is the 67 kDa LBP. This non-integrin protein was initially identified on the surface of highly metastatic breast carcinomas (Barsky et al., 1984). Using immunohistochemical and molecular techniques, the 67 kDa LBP has since been identified in many cancer tissues, including melanoma (Kleinman et al., 1989), lung carcinomas (Martignone et al., 1992), and colonic adenocarcinoma (Mafune et al., 1990). A strong positive correlation has been demonstrated, with expression of the 67 kDa LBP significantly increased in highly metastatic tumors as compared to non-malignant tissues and non-invasive tumors of similar histologic origins (Martignone et al., 1992; Mafune et al., 1990; Marques et al., 1994; D'Errico et al., 1991; Vacca et al., 1993). In the present study, we evaluate the metastatic phenotype of chinese hamster ovary (CHO) cell lines which overexpress the 67 kDa LBP.

Translational regulation of the expression of the 67 kDa laminin binding protein has been proposed by several groups (Rao et al., 1989; Makrides et al., 1988). These predictions were based on consensus sequences found in the 5' untranslated region of the cDNA of the mouse sequence. In this manuscript, we demonstrate that translational regulation is, indeed, a likely mechanism used by cells to control the surface expression of the 67 kDa laminin binding protein. Transfected cells which expressed high levels of the 67 kDa LBP mRNA did not always express correspondingly elevated levels of the 67 kDa protein on their surface.

We have also used the CHO overexpression system to investigate the laminin binding site of the 67 kDa LBP. A nine amino acid sequence located in the β chain of laminin-1 has been identified as a binding site for the 67 kDa LBP. This sequence, CDPGYIGSR, known as peptide 11, has been shown to block the adhesion of tumor cells to laminin *in vitro*, and to interfere with the metastatic cascade *in vivo*. The corresponding ligand binding domain of the 67 kDa laminin binding protein has been shown to reside in the carboxyl terminal half of the protein by blocking with an antibody raised to the truncated product (Wewer et al., 1986). Castronovo *et al.* have demonstrated that a synthetic peptide corresponding to residues 161-180 (Peptide G) of the 67 kDa LBP blocked the adhesion of tumor cells to cultured endothelium (Castronovo et al., 1991b). Adhesion via this domain of the 67 kDa LBP was later shown to be dependent on heparin, another basement membrane macromolecule which also binds laminin (Guo et al., 1992). In the present study, we demonstrate biological activity of a second synthetic peptide, corresponding to residues 205-229 of the LBP, in direct interaction with laminin, and in modulating tumor cell invasion of basement membrane matrix and tumor lung colonization. For this peptide, interaction with laminin could be inhibited with peptide 11.

Materials and Methods

Animals and tumor cell lines

The highly invasive and metastatic B16BL6 murine melanoma cell line

was isolated originally by Dr. I.R. Hart (Poste et al., 1980) and was obtained from the Mason Research Institute, Worcester, MA. B16BL6 cells were propagated in RPMI 1640 medium (Sigma) with 10% fetal bovine serum (Intergen), 5 $\mu\text{g}/\text{ml}$ insulin and penicillin/streptomycin (complete medium). The CHO mutant cell line (DG44) was kindly provided by Dr. Lawrence Chasin of Columbia University. These cells are double negative mutants for the dhfr gene, and rely on the presence of exogenous hypoxanthine and thymidine (HT) in the culture medium for proliferation (Urlaub and Chasin, 1980). Untransfected DG44CHOs (DG44wt) were maintained in αMEM (Sigma) with 10% dFBS, and supplemented with HT, 5 $\mu\text{g}/\text{ml}$ insulin, and cover antibiotics. Selection of dhfr and pcLR transfected DG44CHOs was carried out in αMEM containing 10% fetal bovine serum (Intergen) extensively dialyzed against CMF saline (dFBS) and 400 $\mu\text{g}/\text{ml}$ G418 (Gibco).

Female C57Bl/6J mice, 8 to 12 weeks of age, were used in these experiments. All mice were raised in the Montana State University Animal Resources Center from breeding stock obtained from the Jackson Laboratories, Bar Harbor, ME.

Expression of the laminin binding protein

A cDNA clone of the laminin binding protein (pcLR), isolated from a hamster expression library, was kindly provided by Dr. James Strauss, of the California Technical Institute, in the pcDNA1/neo vector (Invitrogen). This expression vector utilizes a CMV promoter and G418 selectable marker. A

dihydrofolate reductase (dhfr) expression vector, under the control of an SV40 promoter, was obtained from ATCC (Rockville, MD). DG44CHO cells were simultaneously transfected with both plasmids at ratios of 5:1, and 20:1 pcLR:dhfr by calcium phosphate precipitation (Chen and Okayama, 1988). Methotrexate (CalbioChem) was added to amplify the dhfr expression vector, and thus, co-amplify the pcLR vector. Methotrexate treatment was initiated at a concentration of 0.03 μM and increased by three-fold increments approximately every 5th passage, to a maximum concentration of 120 μM . Expression of the laminin binding protein was assessed by Northern and FACScan analysis using the anti-LBP peptide 205-229 polyclonal antiserum described below.

Laminin binding protein isolation

The laminin binding protein was extracted essentially by the method of Wewer *et. al.* (1986). DG44CHO cells, selected for expression of the pcLR and dhfr plasmids, were harvested by rinsing twice with Puck's calcium and magnesium-free Saline G solution, approximately 10 minutes each at 37°C, and a third time with the same solution containing 0.01 mM EGTA, until the cells detached. This harvest technique was used to avoid the use of trypsin and thus optimally preserve membrane proteins. Cells were then centrifuged for 10 minutes at 500 x g, the pellet resuspended in a 10x volume (v/v) of calcium and magnesium-free Dulbecco's PBS, and centrifuged again under the same conditions. Included in this and all subsequent buffers were the protease inhibitors PMSF at 50 $\mu\text{g}/\text{ml}$, N-ethylmaleamide at 1mM, and benzamidine at 5

mM. Cells were then suspended in 2 volumes of ice cold 25mM Tris/0.3 M sucrose, pH 7.4, and sonicated on ice with 4-5 x 5 second bursts using a Fisher Model 50 Dismembrator at 50% power. Nuclei, cytoskeletal proteins, and intact cells were pelleted by centrifugation at 500 x g for 10 minutes at 4°C, the supernatant collected, and the pellet resuspended in 5 volumes of Tris/sucrose buffer. Sonication was repeated, and the supernatant again collected and pooled with the first supernatant. This was repeated three times, or until the majority of the cells appeared to be lysed on microscopic examination. The membrane fraction was then collected on a 0.3 M sucrose cushion by centrifugation at 143,000 x g for 90 minutes at 4°C, and adjusted to a protein concentration of approximately 1 mg/ml with 25 mM Tris/150 mM NaCl/1 mM CaCl₂/3 mM MgCl₂, pH 7.4 buffer. To this was added an equal volume of the same buffer containing 1% NP-40 detergent, and the solution was rotated end over end for 12-16 hours at 4°C. The insoluble material was pelleted by centrifugation at 200,000 x g for 60 minutes, and the supernatant combined with a laminin-sepharose column which had previously been pre-equilibrated with 10 volumes 25 mM Tris/150 mM NaCl/1 mM CaCl₂/3 mM MgCl₂ + 0.05% NP-40 followed by 1 volume of the same buffer containing 400 mM NaCl. The solution was then rotated for 8-12 hours at 4°C. For isolation of high affinity laminin binding proteins, the column was first washed with 50 mM Tris/0.1 M NaCl, pH 7.4 and UV absorbance monitored until it returned to baseline. Approximately one column volume of 50 mM Tris/1.0 M NaCl, pH 7.4, was added and allowed

to stand 15-20 minutes at room temperature prior to elution of high affinity binding proteins. Peptide 11 elution of laminin binding proteins was carried out in the same manner, except that a 1 mg/ml solution of peptide in 50 mM Tris/0.1 M NaCl, pH 7.4, was added and allowed to stand 15-20 minutes before elution of proteins. This treatment was followed by 50 mM Tris/1.0 M NaCl, pH 7.4 to elute any remaining high affinity laminin binding proteins.

Structure prediction and homology studies

Using the predicted amino acid sequence for the 67 kDa LBP published by Yow *et al.*, secondary structure predictions, as well as sequence and functional homologies were determined. Structure prediction programs utilized were a) the program developed by Finer-Moore and Stroud (1984) with default parameters suggested by the authors for a membrane bound protein; and b) the Michigan State University program MSEQ. Structural homology comparisons were done using the ALIMAT program developed by Argos (1987). This program combines two distinct scoring processes in comparing protein sequences including (1) the Dayhoff relatedness odds matrix for amino acid exchange, and (2) a selection of five physical characteristics for amino acid residues: hydrophobicity, turn preference, refractivity index, residue bulk, and anti-parallel strand preference. The sequence was also searched for known functional motifs using the MOTIFS program (Devereux *et al.*, 1984) to examine the ProSite database release 7.0 (Bairoch, 1991), running on the Pittsburgh Supercomputing Center (PSC) Vax system. Protein sequences for comparison

were obtained from Genbank/EMBL and NBRF/PIR databases at PSC.

Peptide synthesis and competitive binding studies

All peptides were synthesized on a Milligen 9050 automated peptide synthesizer employing standard Fmoc chemistry on polystyrene (PAL) resin (Milligen-Biosearch). Crude peptides were purified by preparative HPLC, and purity determined to be >90% by analytical reverse-phase HPLC and FAB and electrospray mass spectrometry.

A highly charged region, predicted by the Finer-Moore-Stroud and MSEQ protein structure prediction programs to form a helical structure (residues 205-229), exists within the carboxyl-terminal half of the cDNA derived amino acid sequence for the 67 kDa laminin binding protein. Since the laminin binding domain of the 67 kDa laminin binding protein has been localized to the carboxyl-terminal half of the protein (Wewer et al., 1986), a peptide including this sequence was synthesized (LBP 205-229; RDPEEIEKEEQAAAEKAVTKEEFQG) and examined for its ability to bind to whole laminin in a plate assay. For this assay, 1 mg laminin was tritiated by reaction with 500 μ Ci [3 H]-acetic anhydride (Amersham) in a buffer consisting of 30mM Tris-HCl/0.2M NaCl/5mM CaCl₂, pH 7.4, for 2 hours at room temperature. Unbound radioactivity was removed by extensive dialysis against the same buffer. The laminin binding protein-derived synthetic peptide was dissolved at 1 mg/ml in neutral buffer, 0.5 ml of the peptide solution was applied to each well of positively charged 24-well Primaria plates (Falcon), and the plates dried under vacuum for 48 hours at room

temperature. Just before use in the binding assay, the peptide-coated plates were washed twice with Tris buffered saline (20 mM Tris/500 mM NaCl, pH 7.5) to remove dried buffer residue, and treated with 10% non-fat milk dried blocking solution for 30 minutes. The binding assay was performed in the same buffer used for laminin binding protein isolation on the laminin affinity column, with the addition of 0.05% Tween-20 to favor only highly specific binding. [³H]-laminin (25 μg laminin, 5000 cpm per well) was added, with or without 500 μg peptide 11, in 250 μl binding buffer to each peptide coated well and to control wells which had not been treated with synthetic peptide. The plates were incubated for 2 hours at 37°C with intermittent shaking. Unbound laminin was then removed with five washes of binding buffer. The material remaining on the plate was harvested using an incubation with 0.2ml 2%SDS for 2 hours at 37°C, and the SDS solution was then measured by scintillation counting in Aquassure (Amersham) to quantitate the amount of bound laminin.

The association of peptide 11 with LBP peptide 205-229 was also monitored by following the transit time of 2 mg of [³H]-gly-peptide 11 through an affinity column composed of sepharose-coupled synthetic LBP peptide. 20 mg of the LBP 205-229 synthetic peptide was coupled to 10 ml CnBr-activated Sepharose using the methods described by the manufacturer (Pharmacia). A radioactive sample of peptide 11, CDPGYIGSR, was synthesized using [³H]-glycine (New England Nuclear) to label the peptide. The radioactive glycine was mixed with cold glycine and derivatized with the Fmoc group using standard

protocols. The [^3H]-glycine preparation was used in both glycine cycles during peptide synthesis, resulting in 11,000 cpm/mg specific activity of the peptide 11. Transit time for the [^3H]-gly- peptide 11 was assessed with and without competition from 2 mg cold peptide 11. The matrix was washed with phosphate buffered saline and collected in 2 ml fractions until radioactivity could no longer be detected.

The ability of the LBP 205-229 derived synthetic peptide to bind [^3H]-laminin was also assayed by this protocol. In this case, 2 mg of [^3H]-laminin was applied to the LBP peptide column, and unlabelled peptide 11 added as competitor. A non-related, highly charged peptide derived from the sequence of cytochrome c was used as a negative control.

Tumor cell invasion of basement membrane matrix

For experiments using B16BL6 cells, an 8 micron pore size polycarbonate separation filter between upper and lower chambers in a 6.5 mm Transwell (Costar) was impregnated with a 1:20 dilution of Matrigel in serum free medium as described by Repesh (1989). Matrigel was prepared in our lab from freshly excised EHS tumor tissue by the method of Kleinman *et al.* (1986). Preliminary experiments with DG44CHO cells indicated that these cells invaded the matrix so rapidly that it was necessary to add additional type IV collagen (Sigma) to the Matrigel matrix in order to slow down invasion and see differential effects. The minimum supplemental concentration of type IV collagen required to sufficiently retard invasion by the DG44CHO cells was determined to be 2.5 mg/ml, and this

concentration was added to the Matrigel for both DG44CHOwt cells, and DG44CHO pcLR/dhfr cells. 5×10^4 monodispersed tumor cells were added to the upper chamber (Transwell insert) in 0.2 ml complete medium. 0.8 ml complete medium was added to the base well. Both chambers contained 100 $\mu\text{g/ml}$ of the specific peptide, and were fed daily with fresh peptide-containing medium. No chemoattractant was used in the lower well. At the end of one week, the loosely adherent cells were washed from the bottom of the inserts into the lower wells, the inserts removed, and colonies allowed to develop. Because of superior performance of Nunc plastic when growing B16BL6 cells from very low numbers, Costar transwells were used in Nunc 24 well plates. Colonies which developed in the lower well were washed, fixed in methanol and stained with hematoxylin for counting. Control experiments indicated that, for cell lines, such as B16BL6, which have very high cloning efficiencies, colony counting gave identical results to those obtained using radiolabelled cells (data not shown). DG44CHO pcLR/dhfr cells appear to have a much longer doubling time than untransfected DG44CHO wt cells. Therefore, for the CHO cell lines, individual cells invading through the Matrigel barrier to the lower chamber were counted on a daily basis to reduce experimental variation resulting from differences in proliferation rates. The day 4 results are shown in figure 3.6. Control experiments indicated that none of the peptides used in these experiments grossly affected cell viability or proliferation rates. Methotrexate was not included in any of the media for invasion assays.

Northern analysis

Total RNA was extracted from pcLR/dhfr transfected cells and untransfected control cells by the guanidine-isothiocyanate method of Chomczynski *et al.* (Chomczynski and Sacchi, 1987), separated in a 1.2% agarose gel containing 29% formaldehyde, blotted to nitrocellulose (Schleicher and Schuell), and probed with the EcoR1 laminin binding protein insert previously labelled with ^{32}P by nick translation. Blots were prehybridized for 2-4 hours at 42°C in 20xSSC, 50% Denhardt solution, 1 M NaH_2PO_4 , 1 mg/ml yeast tRNA, 10% dextran sulfate, and hybridized under the same conditions with 2×10^6 cpm/ml probe, which had been previously denatured by boiling for 15 minutes and cooled on ice (Sambrook *et al.*, 1989). Visualization and quantitation of the blots was performed with a Molecular Dynamics PhosphorimagerTM using ImageQuantTM software. Sample loading was assessed by stripping the blots with boiling water and probing under the same conditions with a human cardiac β -actin probe which had been similarly labelled.

Genomic DNA extraction and Southern blot analysis

Genomic DNA was extracted from untransfected DG44CHO cells, and transfected DG44CHO pcLR/dhfr cells using SDS and proteinase K to liberate the DNA, followed by purification by phenol-chloroform extraction. The DNA was digested to completion with EcoRI and SmaI, and electrophoretically resolved in a 0.8% agarose gel. Following denaturation for 45 minutes in 0.5 N NaOH, and depurination in 0.2 N HCl, DNAs were neutralized for 30 minutes in 1 M Tris/1.5

M NaCl, pH 7.4. DNA was subsequently transferred to a nitrocellulose filter (Schleicher & Schull) by capillary transfer in 20x SSC for 16 hours (Sambrook et al., 1989). The DNA was fixed to the filter by UV crosslinking. Probing and visualization of the blot were done as for the Northern analysis.

Antibody production and Western blotting

The LBP peptide 205-229 (described above) was conjugated to KLH (Sigma) by crosslinking for 2 hours at room temperature in 0.2% glutaraldehyde, and the solution then dialyzed against Dulbecco's PBS for 48 hours with 4 changes of dialysate to remove glutaraldehyde and free peptide prior to immunization. New Zealand white rabbits were immunized with 1 mg of peptide-KLH and Freund's complete adjuvant at multiple subcutaneous sites, and boosted every two weeks with peptide-KLH and incomplete Freund's adjuvant. Thirty days later, a test bleed was obtained and the anti-peptide activity titered by ELISA with KLH, peptide-KLH, and peptide conjugated to ovalbumin used as target antigens. The polyclonal antiserum was purified using a protein A-column (Pierce), and the majority of the anti-KLH activity removed with a KLH-Sepharose affinity column.

Laminin-affinity isolated 67 kDA LBP was separated on a 10% SDS-PAGE gel, and transferred to PVDF membrane (Bio-Rad) using a semi-dry blotter (Ellard Instrumentation). We found that the protein did not adhere well to PVDF membrane. Therefore, it was necessary to fix the membrane in 0.02% glutaraldehyde for 2 hours at room temperature prior to blocking with 5% nonfat

dry milk in DPBS. To ensure that the glutaraldehyde was not introducing false positive results, fixation in 10% acetic acid/25% methanol was also used, and shown to give identical results. Antibody detection was performed using alkaline phosphatase conjugated goat anti-rabbit antibody (Bio-Rad) and BCIP/NBT chromogenic substrate (Kirkegaard Perry Inc.).

FACScan analysis

Cells were harvested for FACScan analysis with three washes of Tyrode's CMF saline solution, followed by very brief trypsin exposure, and rapid trypsin inactivation in serum containing medium. Following recovery in 50:50 Tyrode's CMF:complete medium, the cells were washed with 10 volumes of 50:50 Tyrode's CMF:serum free medium. Unpermeabilized cells were incubated with the primary antibody or PBS in the presence of NaN_3 for 60 minutes on ice. The primary antibody used was Protein A purified rabbit anti-LBP 205-229 peptide immunoglobulin fraction. After pelleting the cells and resuspending them in Dulbecco's PBS, the secondary antibody, FITC labelled goat anti-rabbit Ig (Sigma Immunochemical), was added and allowed to react for 60 minutes on ice in the presence of azide. The cells were then washed by centrifugation, were resuspended in phosphate buffered saline, and filtered through nylon mesh immediately prior to analysis. FACScan analysis was performed on a Becton Dickinson FACScan. In order to evaluate the effects of subculturing the CHO cells on LBP surface expression, transfected cells were harvested by incubating for 10 minutes at 37°C in Tyrode's CMF saline, followed by a brief exposure to

Tyrode's CMF containing 0.05% trypsin, and plated in selection medium. Surface LBP expression was measured by FACScan analysis at 3, 6, 14, and 24 hours post plating. The effects of cell density on LBP expression were measured using cells from subcultures made at various split ratios. All cells were assayed at 16 hours post plating, and split ratios of 1:4, 1:8 and 1:16 were used.

Quantitative lung colony assay

Tumor cells were harvested from subconfluent (60%-80% confluency) cultures using minimal trypsin exposure as described above for FACScan analysis. The cells were harvested in complete medium to allow for serum inactivation of trypsin, then washed three times and resuspended in serum-free medium. Despite the lack of reports in the literature, we have found that mice injected intravenously with excesses of some matrix derived peptides can show evidence of intravascular embolization. Therefore, where possible, we use a modified protocol to avoid injecting excess free peptide. Tumor cell suspensions, harvested as described above, were first incubated with 2 mg/ml peptide for 20 minutes at 37°C. The cells were then pelleted, the supernatant containing excess peptide discarded, the cells washed in buffer consisting of 50% Tyrode's CMF saline: 50% complete RPMI medium, then counted and diluted in the same buffer. After removal from peptide-containing solutions, the cells were held on ice. Prior to injection, the animals were warmed at 37°C for 30 minutes. Tumor cells, 5×10^4 , in 0.2 ml were injected per mouse via the

lateral tail vein. Where the experiment required co-injection of tumor cells with peptide, cells were prepared as indicated above without the preincubation step. 1 mg peptide dissolved in the injection buffer was mixed with the aliquot of tumor cells immediately prior to injection to give a total volume of 0.2 ml. Three weeks after injection, the animals were sacrificed and autopsied. All tissues with suspect tumor colonies were rinsed in a balanced saline solution and fixed for 3 days in Bouin's fixative for gross and histological examination. The number of superficial nodules in the Bouin's-fixed tissues was determined using a dissecting microscope.

Results

Messenger RNA levels of the 67 kDa LBP do not directly reflect the surface expression of the protein

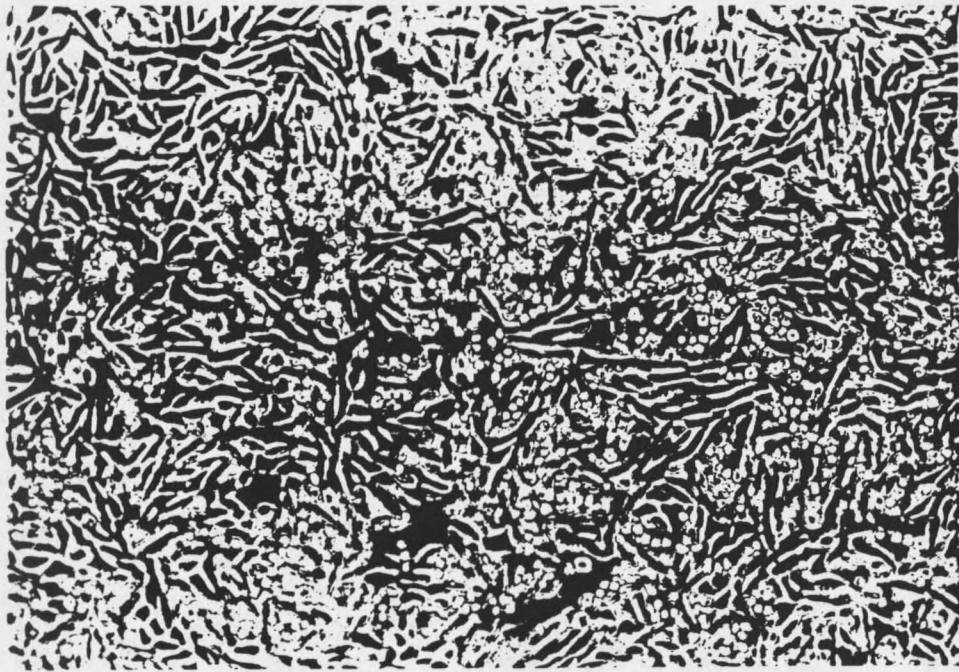
Since Chinese Hamster Ovary (CHO) cells have been shown to express the 67 kDa LBP on their surface (Graf et al., 1987a; Wang et al., 1992), we elected to utilize a homotypic expression system to assess the phenotypic effects of overexpression of this protein. The cell line DG44CHO, which is deficient in dihydrofolate reductase (dhfr), was selected as a methotrexate amplifiable expression system. DG44CHO cells were transfected with the hamster 67 kDa LBP expression vector pcLR, and co-transfected with a dhfr expression plasmid. Although the mechanism is not well understood, it has been shown that methotrexate treatment of such dhfr transfected cell lines results in reduplication of the dhfr plasmid, and concurrent amplification of cotransfected

plasmids.

Two populations of cells were selected from the transfection and propagated for further study. The first population, designated "flats", were transfected at a pcLR:dhfr ratio of 20:1, and were initially selected in HT deficient medium containing G418, but without methotrexate treatment to induce amplification. These cells are characterized by a flattened morphology, form close cell:cell associations, and a majority of the cells display a cobblestone like appearance (Fig. 3.1). The DG44CHO pcLR/dhfr "flats" appeared to have longer doubling times than the parental cell line or the other transfected cell lines. FACScan analysis of the "flats" using the sequence specific anti-LBP peptide 205-229 antibody, revealed the presence of a subpopulation of the cells which showed a substantial surface expression of the 67 kDa LBP protein, which was further increased following later treatment with methotrexate (Fig. 3.2a-c). Northern analysis with a cDNA probe identified two species of 67 kDa LBP mRNA in the "flat" cells, one endogenous product of approximately 1.0 kb, and one slightly larger transcript consistent with the expected product from the pcLR plasmid (Fig. 3.3).

The second population of cells, designated "5:1" were transfected at a pcLR:dhfr ratio of 5:1, and were morphologically similar to the DG44CHOwt parental cell line, which contains many poorly adherent, rounded cells, as well as a population of "fibroblastic" cells. Methotrexate treatment of the transfected cells was initiated at 0.03 μ M methotrexate, and increased in three-fold

a.



b.

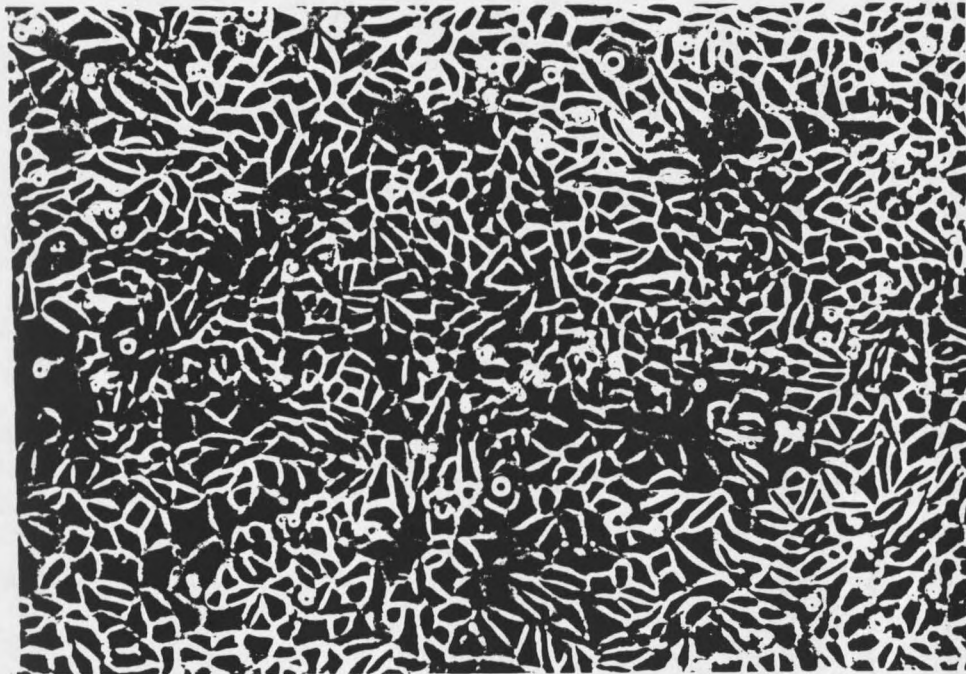


Figure 3.1. Phase contrast photomicrographs of a) DG44CHOwt cells, and b) DG44CHO cells transfected with pcLR and dhfr at a ratio of 20:1. These cells were designated "Flats" due to their spread, cobblestone-like appearance. Magnification is 150x.

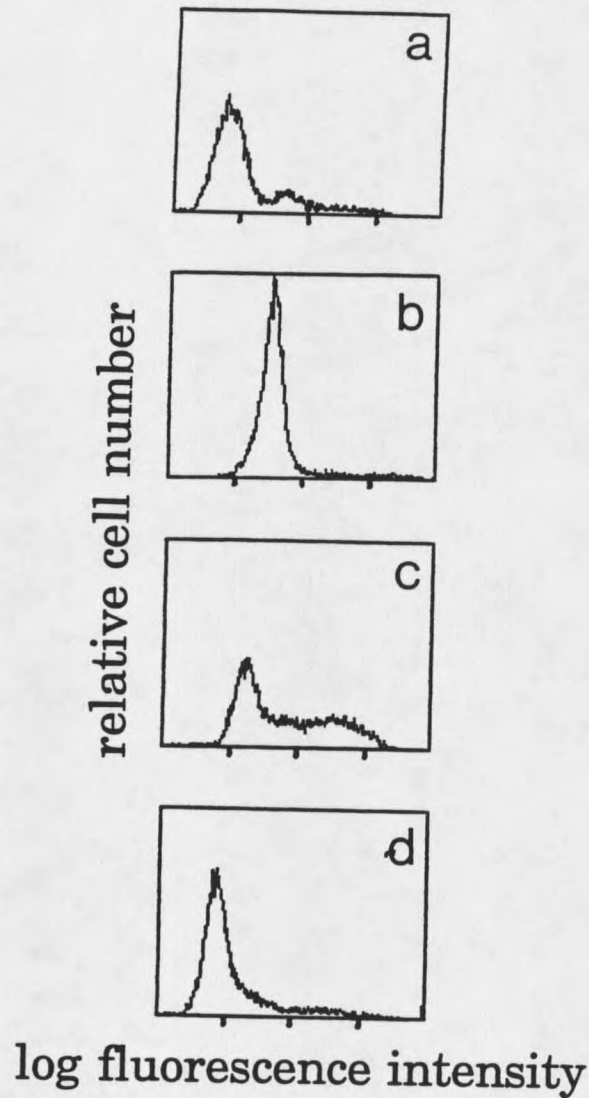


Figure 3.2. FACS analysis of the surface expression of the 67 kDa LBP. Cells were treated with anti-LBP peptide 205-229 antiserum, and stained with FITC conjugated goat anti-rabbit secondary antibody. a) endogenous expression of the 67 kDa LBP in untransfected cells; b) DG44CHO cells transfected with the pcLR and the dhfr plasmid; c) DG44CHO cells transfected with pcLR and the dhfr plasmid, and treated with 1 μ M Methotrexate; d) DG44CHO cells transfected with pcLR and the dhfr plasmid showing adaptation to methotrexate. A constant vertical scale is used to show the relative size of different peaks.

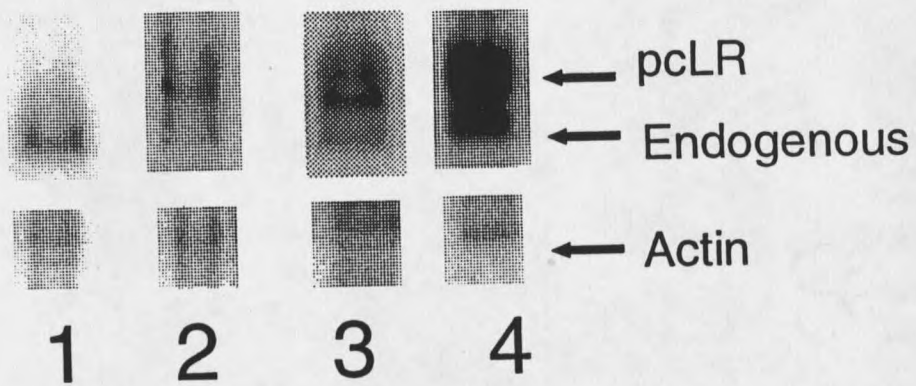


Figure 3.3 Northern analysis of DG44CHO mRNA probed with the coding region of the 67 kDa LBP cDNA. Arrows indicate the transcript of the endogenous gene product, and the transfected plasmid product. a) untransfected DG44CHO cells; b) pcLR/dhfr transfected DG44CHO cells; c) pcLR/dhfr transfected DG44CHO cells treated with 1 μ M Methotrexate; d) pcLR/dhfr transfected DG44CHO cells treated with 12 μ M Methotrexate.

increments approximately every 5th passage. Southern analysis of 5:1 cells which were at a methotrexate treatment level of $0.3 \mu\text{M}$ demonstrated an increased copy number of the pcLR plasmid, as expected (Fig. 3.4).

Treatment of the 5:1 transfected cells with increasing levels of methotrexate resulted in an amplification of the plasmid derived mRNA, while levels of the endogenous transcript remained constant (Fig. 3.3). FACScan analysis of the unpermeabilized 5:1 cell line using the anti-LBP peptide antibody showed that surface expression of the 67 kDa laminin binding protein was initially increased after growth in methotrexate. However, after the cells apparently adapted to higher levels of methotrexate, surface expression of the protein decreased and again approached the levels seen in untransfected cells (Fig. 3.2d). Incremental increases of the level of methotrexate resulted in the 5:1 cells transiently displaying the flattened phenotype, however, following several additional passages in the higher level of methotrexate, the cells again regained the characteristic morphology of the parental cells, and the 67 kDa LBP surface expression was concomitantly decreased. Such cells were designated "methotrexate adapted". mRNA levels remained increased in a methotrexate dose dependent manner even in "methotrexate adapted" cells whose surface expression of the 67 kDa LBP had returned to basal levels, as shown by FACScan analysis (Figs. 3.2 and 3.3). Both late passage transfected CHO cells and methotrexate "adapted" CHO cells expressed surface levels of LBP comparable to untransfected cells. However, when the same cultures were

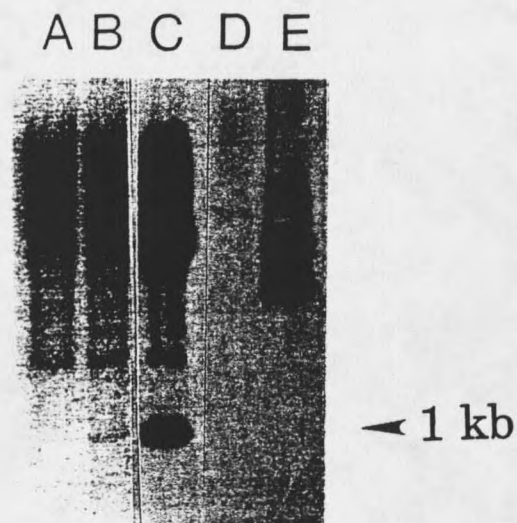


Figure 3.4 Southern analysis of DG44CHO transfected cell lines. Genomic DNA was digested with EcoR1 (lanes A-C) or Sma1 (lanes D and E) and probed with the coding region of the 67 kDa LBP cDNA. Lane A, untransfected DG44CHO cells; Lanes B and D, DG44CHO cells transfected with pCLR and dhfr at a ratio of 5:1; Lanes C and E, DG44CHO cells transfected with pCLR and dhfr at a ratio of 5:1, and treated with Methotrexate to 0.3 μ M.

sparsely plated and less than 24 hours old, a substantial proportion of the population expressed high levels of the 67 kDa LBP. The largest proportion of cells expressing high levels of the LBP was observed at 14 hours after subculture (Fig. 3.5, Panel A). Cell density was also found to effect the proportion of high LBP expressing cells. Only 5% of the cells subcultured at a 1:4 split ratio were high expressors, whereas 40% of the cells subcultured using a 1:8 split ratio, and 60% of the cells subcultured using a 1:16 split ratio were high expressors (Fig. 3.5, Panel B).

Cells exposed to methotrexate for extended periods of time express a 37 kDa laminin binding protein

The 67 kDa laminin binding protein, isolated from membrane extracts of "5:1" DG44CHO pcLR:dhfr cells, was eluted from a laminin-sepharose column with high ionic strength salt buffers and shown to be a single band at 67 kDa by silver stained SDS-PAGE. This product was specifically recognized by the anti-LBP peptide antibody in a Western blot. Furthermore, antibody binding in the Western blot was completely inhibited by excess LBP peptide (data not shown). However, extraction and affinity purification of laminin binding protein under identical conditions from "methotrexate adapted" 5:1 pcLR/dhfr cells resulted in the isolation of a laminin binding protein with apparent molecular weight of 37 kDa on reduced SDS-PAGE (data not shown). This protein was isolated in significantly lesser quantities than the 67 kDa product, and was never found in the same preparation along with the 67 kDa protein. However, laminin binding affinity appeared to be similar to that of the 67 kDa protein, as evidenced by

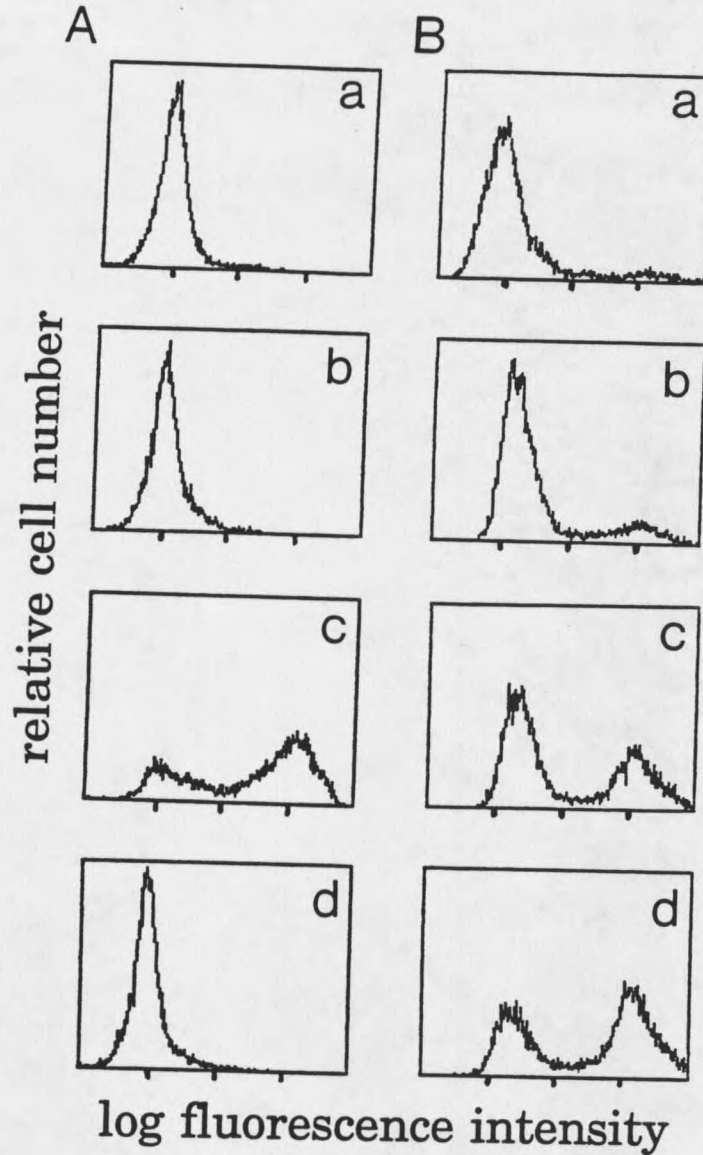


Figure 3.5. FACS analysis of 67 kDa LBP expression at various times and densities post plating. DG44CHO "flats" cells were harvested with Tyrode's CMF and Tyrode's CMF with 0.05% trypsin, and plated in selective medium. The effects of time and density were assayed after staining with anti-LBP peptide 205-229 antiserum, and FITC conjugated goat anti-rabbit secondary antibody. A constant vertical scale is used to show the relative size of the peaks.

Panel A: a) 3 hours post plating; b) 6 hours post plating; c) 14 hours post plating; d) 24 hours post plating.

Panel B: a) high cell density; b) low cell density after subculturing at a split ratio of 1:4; c) split ratio of 1:8; and d) split ratio of 1:16.

elution with high ionic strength buffers. The 37 kDa species was also shown to be specifically eluted from the laminin-sepharose affinity column by the laminin derived peptide 11.

A new ligand binding domain in the 67 kDa LBP

Peptide 11 has been previously shown to block the adhesion of tumor cells to laminin-1, presumably, by interfering with the interaction of the 67 kDa laminin binding protein with laminin (Graf et al., 1987a; Graf et al., 1987b). Computer analysis of the predicted amino acid sequence of the 67 kDa LBP using the Stroud and MSEQ structural prediction programs (Finer-Moore and Stroud, 1984), allowed us to identify a region of highly charged amino acids in the carboxyl terminal half of the molecule (residues 205-229) which are predicted to form an alpha helical structure. We hypothesized that the highly charged nature of this domain could contribute to a direct interaction with laminin. A synthetic peptide with a sequence corresponding to this region did show laminin binding activity in a plate assay. This activity was directly competed by peptide 11 (Table 3.1), however, as expected for peptide:peptide interactions, direct interaction between the LBP peptide 205-229 and the laminin derived peptide 11 proved to be very difficult to demonstrate. When radiolabelled peptide 11 was added to a LBP peptide-Sepharose column, the transit time for radiolabelled peptide was only marginally affected by the presence of unlabelled peptide 11. Slightly over 10% of the peptide 11 counts appeared in earlier fractions of the column eluate when cold additional Peptide 11 was present (data not shown).

Table 3.1.

Laminin bound per well per mg synthetic peptide²

Laminin bound in the absence of peptide 11	13.9 ± 1.07 μ g
Laminin bound in the presence of peptide 11	0.97 ± 0.5 μ g

¹Wells were coated with peptide 205-229 and evaluated for their ability to bind tritiated laminin. 500 μ g peptide 11 and 25 μ g laminin were used per well, and the data shown are averaged from 4 replicate wells. The experiment was run in duplicate.

²Residues 205-229 from the laminin receptor predicted sequence.

However, the binding of whole laminin to the LB peptide-Sepharose column was clearly decreased by the presence of peptide 11, (Table 3.2) indicating there is, at least, a destabilizing interaction between these two molecular domains. No significant effect was seen with an unrelated, highly charged peptide. These data were highly reproducible, and indicate that the highly charged LBP domain (residues 205-229) is involved in the association of the 67 kDa LBP with laminin.

Table 3.2. Binding of laminin to 67 kDa LBP peptide 205-229

Peptide added	³ H-laminin bound by 2 mg peptide 205-229 coupled to a sepharose matrix	
	<u>% of control</u>	<u>c.p.m. bound</u>
None	100.0	25,182
Peptide 11	50.5	12,723
Control peptide	84.9	21,400

¹140 μ g tritiated laminin were incubated with 2 mg peptide 205-229 in the presence and absence of 2 mg peptide 11 or an unrelated peptide, KISSWGKIKEC derived from the sequence of cytochrome c. The sepharose was washed until no unbound radioactivity was apparent, then the matrix bound counts were quantitated using scintillation counting in 20 ml Aquassure.

Extended exposure of B16BL6 melanoma cells to LBP 205-229 enhances their metastatic capability

The LBP peptide showed biological activity both *in vivo* and *in vitro*. In a tumor lung colony assay, injection of the peptide along with the mouse melanoma B16BL6 tumor cells resulted in a reduction of experimental metastasis to 66% of the control value (Table 3.3). However, when the peptide was preincubated with the tumor cells and excess peptide removed prior to injection, lung colonization was enhanced to 156% of the control. This is in contrast to the inhibitory effects of the laminin derived peptide 11, which were seen whether the tumor cells were pretreated with the peptide, or co-injected.

Table 3.3.

Effect of synthetic peptides on tumor lung colonization by B16BL6 melanoma cells

Peptide used:	Preincubated with tumor cells	Injected with tumor cells	Average number of tumor lung colonies per mouse (% of control)
None	--	--	100
LBP peptide 205-229	yes	--	156 (p=0.08) ²
LBP peptide 205-229	--	yes	66 (p=0.06)
Laminin peptide 11	yes	--	63 (p=0.04)
Laminin peptide 11	--	yes	60 (p=0.007)

¹ Mice were injected intravenously with tumor cells as described in "Methods". After 3 weeks, the animals were killed and tumor lung colonies enumerated. Data shown are from groups of mice consisting of 7-13 individuals per data point, and represent the results of 6 separate experiments.

² Statistical differences between the experimental and control groups were evaluated using the Mann Whitney 2-tailed test.

In vitro, the LBP peptide enhanced the invasion of the B16BL6 mouse melanoma tumor cells through a basement membrane matrix (Fig. 3.6). The invasion of the B16BL6 cells was increased by four fold over control values. In contrast, the invasion of DG44CHO and DG44CHO pcLR/dhfr "flats" cells was not enhanced by the LB peptide, but rather, both cell lines were inhibited. Peptide 11 inhibited the invasion of Matrigel by B16BL6 melanoma and both DG44CHO cell lines.

