THE ROLE OF DOMAIN I IN LAMININ CHAIN ASSEMBLY

DISSERTATION

Submitted to the Graduate College
of
Marshall University
In Partial Fulfillment of the Requirements for
The Degree of Doctor of Philosophy
by

George D. Kamphaus

Huntington
West Virginia
1998

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Introduction

Basement Membrane

Surrounding tissues such as muscle, nerve and adipose is a complex of proteins and carbohydrates which forms the extracellular matrix (ECM). The region of the ECM directly adjacent to a cell population is known as the basement membrane. This term arose from microscopic examination of the extracellular matrix and was originally used to designate a layer which was stained by the periodic acid-Schiff reaction. Electron microscopy revealed subdivisions of this layer, termed basal laminae, which contained an electron dense core (lamina densa) sandwiched by less dense layers (lamina lucida). The lamina lucida was determined to be in contact with the cellular component of the tissue (Junqueira 1989). Studies of the molecular composition of the basement membrane have revealed, among other glycoprotein components, a unique type of collagen (Type IV), heparan sulfate proteoglycans and the glycoprotein Laminin (Ln) (reviewed in Timpl 1989).

Characterization of the laminin molecule has shown it to be both abundant and ubiquitous. It is the second most plentiful glycoprotein of the extracellular matrix after collagen. Biochemical and ultrastructural studies of polymers of extracellular matrix components have shown that Ln is capable of binding with a number of components, making it a probable initiator for matrix formation (Yurchenco et al. 1992). Furthermore, a number of different Ln variants have been discovered and characterized in tissues

throughout the body. Seven mammalian isoforms, Ln-1 through Ln-7 (Burgeson et al. 1994), have been characterized as to their protein composition and tissue distribution. Four more laminins are newly described and named Ln-8 through Ln-11 (Miner et al. 1997). Laminin is now generally referred to as a "family" of glycoproteins (Tryggvason 1993).

Laminin Structure

The laminin glycoprotein is comprised of three chains: α, β, and γ (Figure 1). Electron microscopic images of the Ln molecule show a cruciform shape which is divided into a long arm and three short arms. The long arm has been measured at 77 nm and the short arms measure 36 ± 6 nm for the Ln-1 glycoprotein (Beck et al. 1990). The C-terminal regions of these three chains combine to form the rod-like long arm, which is stabilized at either end by disulfide bonds, and the N-terminal portion of the chains each form a short arm (Deutzmann et al. 1988; Beck et al. 1990; Antonsson et al. 1995). All Ln isoforms described to date contain the characteristic α, β, γ chain complement (Burgeson et al. 1994). To date five α chains, three β chains and two γ chains are cloned and the primary protein structure is known (reviewed in Burgeson et al. 1994; Miner et al. 1995; Iivanainen et al. 1997). These chains assemble into 11 Ln variants (Burgeson et al. 1994; Miner et al. 1997; and see Figure 2).

Figure 1.

Laminin-1 Trimeric Structure

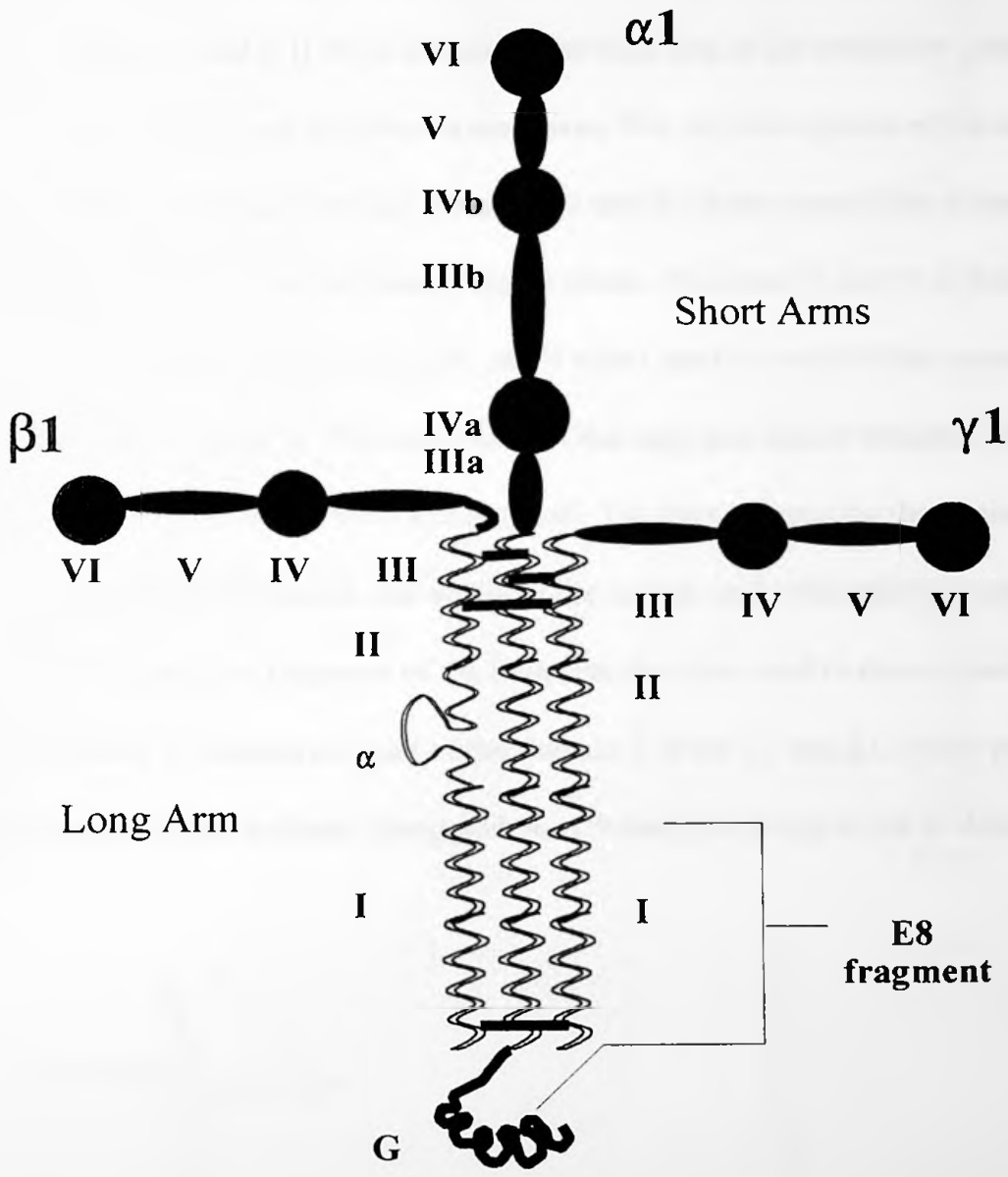


Figure 1. Ln-1 (formerly EHS [Engelbreth-Holm-Swarm tumor] laminin) is depicted as a representative example of the trimeric structure of the Ln glycoprotein. The structural domains are labeled for each chain. In order from the C-terminus they are: I-VI for γ1; I, α, and II-VI for β1; G, I/ II, IIIa, IVa, IIIb, IVb, V and VI for α1. Domains I and II of γ1 and β1 plus domains G and I/ II of α1 comprise the long arm of the cruciform protein. Domains III - VI of each chain comprise a short arm. The circular regions of the short arm depict globular domains; these are domains IV and VI in the case of the γ1 and β1 chains, and IVa, IVb and VI in the case of the α1 chain. Domains III and V of both the γ1 and β1 chains as well as domains IIIa, IIIb, and V of α1 are rich in EGF-like repeats (represented as oblong shapes). The wavy lines of the long arm depict domains with high α-helical content that interact to form a coiled-coil. The bars connecting the chains in the long arm represent disulfide bonds that stabilize the coiled- coil. Also shown is the region comprising the E8 protease fragment of the long arm that was used in the original studies of trimer formation. It consists of most of the domain I of the γ1 and β1 chains plus the homologous region of the α chain, along with 4 of 5 disulfide loops of the G domain .

Figure 2.
Laminin Variants

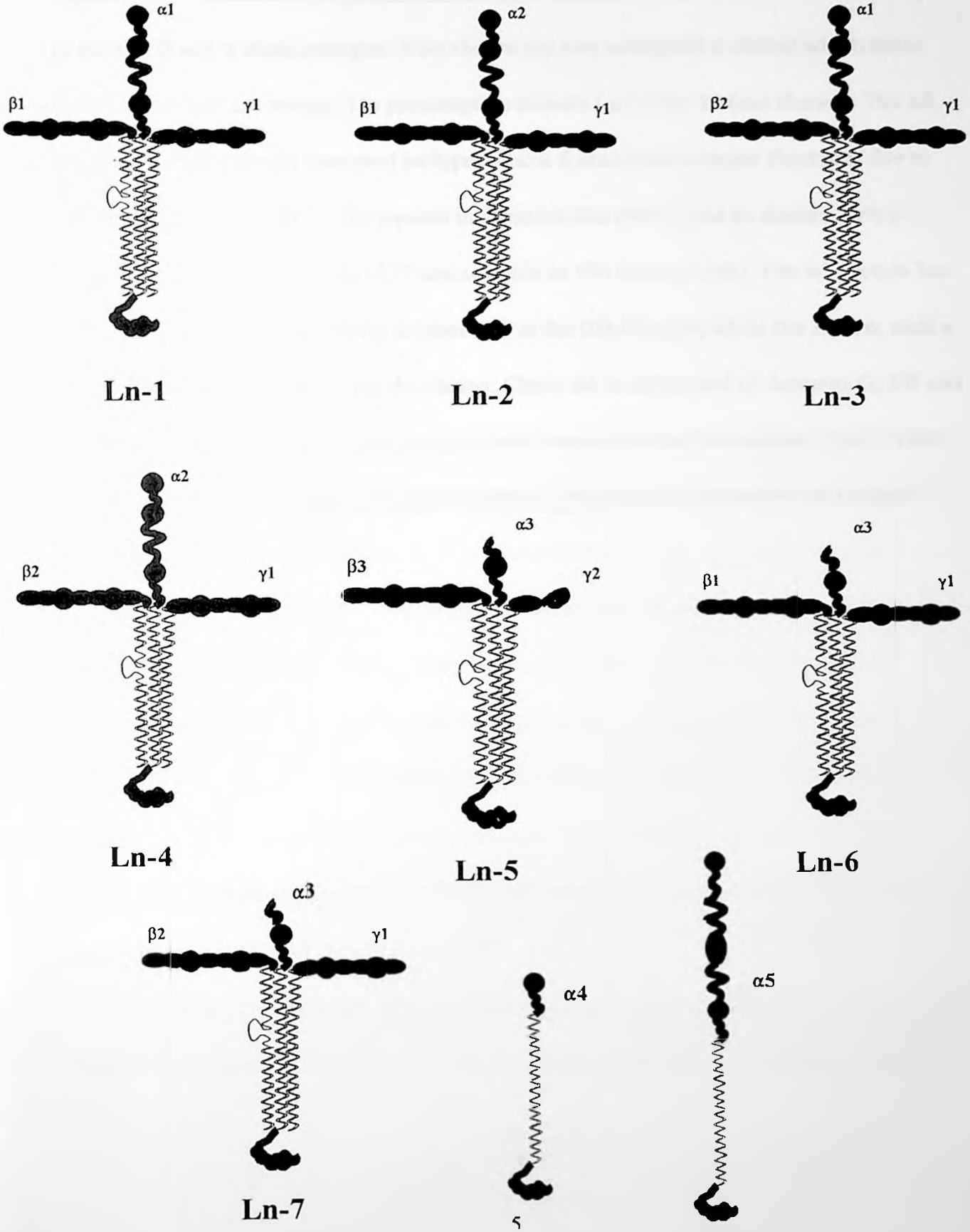


Figure 2. The seven recognized laminin variants (Ln-1 - Ln-7) are shown with respect to their α, β and γ chain isotypes. Also shown are two additional α chains which have been cloned, and are assigned to presumptive trimers Ln-8 - Ln 11 (not shown). The α3, α4 and γ2 chains are all truncated isotypes.The α 5 chain has a longer short arm due to increased numbers of EGF-like repeats in domains IIIa and V, and its domain IVb is larger than those of α1 and α2 (577 amino acids to 196 amino acids). The α3 protein has two forms, A and B. The A form is truncated at the IIIb domain, while the B form adds a small domain IV, unique among the chains. Chain α4 is comprised of domains G, I/II and III with 34 extra amino acids (not assigned to a domain) at the N-terminus. The γ2 chain is truncated within its domain IV. These trimeric glycoproteins comprise the Laminin "family".

Using computer-generated models of protein secondary structure, which are based on the known cDNA sequences of the individual chains, the β and γ chains have been divided into six domains, I - VI, which correspond to structural motifs or groups of structural elements (Sasaki et al. 1987a-b). In the case of the β chains, there is an additional domain, α, between domains I and II (Sasaki et al. 1987a). Similarly, the α chain is divided into domains I, II, IIIa, IVa, IIIb, IVb, Vand VI with a unique G (globular) domain at the C-terminus (reviewed in Beck et al. 1990).

The long arm of the laminin trimer is formed by interaction of domains I and II of the chains; these domains have a secondary protein structure which is primarily α-helical (Barlow et al. 1984). The short arms vary according to which chain isotypes comprise the trimer. For instance, the α3, α4, and γ2 chains are all truncated within their short arms at domain IIIa, IVa and III, respectively (reviewed in Miner et al. 1997). In the full length chain isotypes the short arms have globular domains (IV and VI) and a number of repeated EGF-like motifs which have since been found in other extracellular matrix proteins and are now classified as Ln- EGF-like domains. These repeats, named for their homology to epidermal growth factor, consist of 50 - 60 amino acid residues which are predicted to form four disulfide-bonded loops based on placement of cysteines and the known structure of EGF (Mayer et al. 1995).

The final characteristic structural motif of the laminin chains is the globular G-domain, found at the C-terminus of all known α chains, which contains five loops formed

by intramolecular disulfide bonds. This domain of the Ln protein has been extensively studied for its cellular adhesion properties. However, binding sites for other basement membrane glycoproteins as well as for cell surface receptors have been mapped to sites throughout the trimeric structure of Ln-1 (reviewed in Timpl 1989 and Beck et al. 1990). This suggests that Ln was formed by the incorporation of a number of functional motifs into a multifunctional glycoprotein. With the globular domains of the short arms and the G-domain of the α chain at the C-terminus of the long arm, the trimeric molecule looks like a cross with beads at its ends and along the crossbeam when visualized by rotary shadowing and electron microscopy (Beck et al. 1990). This molecular anatomy of three short arms and a rod-like long arm projecting out in three dimensions may allow laminin to form a complex independent network which acts as a foundation for basement membrane formation (Yurchenco et al. 1992).

Alpha-Helical Secondary Structure in Proteins

Perhaps the most well studied and recognizable secondary structure within proteins is the alpha-helix. In the past decade the number of known crystalline structures of proteins has become large enough such that a number of proteins containing α-helix secondary structure have been characterized and their dimensions measured. With the advent of techniques that allow cDNAs to be rapidly isolated and sequenced, the primary amino acid sequence of a protein can be deduced. By comparing crystallographic data of

a protein to its deduced amino acid sequence, protein chemists have created theoretical algorithms that can now predict secondary structure from primary amino acid sequence with fairly high accuracy (Lupas et al. 1991; Cohen and Parry 1994). Therefore, the secondary structure of a gene product can be determined before the purified protein is isolated. Once the protein is isolated and purified, it can be subjected to biophysical measurements that will in turn test the efficacy of the algorithms which predicted its secondary structure.

These algorithms have allowed researchers to determine the amino acid sequences that constitute an α-helix. Pauling first described the α-helix as a recognizable protein secondary structure, and it has since been determined that α-helical regions contain patterned repeats of seven amino acids which complete two turns of a helix (reviewed in Cohen and Parry 1990). This secondary structure is stabilized by the formation of hydrogen bonds between the C = O bond of the *n* amino acid and the amino (NH) group of the *n+4* amino acid (Branden and Tooze 1991).

The amino acids within the heptad repeat are designated by lower case letters, *a* - *g*, thus an α-helix can be written as $(abcdefg)_n$. Within this repeat, charged amino acids occur with high frequency at the "e" and "g" positions and apolar residues occur in the "a" and "d" sites (Cohen and Parry 1994; Monera et al. 1996). This arrangement of hydrophilic and hydrophobic amino acids creates "faces" down the sides of α-helices (Beck et al. 1993). Often proteins will be folded so that the hydrophobic face is tucked

into the interior of a protein, thus minimizing the interaction with the aqueous environment (reviewed in Branden and Tooze 1991). In the case of laminin chains, it is believed that the charged (hydrophilic) face plays an important role when α-helices interact during protein subunit assembly (Beck et al. 1993; Nomizu et al. 1996).

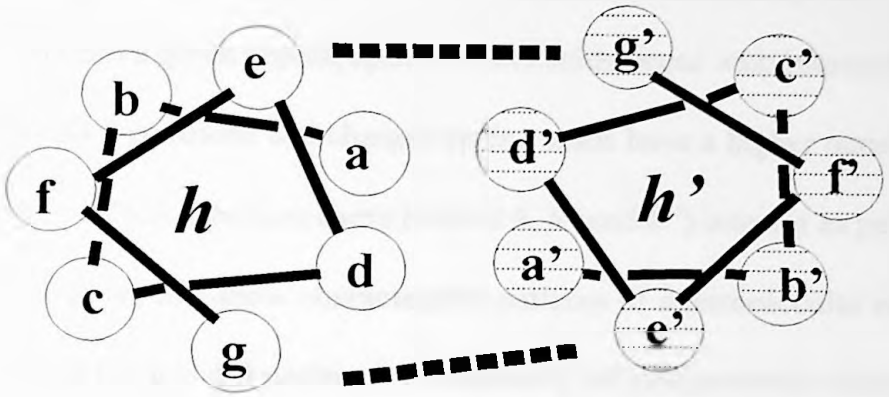
Cohen and Parry (1990) defined the interaction of two (or more) right-handed α-helices which wrap around each other to form an even more stable left-handed supercoil, as a coiled-coil. These coiled-coils are ubiquitous motifs for assembling proteins into tertiary and quaternary structures. Transcription factors, intermediate filaments and extracellular matrix proteins all have examples of subunits interacting via coiled-coils (Cohen and Parry 1990; Lumb and Kim 1995). Examination of a large number of GenBank sequences and comparison of coiled-coil proteins to those proteins having little or no α-helical structure allowed one group of researchers to compile a coiled-coil index. This gives a predictive score for the presence of a particular amino acid in each of the seven positions of a heptad repeat that interacts with another α-helix (Lupas et al. 1991). Some notable findings are: charged amino acids (Lys, Arg, Glu, Asp) have high relative occurrence at the "e" and "g" positions (as expected), leucine residues occur with high frequency at the "a" and "d" positions, and proline and tryptophan, which disrupt the α-helical structure, are almost completely absent. This study noted that the region from amino acid 1027 to 1559 of the Ln γ1 chain was clearly a coiled-coil, but did have regions of "low stability", i.e. it was not rigidly coiled-coil (Lupas et al. 1991). Using

computer algorithms and statistical studies of deduced amino acid sequences some differences in the amino acid composition have been found in α-helices which interact in pairs versus those which interact in triplets; particularly, charged amino acids and branched side chain amino acids have different distributions within the heptad repeat (Cohen and Parry 1994). Another feature of coiled-coil interactions, gleaned from studies of *de novo* synthesized peptides, was that placement of alanine amino acids in the heptad repeats was different in parallel versus anti-parallel coiled coils (Monera et al. 1996). (α-helices which come together at their N-terminal ends or C-terminal ends are parallel coiled coils; those with the N-terminal end of one α-helix interacting with the C-terminal end of the second α-helix are anti-parallel).

A number of biophysical studies of full-length trimeric Ln and chain fragments have confirmed that the Ln trimer long arm is a three chain, parallel coiled-coil (I. Hunter et al. 1990; Antonsson et al. 1995; Nomizu et al. 1996). Interactions of α-helical units which produce higher ordered structures have led to predicted schemes for amino acid alignment which produce two- and three-stranded coiled-coils (McLachlan and Stewart 1995). Two α-helices *h* and *h'*, with heptad repeats *a-g* and *a'-g'*, respectively, are predicted to align such that the *e* amino acids interact with the *g'* amino acids and the *g* amino acids interact with the *e'* amino acids (see Figure 3). The addition of a third helix *h"* (heptads *a"-g"*) forms a three-stranded coiled-coil where the pairs *e"-g'*, *e'-g*, and *e-g"* interact to align the chains (Cohen and Parry 1990; Beck et al. 1993).

Figure 3. Heptad Repeat Interactions in Two and Three-Stranded Coiled-Coils

A



B

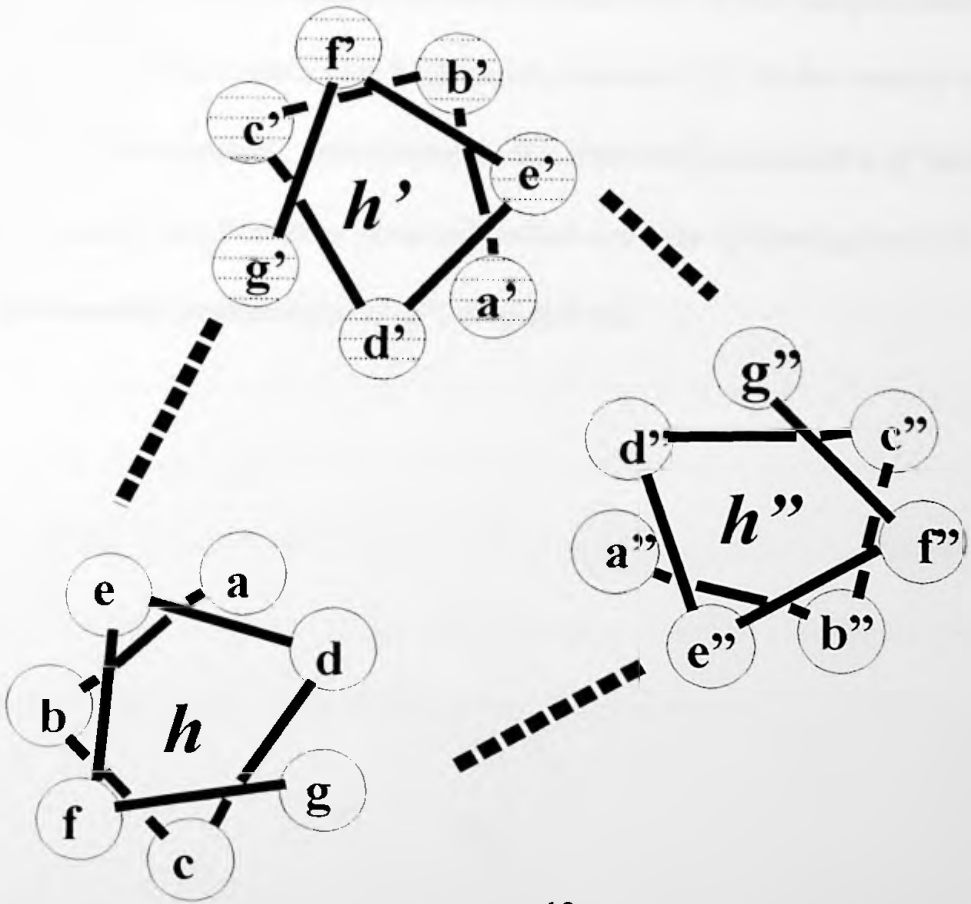


Figure 3. A top down view of the heptad repeat structure of α-helical protein domains. Letters "**a**"through "**g**" represent amino acid positions within the heptad repeat. In three dimensions the **a** position is at the bottom with the **b** through **g** positions spiraling upward as a coil. Within a given repeat, apolar amino acids occur with increased frequency at the **a** and **d** positions and charged amino acids have a higher occurrence at the **e** and **g** positions. When α-helices (here labeled *h*, *h'* and *h''*) interact as part of coiled-coils the heptad repeats show characteristic patterns of intermolecular interactions. The apolar residues at the **a** and **d** positions of opposing helices generally face each other allowing strong electrostatic interactions (dashed lines) to occur between the charged amino acids at the **e** and **g** positions to occur. The combined effects of hydrophobic interactions of apolar residues and electrostatic interactions of the charged residues can produce chain interactions with a very high dipole moment. (A) In the case of a two α-helices coiled-coil, electrostatic interactions of the intermolecular pairs **e-g'** and **e'-g** stabilize the structure. (B) For three stranded coiled-coils the following pairs of amino acids form electrostatic interactions: e'-g", e"-g and e-g'.

In the two-stranded alignment the *a* and *d* apolar residues face the *d'* and *a'* residues, respectively. In the three-stranded coiled-coils the closest intermolecular apolar pairs are *a''-d*, *a'-d''* and *a -d'* (Figure 3-A and B) This packing of apolar residues accounts for the stability of the coiled-coil structure (Cohen and Parry 1990). These interactions are diagrammed in Figure 3.

Laminin Trimer Assembly

It is known that certain cell types, such as skeletal and smooth muscle, can synthesize and secrete more than one Ln variant (Green et al. 1992; Glukhova et al. 1993; Martin et al. 1995; Walker-Caprioglio et al. 1995) . How a cell correctly assembles the various chains to form the different Ln isoforms, and what sequences are involved in specific chain interactions, are still largely unknown. To answer these questions researchers have focused on the C-terminal α-helical regions of domain I within the chains.

Results from a number of biophysical studies of chain interactions support the α-helical nature of domains I and II of the Ln chains (Deutzmann et al. 1988; Beck et al. 1990; I. Hunter et al. 1990 & 1992). Analysis of the amino acid sequence of these domains showed the presence of the characteristic heptad repeat of an α-helix. After alignment of these heptad repeats using two different theoretical paradigms, Beck et al. (1993) predicted both strong interactions between the β and γ chains and the formation of

α-β-γ trimers, based on proposed ionic interactions of the charged amino acids on adjacent faces of the interacting laminin chains. Homodimers of two β chains or two γ chains (β-β or γ-γ) were predicted to interact poorly in this model.

Experimental support for this theoretical model was described by I. Hunter et al. (1990), using the E8 C-terminal protease fragment of Ln (Figure 1). This fragment contains most of domain I of all three chains of Laminin-1 plus a large portion of the G domain of the α1 chain. When dissociated in urea and then reconstituted, this three chain fragment reassembled into a trimer indistinguishable from native E8, but only if the β and γ fragments were first separated from the α fragment and allowed to form dimers. These results were evidence that the order of trimer formation was β plus γ, then α.

Bacterially expressed peptides (~100 to ~ 200 amino acids), corresponding to the C-terminal region of each of the chains, were used to show interactions of β with γ as well as interactions of β-γ with α. The same studies detected no β-β or γ-γ interactions. Furthermore, deletion studies of the γ1 chain localized two separate C-terminal sites of approximately 10 amino acids each, one involved in dimer formation and the other in trimer formation (Utani et al. 1994). *In vitro* mixing experiments suggested that the most thermostable peptide dimer was β1-β1. However, since β1 and γ1 peptides when mixed together formed predominantly β1-γ1 dimers, it was concluded that kinetics favored heterodimer and heterotrimer formation (Nomizu et al. 1994). Recently a more detailed hypothesis of Ln chain assembly has been proposed. In this model the β and γ chains

interact to form a heterodimer with an "acidic pocket", which is a less stable structure, then the α chain fits into the "pocket" via a basic site to create the stable coiled-coil heterotrimer (Utani et al. 1995; Nomizu et al. 1996). Kammerer et al. (1995) confirmed the high thermostability of the β-γ and β-β recombinant peptide dimers, but could not confirm either the presence of a dimerization site or that interactions of the C-terminal 100 amino acids alone could account for the specific Ln chain interaction. These biochemical and biophysical studies of laminin fragments strongly implicate the C-terminal amino acids of the laminin molecule as the determining factors in the specificity of chain assembly.

The cloning of numerous full-length cDNAs for the various chain isoforms, some from more than one species, and the ability to express recombinant full-length Ln chains has led to the study of intracellular assembly of both full-length and truncated laminin chains. Yurchenco et al. (1997) showed that the α1 chain can be secreted by itself and that β1-γ1 heterodimers can form intracellularly, but these heterodimers are not secreted unless assembled into a trimer containing the α1 chain. Matsui et al. (1995) showed formation of recombinant β3-γ2 heterodimers in a human squamous cell carcinoma line (SCC-25). Niimi et al. (1997) reported that homodimers of recombinant laminins could form in COS-1 cells and that N-terminal truncations of either recombinant β1 or γ1 did not prevent assembly of heterodimers or heterotrimers with full-length constitutively expressed laminins.

Early work examining intracellular laminin subunit assembly utilized cell lines which constitutively expressed laminin chains. Intracellular heterodimers of β-γ chains and trimers of β-γ-α could form, but only the trimers could be secreted into cell culture medium (Morita et al. 1985; Matsui et al. 1995). Studies of cell lines also led to the determination of the kinetics of N-linked glycosylation and secretion. It was found that glycosylation was not necessary for subunit assembly (Morita et al. 1985) but was necessary for secretion of the trimer (Green et al. 1992).

Biology of Laminin

Laminin is expressed as early as the two-cell stage of mammalian embryogenesis, making it the earliest expressed ECM component (Dziadek and Timpl 1985). *In vivo* studies of developing organs show dynamic patterns of Ln isoform expression and distribution during embryogenesis which are temporally and spatially regulated in apparent coordination with organogenesis. Some immunohistochemical results are contradictory in relation to the Ln chain isotypes present in structures such as the neuromuscular junction (NMJ) of muscle or the glomerular basement membrane (GBM) of the kidney. For instance, Patton et al. (1997) described the Ln β2 chain as expressed only in the synapse of the neuromuscular junction (NMJ) while Wewer et al. (1997) reported the extrasynaptic expression of the β2 chain in muscle cells using different antibodies. What is clear is that the Ln expression pattern does change with development

of organs such as the kidney and muscle. Studies of *in vitro* kidney development show expression of the Ln α1 chain protein (and therefore the secreted Ln trimer) is concomitant with tubule epithelial development, while the expression of the β1 and γ1 chains is detectable before this early developmental stage (reviewed in Sorokin and Ekblom 1992). *In vitro* examination of cultured C2C12 muscle cell lines detected increasing levels of Ln α2 chain mRNA throughout differentiation into myotubes, and a transient increase followed by the loss of signal for α1 chain mRNA during the same 6 day culture period that corresponded to myotube formation (Vachon et al. 1996).

The necessity of the Ln α2 chain protein for the survival of myotubes in cell culture is seen in embryonic stem cell lines with disrupted α2 chains. These cells form unstable myotubes that dissociate and collapse with the onset of the contractile phenotype (Kuang et al. 1998). Evidence that Ln α2 is important for *in vivo* myotube stability comes from the study of muscular dystrophies. One severe form of congenital muscular dystrophy (CMD), now known as merosin-deficient CMD, has been found to be due to mutations in the LAMA2 gene which codes for the α2 chain (reviewed in Voit 1998).

In other diseases, alteration of Ln expression leads to severe consequences for organ development. The congenital, inherited form of the skin malformation known as epidermolysis bullosa has been determined to be caused by a mutation in the LAMA3 gene. This leads to non-expression of the α3 laminin chain which, in turn, prevents the secretion of the laminin-5 isoform (Ln-5) from keratinocytes. This disrupts the epidermal

adhesion to the basement membrane at the dermal-epidermal interface. Recent studies have shown that transfection of the wild type α3 chain gene into keratinocytes isolated from an epidermolysis bullosa patient can restore the ability of those cells to assemble and secrete the Ln-5 isoform (Dellambra et al. 1998). Clearly, the study of the normal physiology and biochemistry of Ln has implications for characterization of, and possible therapies for, specific diseases.

In other work, Ln isoforms have been implicated as mediators of cell differentiation *in vitro*. Studies of mammary epithelium have shown that Ln is capable of activating transcription and inducing differentiation (or at least markers of differentiation). The addition of Ln to cultures of primary isolated epithelial cells increases levels of the milk protein β-casein via a pathway that increases transcriptional activity at the β-casein promoter (Streuli et al.1995a). Stat5 transcription factor was later shown to mediate transcriptional increases of milk protein messages in mammary epithelial cell on Ln-1 containing matrix (Streuli et al. 1995b). Studies of this nature show clear evidence of laminin's ability to activate cell metabolism and alter cellular function.

Biology of the β2 Chain : A Unique Laminin Chain

The Ln β2 chain (formerly s-laminin) was initially characterized as a laminin-like glycoprotein which was enriched in the NMJ synaptic cleft (D. Hunter et al. 1989a). It has subsequently been found to have restricted expression patterns in various organs such as

the developing brain, kidney, and blood vessels (D. Hunter et al. 1992; Durjeeb et al. 1996). It was discovered after initial characterization of the β2 chain that chick ciliary neurons would adhere to the partially purified protein. Later studies verified that motor neuron-like cells (NSC-34 cell line), but not sensory neurons or a neuron-like cell line (PC12), would bind to a purified fragment of the β2 laminin chain (D. Hunter et al. 1989b, 1991). Deletion studies indicated that the motor neurons were binding to a tripeptide epitope, L-R-E, which could inhibit neurite outgrowth in these cells. This led to the proposal that the Ln β2 chain may act as a stop signal for motor neurons at the NMJ (D. Hunter et al. 1991; Porter et al. 1995).

The studies of neuronal adhesion to the β2 fragment have been refuted as artifactual, and experiments utilizing trimeric Ln-2 and Ln-4 (that contain the β2 chain) showed that motor neurons did extend neurites on these glycoproteins. The contention is that the L-R-E peptide in the C-terminus of laminin β2 chain is masked in the trimeric molecule and therefore unavailable to cellular adhesion molecules of the neurons (Brandenberger et al. 1996). Whether or not this is true *in vivo* has not been studied.

The Ln β2 chain has other apparent functions beyond its neuronal adhesion properties. Transgenic mice which have had both copies of the laminin β2 chain gene deleted die soon after birth. They exhibit slightly abnormal neuromuscular junctions and have severely impaired kidney function (Miner et al. 1995). These abnormalities coincide with the expression of the β2 chain in both neuromuscular junction and kidney glomerular

basement membrane and implicate the laminin β2 isoform in proper function, if not proper development, of these organs. The presence of the β2 chain at specialized structural features of several organs makes this an interesting protein for study. Furthermore, the ability of the muscle to spatially restrict the expression of the β2 chain to the NMJ and the β1 chain to the extrasynaptic basement membrane has prompted the usage of the β2 chain in these studies.

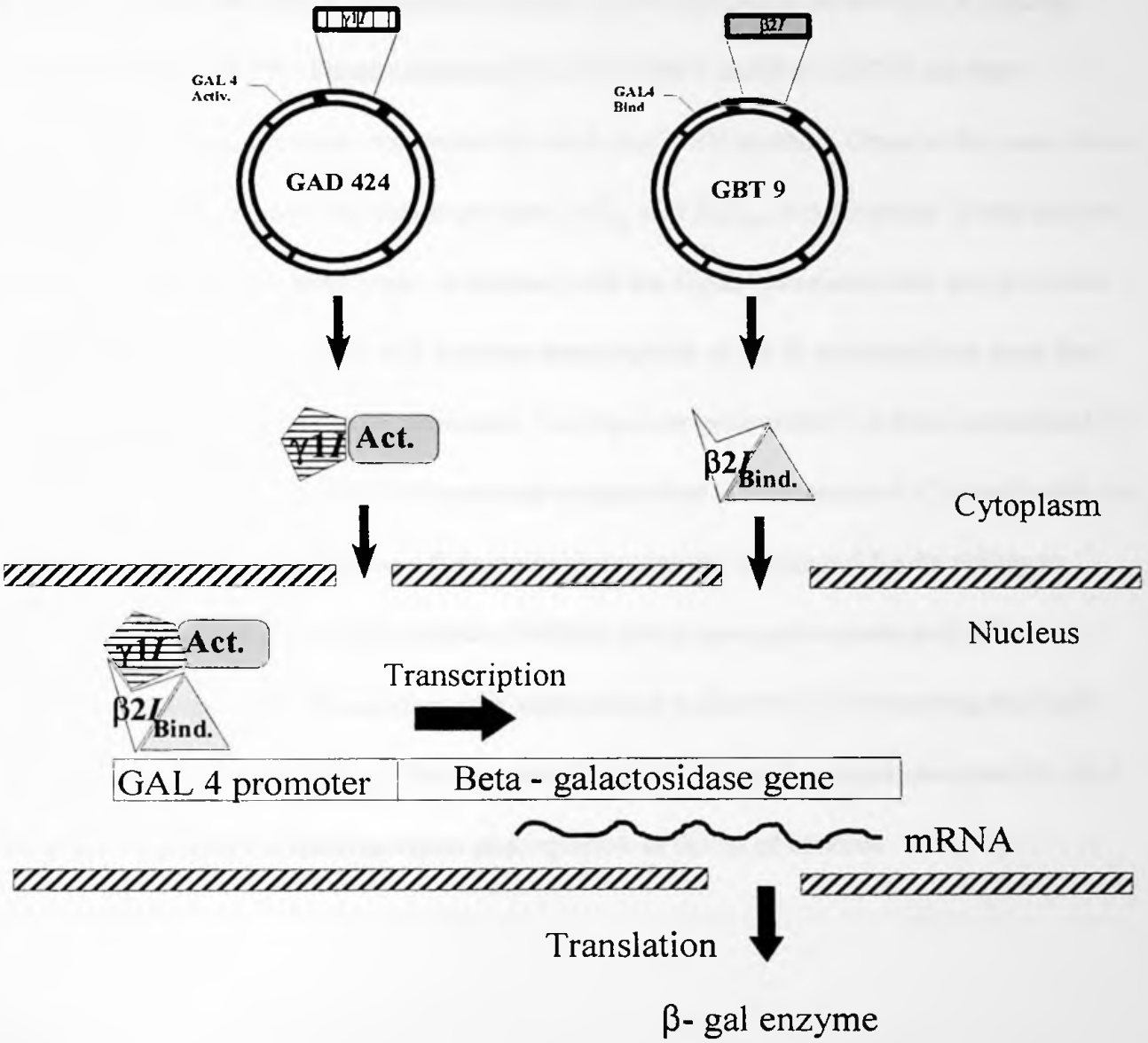
Yeast Two-hybrid Assay

To examine and measure the interactions of the Ln chains, including the amino acid sequences required, the yeast two-hybrid system was utilized. This ingenious technique was developed by Fields and colleagues (1989) to study interactions (or potential interactions) of protein pairs. The developers recognized the ability of the yeast GAL4 transcriptional activator protein to be separated into domains. One domain could function independently as a DNA binding element, which recognized a specific sequence (the GAL4 promoter). The second domain could activate gene transcription but not bind DNA. The gene fragments coding for these separate domains were then placed into separate plasmid vectors. The plasmids, GBT9 (containing the DNA binding domain) and GAD424 (containing the transcriptional activation domain), have multiple cloning sites which allowed genes of interest to be ligated into the plasmid so that the reading frame of the GAL4 domain is maintained, thereby creating a hybrid gene.

The transcriptional activator domain and the DNA-binding domain have no ability to interact with each other. But if protein *x* cloned into the GAD424 vector did interact with protein *y* in the GBT9 vector, the two hybrid proteins bound together could then form a viable transcription factor that would activate transcription of genes under the control of the GAL4 promoter. In the Matchmaker® two-hybrid system (Clontech; Palo Alto, CA) the yeast strain SFY 526 contains a reporter gene (β-galactosidase) under the control of the GAL4 promoter. This allows detection of a protein-protein interaction of the two hybrid constructs by a simple colorimetric assay. A schematic representation of the two-hybrid assay is shown in Figure 4.

The two-hybrid assay was originally used to detect interactions of known transcription factors with potential regulating factors or other transcriptional activators (Fields et al. 1989; Chien et al. 1991). It has since been utilized by researchers to screen libraries for gene products which are able to interact with a protein of interest. To date a wide variety of protein-protein interactions have been detected and characterized, such as the interaction of transcription factor dE2F with cyclins (Du et al. 1996; Jordan et al. 1996), estrogen receptor dimerization (Wang et al. 1995), and intermediate filament interactions (Meng et al. 1996). A novel use for the system, the evaluation of the interactions of an extracellular matrix molecule, is described here. By using this technique, dimer formation by pairs of Ln chain fragments can be quantified in an *in vivo* (yeast) model.

Figure 4. Schematic of Two-Hybrid Assay



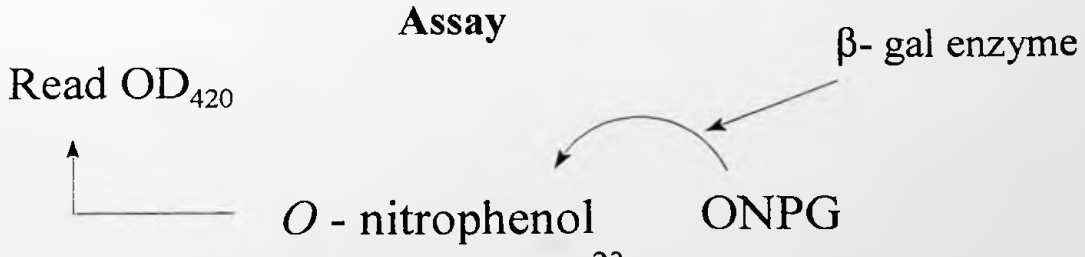


Figure 4. Schematic diagram of the events in a two-hybrid assay. The example shown is of the transformant $\gamma 1I_{act} + \beta 2I_{bind}$. The first step is insertion of the Ln chain fragment ($\gamma 1I$ or $\beta 2I$) into the GAL4 activation domain vector (GAD424) or the GAL4 binding domain vector (GBT9). These constructs ($\gamma 1I$/GAD424 and $\beta 2I$/GBT9) are then transformed into yeast made competent by the LiAc/ PEG method. Once in the yeast these constructs are translated into fusion proteins $\gamma 1I_{act}$ and $\beta 2I_{bind}$, respectively. These fusions must cross the nuclear membrane to interact with the GAL4 promoter site, and provided the two fusions interact, they will activate transcription of the β-galactosidase gene that has been attached to the GAL4 promoter. The reporter gene mRNA is then transcribed by the cellular machinery to yield a functional enzyme that is then assayed. Cultured cells are lysed by a rapid freeze/ thaw and β-galactosidase activity is assayed for its ability to cleave *o*-nitrophenylpyranogalactoside (ONPG) into pyranogalactoside and yellow-colored *o*-nitrophenol. The amount of *o*-nitrophenol is detected by measuring the light absorbance at 420 nm (OD_{420}). The amount of activity for each sample was then divided by that of a positive control reaction and reported as the % of control.

Research Aims

Many of the laminin chain assembly experiments described above have been performed under controlled conditions; i.e., in solution,and not in a cellular environment. This work examines the ability of domain I, and fragments of domain I, to interact within the cellular milieu by using the yeast two-hybrid assay to detect and measure protein-protein interaction of fusion proteins containing recombinant fragments of the laminin β2, γ1, and α1 chains. This assay replicates the recombinant studies described above, but in a cellular context.

If only domain I of the laminin chains is necessary for dimer formation, then a full-length laminin chain should interact with another chain only through domain I. To test the ability of domain I to drive specific β-γ dimer formation, two unique hybrid laminin chains were created. One fused domain I of β2 to the domains II through VI of γ1, and the second fused domain I of γ1 to the domains α through VI of β2. These hybrids allow the assembly role of domain I to be assessed as part of a full-length laminin protein, and within a cellular environment. As a whole, this research is intended to extend previous knowledge of laminin chain assembly by studying chain interactions within cells.

Materials and Methods

Subcloning of cDNA

Standard protocols for subcloning cDNA, including ligation into plasmid vectors, transformation of competent bacteria, and screening for correct constructs was performed for all plasmids constructed. Modifications in these protocols, as well as specific enzymes used create a particular vector, are noted below for the individual constructs. All cDNAs described here were originally cloned by other laboratories and donated by researchers as noted.

The initial step in subcloning required either digestion of the plasmid incorporating the full-length cDNA or amplifying a fragment of the cDNA by PCR. After a ligation reaction that created a circular plasmid DNA (which is then capable of replication in a bacterium), it was transformed into competent bacteria. For most ligations, the entire reaction (approximately 200 ng of DNA) was added to a tube containing 200 µl of competent XL-1 Blue (Stratagene, La Jolla, CA) bacteria and placed on ice for 30 minutes. Subsequently, 800 µl of LB medium (10 $^{g}/_{L}$ Bacto® peptone, 5 $^{g}/_{L}$ yeast extract, 85.5 mM NaCl. pH 7.4) was added to the tube, which was placed in a heat block at 37° C for one hour. From this 1 ml solution, 250 µl was removed and spread onto a LB agar plate containing 50 $^{\mu g}/_{ml}$ of ampicillin (LB-amp). All plasmids used contained the amp^{R} selection marker. This selective plate was placed overnight at 37° C. A number of the

colonies which grew on the plate were removed, placed in a 2 ml culture of LB-amp medium, and incubated overnight at 37° C. The next day rapid plasmid isolation using the method of Berghammer and Auer (1993) was performed. From the culture tube, 1.5 ml of bacterial culture was moved to a 1.5 ml microcentrifuge tube while the rest was placed at 4° C for storage. Bacteria were pelleted by centrifugation at maximum speed in a microcentrifuge for 30 seconds and the supernatant was removed. This pellet was resuspended in 75 µl of "EZ-prep" lysis solution [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 15 % w/v sucrose, 2 $^{mg}/_{ml}$ lysozyme, 0.2 $^{mg}/_{ml}$ pancreatic RNase, 0.1 $^{mg}/_{ml}$ BSA], shaken for 5 minutes, boiled for 1 minute and placed on ice for 1 minute. Cell debris, (including genomic DNA) was pelleted by centrifugation at 14,000 ×g in a microcentrifuge for 10 - 15 minutes. The supernatant contained relatively pure, mostly supercoiled plasmid DNA. To check for the presence of insert cDNA, 10 - 15 µl of this supernatant was used in a restriction digestion, and the products were separated on a 0.7 or 1 % agarose minigel. Reserve bacteria containing these positive constructs were cultured in 5 - 8 ml of LB-amp medium overnight at 37° C and a second plasmid isolation, using the Wizard® miniprep columns (Promega, Madison, WI), was performed in order to isolate enough purified supercoiled plasmid to sequence. Sequenced plasmids were either used for subsequent subcloning steps or used in experiments. To purify large amounts of plasmid DNA, reserved bacteria from the initial isolation were streaked onto an LB-amp plate, then a single colony removed and cultured in 30-50 ml of LB-amp

medium. Plasmids were isolated from these bacteria using a Qiagen (Santa Clarita, CA) midiprep kit, following manufacturer's instructions. These generally yielded 1 - 3 μg of DNA per ml of culture.

DNA Sequencing

DNA constructs were sequenced using the Sequenase 2.0 sequencing kit (Amersham, Arlington Hts, IL) following manufacturer's instructions.

DNA Constructs

Two-hybrid constructs

All PCR reactions were done using *Pfu* polymerase from Stratagene (La Jolla, CA) unless otherwise noted. For all activation domain constructs the expression vector was GAD424 (Bartel et al. 1991), and for binding domain constructs the expression vector was GBT9 (Bartel et al. 1991). The cDNA fragments generated for use in the two-hybrid assay are diagrammed in Figures 5 and 6. Constructs created by PCR or restriction digestion and placed into both two hybrid vectors, are shown in Table 1.

Figure 5.

β2 Chain Fragments Used in Two-Hybrid System

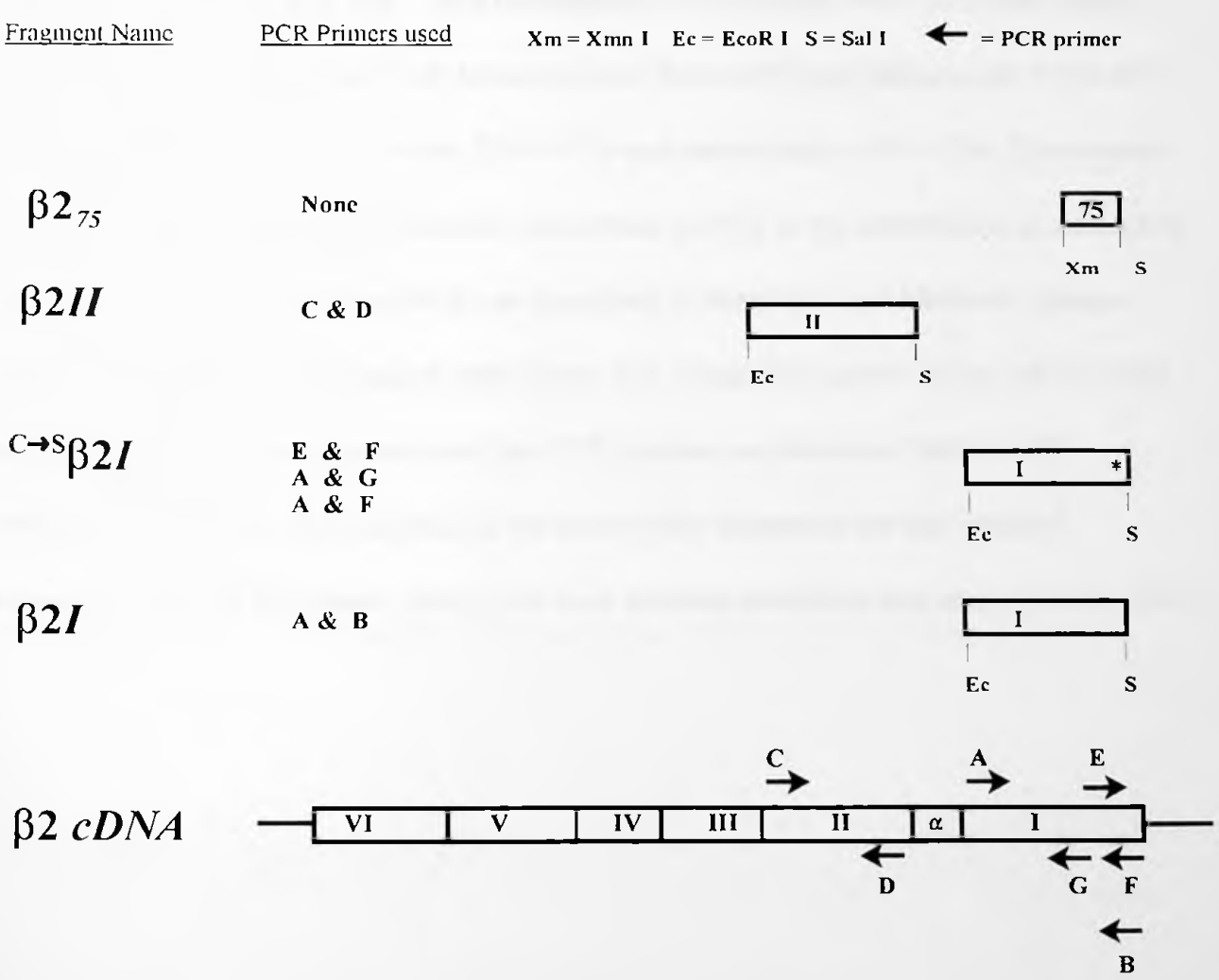
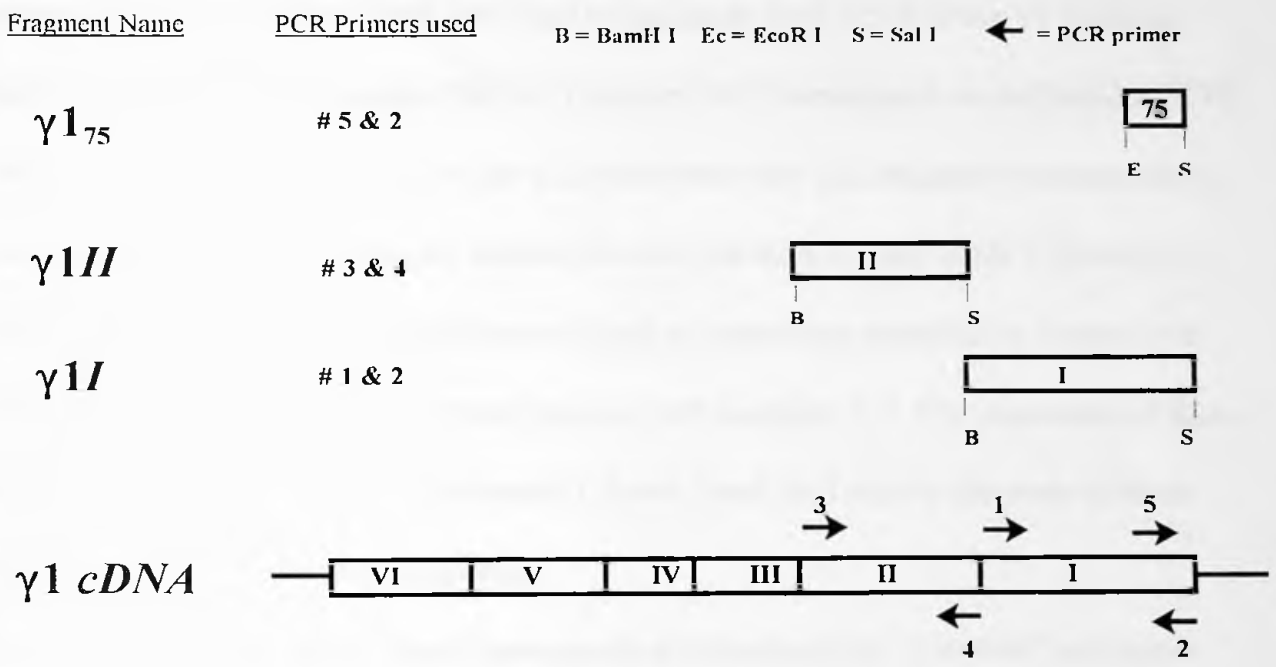


Figure 5. PCR or restriction digestion was utilized to create long arm fragments of the β2 chain. For all PCRs shown the cDNA was the full-length β2 cDNA of Green et al. (1992). Roman numerals designate structural domains as per standard nomenclature. Fragments correspond to the following nucleotides of the cDNA and amino acids of the mature protein as determined from the primary amino acid sequence reported by D. Hunter et al. (1989a): β2*I* and $^{C\rightarrow S}$β2*I* correspond to nucleotides 4401-5550 and amino acids 1410-1766, β2*II* corresponds to nucleotides 3648-4301 and amino acids 1159-1377, and $\beta 2_{75}$ corresponds to nucleotides 5245-5550 and amino acids 1691-1766. The asterisk (*) in the $^{C\rightarrow S}$β2*I* fragment indicates the single base pair (T to A) substitution at nucleotide 5466 that was created via three PCRs as described in *Materials and Methods*. Arrows represent PCR primers designated with letters A-F. Construct names on the left are used throughout the text. The sequences of the PCR primers are shown in Table 1. The restriction sites for EcoR I and Sal I at the ends of the fragments are the result of sequences in the PCR primers. Xmn I site is an internal restriction site at nucleotide 5245.

Figure 6.

A γ1 Chain Fragments Used in Two-hybrid System



B α1 Ln Chain Construct Used in Two-hybrid Assays

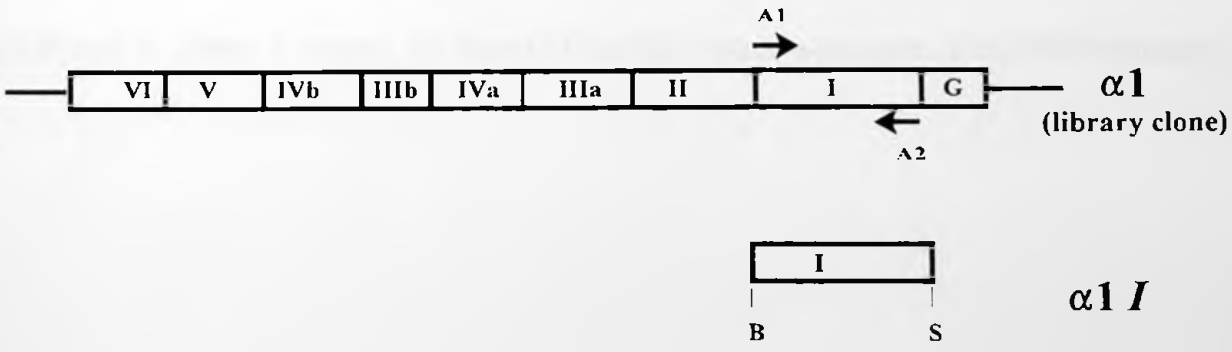


Figure 6. (A) Fragments of the γ1 chain were created by PCR. The template for the γ1*I* PCR was the cDNA provided by Dr. Y. Yamada. For the γ1*II* and $\gamma 1_{75}$ fragment PCRs the template was the cDNA provided by Dr. A. Chung. The γ1*I* fragment corresponds to nucleotides 3907-5067 and amino acids 1188-1574 of the γ1 chain as reported by Sasaki and Yamada (1987b). Fragment γ1*II* corresponds to nucleotides 3271-3840 and amino acids 993-1183 of the γ1 chain, while the $\gamma 1_{75}$ fragment corresponds to nucleotides 4782-5007 and amino acids 4992-5067 of the γ1 chain. Both γ1*II* and $\gamma 1_{75}$ nucleotides and amino acid sequences are based on sequences reported by Durkin et al. (1988). Arrows represent PCR primers labeled with numbers 1-5. The sequences of these primers are shown in Table 1. The BamH I, EcoR I and Sal I sites at the ends of these fragments are primer derived sequences.

(B) A fragment of the α1 chain corresponding to nucleotides 5230-6447 and amino acids 1694-2109 of the full-length cDNA and protein sequences, respectively (based on sequence data of Sasaki et al. [1988]) was generated by RT- PCR. The template for the RT-PCR was mouse kidney poly-A^+ RNA. The product of this reaction was used as the template in a subsequent PCR using primers A1 and A2. The sequences of these primers are shown in Table 1. Again, the BamH I and Sal I sites were part of the PCR primers.

Table 1. Laminin Fragments Subcloned into Two-hybrid Vectors

Ln Fragment	PCR	Product Size (base pairs)	PCR Template	Cloning Restriction Sites	corresponding cDNA nucleotides
β2*I*	94°C - 1", 61°C - 1", 72°C - 2" (20 cycles)	1,149	T. Green, β2 cDNA	EcoR I Sal I	4401-5550 : D. Hunter et al. (1989)
γ1*I*	94°C - 1", 59°C - 1", 72°C - 2" (20 cycles)	1,179	Y. Yamada, γ1 cDNA	BamH I Sal I	3907-5067 : Sasaki and Yamada (1987b)
β2*II*	95°C - 1", 60°C - 1", 72°C - 2" (30 cycles)	675	T. Green, β2 cDNA	EcoR I Sal I	3648-4301 : D. Hunter et al. (1989)
γ1*II*	95°C - 1", 60°C - 1", 72°C - 2" (30 cycles)	589	A. Chung, γ1 cDNA	BamH I Sal I	3271-3840 : Durkin et al. (1988)
$\beta2_{75}$	Not Applicable	310	N/A	Xmn I Sal I	5225-5550 : D. Hunter et al. (1989)
$\gamma1_{75}$	95°C - 1", 54°C - 1", 72°C - 2" (30 cycles)	310	A. Chung, γ1 cDNA	EcoR I Sal I	4782-5007: Durkin et al. (1988)

Table 2. PCR Primers Used in Creating the Two-Hybrid Constructs

Domain I of β2

upper primer (A) = 5'-**gaattc**tgcagtggagcagcagccaca-3'

lower primer (B) = 5'-**gtcgac**agccaagagctctttaatgtc-3'

Domain I of γ1

upper primer (1) = 5'-**ggatcc**tcaatcaaactgcgctggaga-3'

lower primer (2) = 5'-**gtcgac**gggcttctcgataga-3'

Domain I of α1

upper primer (A1) = 5'-cccg**ggatcc**gaccttgagctcaaggctgct-3'

lower primer (A2) = 5'-cccggg**gtcgac**gtctctgtctgcagacacggcgact-3'

Domain II of β2

upper primer (C) = 5'-cccggg**gaattc**catgcatgctttgcag-3'

lower primer (D) = 5'-acgc**gtcgac**cagctcatttactcc-3'

Domain II of γ1

upper primer (3) = 5'-cg**ggatcc**ttgagtgtccggcttgt-3'

lower primer (4) = 5'-acgc**gtcgac**ttctcctgccagggt-3'

C-terminal 75 amino acids of γ1

upper primer (5) = 5'-**gaattc**ctcaatgagatcgaa -3'

lower primer (6) = 5'- **gtcgac**gggcttctcgataga -3'

Table 2. PCR Primers Used in Creating the Two-Hybrid Constructs

Cys to Ser mutation of β2 Domain I

PCR-I	upper primer (E) = 5'-cagatctacaacaccAgccagtga-3'
	lower primer (F) = 5'-atcgatgtcgacgctagccaagagctc-3'
PCR-II	upper primer (A) = 5'-gaattctgcagtggagcagccaca -3'
	lower primer (G) = 5'-agggagtggtcactggcTggt gtt-3'
PCR-III	upper primer (A) = 5'-**gaattc**tgcagtggagcagccaca -3'
	lower primer (F) = 5'-atcgat**gtcgac**gctagccaagagctc-3'

Table 2. Primers above were utilized to amplify portions of a particular Ln chain cDNA by PCR. Letters and/or numbers in parentheses denote the primer designation in Figure 6. Bold letters represent the restriction sites used to subclone these fragments.

Table 3. PCR Primers Used in the Creation of Ln Chain Expression Constructs

Upstream Region of β2 cDNA

upper primer 5'-cttgatatcgaattcagtgactgctggtcggacc-3'

lower primer 5'-tccaaagcatgcatgggccaatgtggcagccagcac-3'

Creation of Hybrid Ln Chain $\beta2(\gamma1)_I$

PCR-I upper primer (H3) = 5'-ccccgctgtggcctcggtaatcaaactgcgctggagatt-3'

lower primer (H4) = 5'- gcgatcctctctgcctcatgggccttgttc-3'

PCR-II upper primer (Hc) = 5'-cccgcaacacctcagctgcgtctactgcaa-3'

lower primer(Hd) =5'-aatctccagcgcagtttgattaccgaggccacagcggggttg-3'

PCR-III upper primer (Hc) = 5'-cccgcaacacctcagctgcgtctactgcaa-3'

lower primer (H4) = 5'- gcgatcctctctgcctcatgggccttgttc-3'

Creation of the Hybrid Ln Chain $\gamma1(\beta2)_I$

PCR-I upper primer (H1)=5'-*ttgaggaccctggcaggagaa*tgcagtggagcagcagcagccaca-3'

lower primer (H2) = 5'-tgcttggagacaaggctaaaccctagggagtggtca-3'

PCR-II upper primer (Ha) = 5'- ctgattgagatcgcctccagggagctcgagaaa-3'

lower primer (Hb) = 5'-*tgtggctgctgctccactgca*ttctcctgccagggtcctcaa-3'

PCR-III upper primer (Ha) = 5'- ctgattgagatctgcctccagggagctcgagaaa-3'

lower primer (H2) = 5'-tgcttggagacaaggctaaaccctagggagtggtca-3'

Table 3. PCR Primers used in the Creation of Ln Chain Expression Constructs

	Creation of Domain IV-V Deleted β2 Ln Chain
PCR-I	upper primer = 5'-ggtaccgaattcccctgccagtgtgac-3'
	lower primer = 5'-atcgatgtcgacgctagccaagagctc-3'
PCR-II	upper primer = 5'-gcgccccggggcggccggagtgactgctggtc-3'
	lower primer = 5'- gggcccgaattcgcggatgacaagttcatagagagc-3'

Table 3. Hybrid Ln chains were created by utilizing PCR to fuse domain I of β2 with domain II of γ1 and to fuse domain I of γ1 with domain II of β2. To do this two PCR reactions were performed, and the products of each were fused to create a template for a third PCR, which created the hybrid product. These reactions are described in *Materials and Methods* and diagrammed in Figures 7 and 8. Letters and numbers in parentheses represent the designations for the primers in Figures 7 and 8.

$\alpha 1_I$/GAD424 and $\alpha 1_I$/GBT9: *Domain I of Ln α1 chain in yeast two-hybrid vectors.*

Domain I of α1 was generated via RT-PCR using mouse kidney poly-A^+ RNA (CLONTECH, Palo Alto, CA) as the template. 100 ng of RNA was reverse transcribed with 40 U of M-MLV RT (Life Technologies, Grand Island, NY) in 50mM Tris [pH 8.3], 75mM KCl, 3mM $MgCl_2$, 0.5mM dNTPs, and 30 U RNasin (Promega, Madison, WI) at 42° C for 1 hour. The α1 cDNA was amplified by PCR with the upper primer 5'-cccgggatccgacttgagctcaaggctgct-3' and the lower primer 5'- cccggggtcgacgtctctgtctgcaga cacggcgact-3'. This 1,241 base pair fragment corresponds to nucleotides 5230-6447 and amino acids 1694-2109 of α1, based on domain sequences reported by Sasaki et al. (1988). This PCR fragment was digested with the BamH I and Sal I restriction endonucleases and ligated into BamH I and Sal I -digested GAD424 and GBT9 two-hybrid vectors.

$^{C\rightarrow S}\beta 2_I$/GAD424 and $^{C\rightarrow S}\beta 2_I$/GBT9: *Cysteine to serine mutation of amino acid 1765 within domain I of Ln β2 chain in yeast two-hybrid vectors.*

To create a β2 domain I cDNA construct with a T to A substitution mutation at nucleotide 5466 (which changes amino acid 1765 from cysteine to serine), three PCR reactions were performed using Taq polymerase and full-length β2 cDNA as the template. The first amplified the 3' end of domain I (nucleotides 5451-5553 of the cDNA) using the upper primer 5'-cagatctacaacaccAgccagtga-3' (capital A represents the altered nucleotide)

and the lower primer 5'-atcgatgtcgacgctagccaagagctc-3'. After an initial 3 minute incubation at 94° C, 25 cycles of 94° C for 1 minute, 54° C for 1 minute, and 72° C for 1 minute were done. The resulting product was 115 base pairs long and for reference will be called PCR-I.

The second PCR from this construct amplified the region from nucleotides 4400-5483 of the β2 domain I sequence. The upper primer was 5'-gaattctgcagtggagcagccaca -3' and the lower primer was 5'-agggagtggtcactggcTggt gtt-3' (capital T represents the altered nucleotide). The 1,089 base pair PCR product (PCR-II) was amplified by incubating for 3 minutes at 94° C, then performing 25 cycles of 94° C for 1 minute, 62° C for 1 minute, and 72° C for 2 minutes. In the third PCR, equimolar amounts of the PCR-I and PCR-II fragments were denatured at 94° C for 3 minutes and reannealed to one another at 25° C for 5 minutes, then the complementary strands were filled in by Taq polymerase at 72° C for 7 minutes. The product was amplified by PCR for 25 cycles of 94° C for 1 minute, 63° C for 1 minute, and 72° C for 2 minutes. The upper primer was the same as the upper primer of PCR-II and the lower primer was the lower primer of PCR-I. This created a 1,171 base pair fragment corresponding to β2 domain I sequence, with a one base pair substitution.

This PCR product was purified and digested with Nde I and Sal I, which yielded a 203 base pair fragment. This fragment was then ligated into Nde I and Sal I -digested pGEM-T. The Nde I - Sal I fragment was subsequently removed and subcloned into wild-

type β2 domain I (in the pSK^+ vector [Stratagene, La Jolla, CA]), which had also been digested with Nde I and Sal I. The resulting plasmid was named $^{C\rightarrow S}\beta 2_I$ /SK. This plasmid was digested with EcoR I and Sal I and the mutant domain I was subcloned into GAD424 and GBT9 at the EcoR I and Sal I sites.

Cell Culture Expression Constructs

β2 IαII-up / SP72: *A truncated cDNA containing the sequence of the long arm domains of the β2 chain, in-frame with the signal sequence of the β2 cDNA.*

The 5' upper region of the β2 chain was created from the full-length cDNA via PCR. The reaction consisted of 30 cycles of 94° C for 1 minute, 64° C for 1 minute, and 72° C for 1 minute, and utilized the upper primer 5'-cttgatatcgaattcagtgactgctggtcggac cct-3' and the lower primer 5'-tccaaagcatgcatgggccaatgtggcagccagcac-3'. The PCR product was 203 base pairs long and corresponded to bases 1- 173 of the β2 chain cDNA. The generated product was digested with EcoR I and Nsi I, then ligated into EcoR I and Nsi I digested SP72/β2 plasmid. The digestion of the SP72/β2 cut out the sequence for the domains III - VI, leaving only the long arm sequences of β2 (domains I-α-II) still in the vector. This construct was used later for creation of other β2 fragments.

β2 /cDNA1: *Full-length Ln β2 chain in the CMV promoter- containing mammalian expression vector.*

The full-length cDNA for the Ln β2 chain (Green et al. 1992) was digested with EcoR I and Nhe I and ligated directionally into EcoR I - Xba I -digested pcDNAI/Amp expression vector (Invitrogen, San Diego, CA).

γ1 /cDNA1: *Full-length Ln γ1 chain in the CMV promoter- containing mammalian expression vector.*

A full-length cDNA coding for the mouse Ln γ1 chain was a gift of Dr. Albert Chung. This cDNA was digested with Hin*d* III and Dra I and subcloned into pSP72, digested with Hin*d* III and Sma I. This removed the polyadenylation site from the 3' end of the cDNA but left the 5' signal sequence, the entire open reading-frame and some of the 3' untranslated region. Initial sequencing of the construct showed the clone was correct, but later sequencing revealed a one base pair substitution from the published sequence at nucleotide 4958. It is unclear if this mutation occurred in our laboratory or if the nucleotide sequence was misreported. The nucleotide substitution would lead to a Leu →Met amino acid change; based on studies of amino acid composition of alpha-helices, this change would not disrupt the secondary structure nor the charge interactions of domain I. To ligate the γ1 chain cDNA into the pcDNAI expression vector, the newly created SP72/γ1 plasmid was digested with Hin*d* III and Eco RV and subcloned into pcDNAI/ Amp (Invitrogen, San Diego, CA) digested with the same two restriction endonucleases.

$\beta 2(\gamma 1)_I$ /cDNA3: *Hybrid Ln chain of Domain I of γ1 fused to Domains α-VI of β2 in the CMV promoter- containing mammalian expression vector.*

Creating a Ln chain which fused domain I of γ1 with domains α- VI of β2 required three initial PCR steps (Figure 7). All three utilized Taq polymerase enzyme. The first PCR utilized a cDNA of the γ1 chain donated by Dr. Y. Yamada to amplify the domain I region. The upper primer was 5'-*ccccgctgtggcctcggt*aatcaaactgcgctggagatt-3'; the italicized letters correspond to nt 4380 - 4400 of the β2 cDNA and the last 21 nucleotides correspond to nt 3907 - 319927 of the γ1 cDNA. The lower primer of this PCR was 5'- gcgatcctctctgcctcatgggccttgttc-3', corresponding to nt 4538 - 4509 of the γ1 sequence. This reaction consisted of 20 cycles of 94° C for 1 min., 63° C for 1 min., and 72° C for 2 min. A 653 base pair (bp) fragment was isolated.

The second PCR utilized the β2 cDNA as a template, with the upper primer 5'-cccgcaacacctcagctgcgtctactgcaa-3', which anneals at nt 3817 - 3847 on the β2 sequence, and the lower primer 5'-*aatctccagcgcagtttgatt*accgaggccacagcggggttg-3'. The italicized letters of the lower primer correspond to nt 3927 - 3902 in the γ1 cDNA and the remaining 21 nt correspond to nt 4400 - 4377 in the β2 cDNA. The reaction was run for 20 cycles of 94° C for 1 min., 64° C for 1 min., and 72° C for 2 min. This created a 605 bp fragment corresponding to domain II and α of the β2 chain with the sequence of γ1 as an overhang. The 5' end of the upper primer in PCR 1 is complementary to the 3' end of the lower primer in PCR 2. The third PCR utilized the PCR products from the first two

reactions as the template; the upper primer was the same as the upper primer of reaction 2 and the lower primer was the same as the lower primer of reaction 1.

Equimolar amounts of the double-stranded PCR products used as templates were denatured and cooled to allow the two complementary ends to anneal. The DNAs were heated to 94° C for 3 minutes, cooled to 25° C for 2 minutes, then Taq polymerase was added and the reaction was reheated to 72° C for 5 minutes to fill-in overhanging ends. The product was cycled 20 times at 94° C for 1 minute, 64° C for 1 minute, and 72° C for 2 minutes. The resulting 1217 bp product was a hybrid molecule of domains II and α of the β2 chain in the same reading frame as domain I of γ1. This third PCR product was ligated into the vector pGEM-T utilizing the 3' adenine residue added by Taq polymerase enzyme. This construct was called $\beta2_{II}(\gamma1)_I$ / GEM-T.

To facilitate the subcloning of the $\beta2(\gamma1)_I$ chain, the β2 cDNA was subcloned into the pBS vector (Stratagene, La Jolla, CA) using the EcoR I and Hin*d* III sites which cut at nt 1 and 3846, respectively. To create a full-length hybrid, the $\beta2_{II}(\gamma1)_I$ / GEM-T DNA was digested with Hin*d* III and Sal I enzymes, and the hybrid portion was ligated to β2 in pBS vector, also digested with Hin*d* III and Sal I. This plasmid was termed $\beta2(\gamma1)_I$ /pBS. This cDNA did not contain a termination codon, and therefore the region containing the termination codon was removed from the γ1 cDNA with BamH I and Dra I and ligated into the BamH I and EcoR V sites of pSK^+ vector. This fragment was removed again with

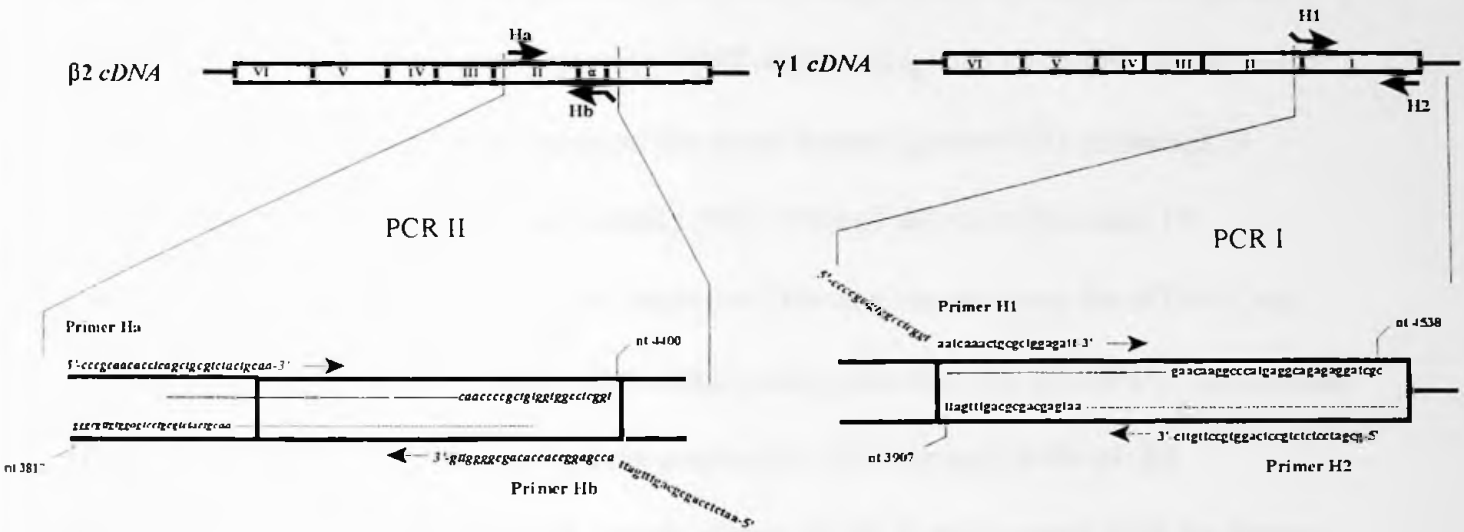
Nde I and Sal I enzymes and subcloned into Nde I - Sal I digested $\beta2(\gamma1)_I$/pBS DNA to create a full-length (termination codon included) hybrid laminin chain. To make the final product, a vector capable of expressing this hybrid laminin in mammalian cells, this DNA was cleaved with EcoR I and Sal I, then ligated into the pcDNA3 vector digested with EcoR I and Xho I. This construct was called $\beta2(\gamma1)_I$/cDNA3.

$\gamma1(\beta2)_I$ / SK: *Hybrid Ln chain of Domain I of β2 fused to Domains II-VI of γ1 in pSK$^+$.*

As with the $\beta2(\gamma1)_I$/cDNA1 construct, three PCR steps were needed to create the hybrid portion of this laminin chain (Figure 8). The first PCR utilized the full-length β2 cDNA as a template to create a 1,128 base pair fragment corresponding to domain I of the β2 chain which could anneal to nucleotides 3886-3906 of domain II of the γ1 chain. In the reaction, the upper primer was 5'-*ttgaggaccctggcaggagaa*tgcagtggagcagcagcagccaca-3'. The italicized letters correspond to nucleotides 3886-3906 of domain II of the γ1 cDNA and the normal characters represent nucleotides 4401- 4421 at the 5' end of domain I in the β2 chain sequence. The lower primer was 5'-tgcttggagacaaggctaaaccctag ggagtggtca-3', which correspond to nucleotides 5507-5472 in the 3' untranslated region of the β2 cDNA. The PCR was run for 20 cycles of 95° C for 1 minute, 65° C for 1 minute, and 75° C for 2 minutes.

Figure 7.

PCRs Performed in Creating the β2(γ1)$_I$Laminin Chain



PCR II product

3'-gggcgttgtggagtcctgcgtctactgcaa ------/

5'-cccgcaacacctcagctgcgtctactgcaa-3'

Primer Ha

PCR III

/———gttggggcgacaccaccggagccat tagtttgacgcgaccctctaa-5'

5'-cccgctgtggccacggta atcaaactgcgctggagatt-------/

PCR I product

Primer H2

3'-ctlgtlccgtggactccgtctctcctagcg-5'

/———gaacaaggcccatgaggcagagaggatcgc-3'

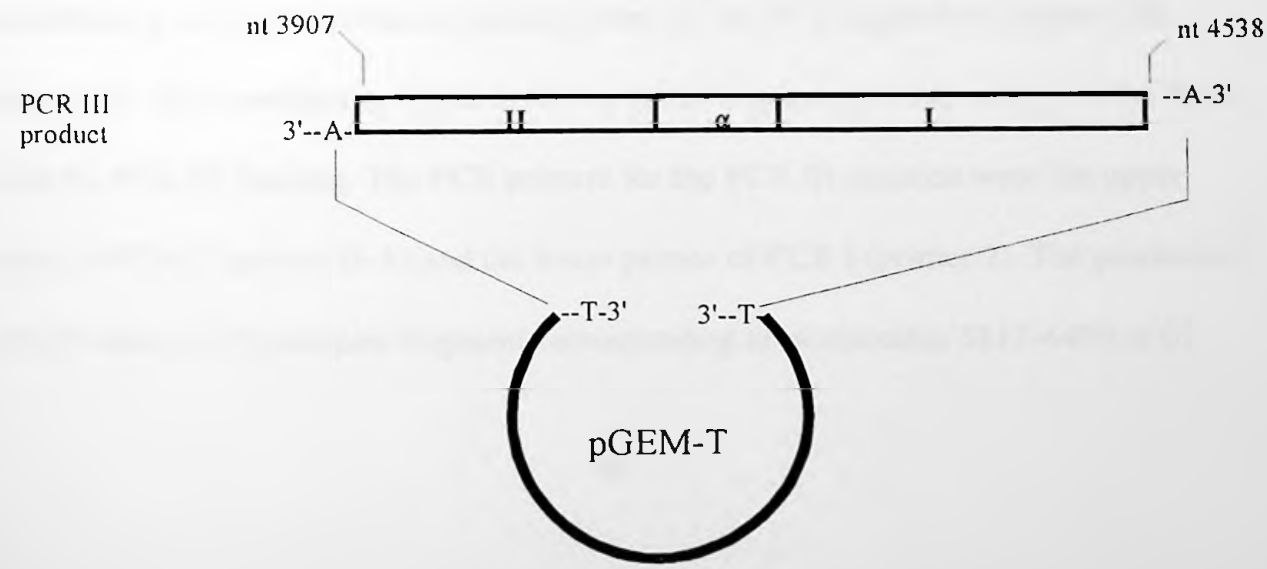
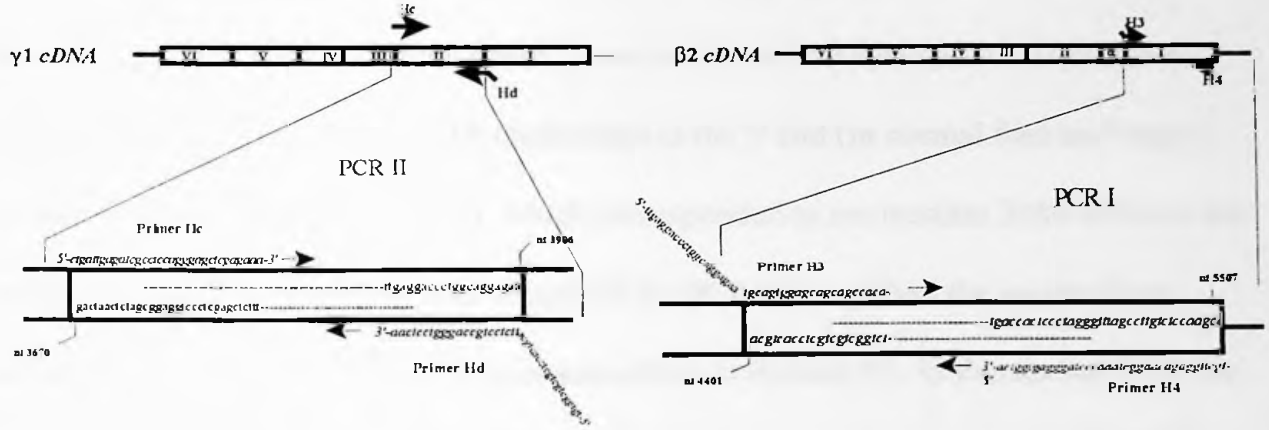


Figure 7. The hybrid laminin chain $\beta2(\gamma1)_I$, which fuses domain I of the γ1 chain to domains α-VI of the β2 chain was created in part by PCR. Shown is a schematic of the three PCRs, which enabled fusion of the two chain fragments. The first PCR (PCR I) amplified the domain I of γ1 (nucleotides 3907-4538) using the cDNA donated by Dr. Y. Yamada as the template. In this reaction the upper primer (primer H1) contained 21 nucleotides, which annealed to nucleotides 3907-3928 of the γ1 cDNA plus 18 nucleotides at the 5' end (in italics and angled off the box representing the cDNA); this corresponded to nucleotides 4380-4400 of the coding strand of the β2 cDNA. The second PCR (PCR II) amplified the region from nucleotide 3907 through 4400 of β2 (corresponding to part of domain II and all of domain α). In this second PCR the lower primer (primer HB) contained 24 nucleotides that annealed to nucleotides 4377-4000 of the β2 cDNA (italics) and 21 nucleotides at it 5' end (in normal font and angled off the box representing the cDNA), which were complementary to nucleotides 3907-3927 of the coding strand of γ1 cDNA. Therefore, the 38 nucleotides at the 5' end of the coding strand of the PCR I product (primer H1 sequences) were complementary to the 38 nucleotides at the 5' end of the non-coding strand of the PCR II product (primer HB sequences). This overlapping region is shown as the sequences in the middle of the figure under the PCR III heading. The PCR primers for the PCR III reaction were the upper primer of PCR II (primer H-A) and the lower primer of PCR I (primer 2). The product of PCR III was a 1217 base pair fragment corresponding to nucleotides 3817-4400 of β2

sequence fused to nucleotides 3907-4538 of the γ1 sequence. This product, shown diagrammatically at the bottom of the figure, was ligated into the pGEM-T vector using the overhanging "A" nucleotide left by the Taq polymerase. Subsequent subcloning to create the full-length hybrid chain is described in *Materials and Methods*.

Figure 8.

PCRs Performed in Creating the γ1(β2)$_1$ Hybrid Laminin Chain



PCR III

Primer IIc

5'-ctgattgagatcgcctccagggagctcgagaaa-3'

3'-gactaactctagcggaggtccctcgagctctt----------/

PCR II product

PCR I product

5'-ttgaggaccctggccaggagaac tgcagtggaacagcagccagccaca----------' /----------tgaccactccctagggttagcctgtctccaagca-3'

3'-acgtcaccctgtcgtcggtct-5'

5'

Primer H4

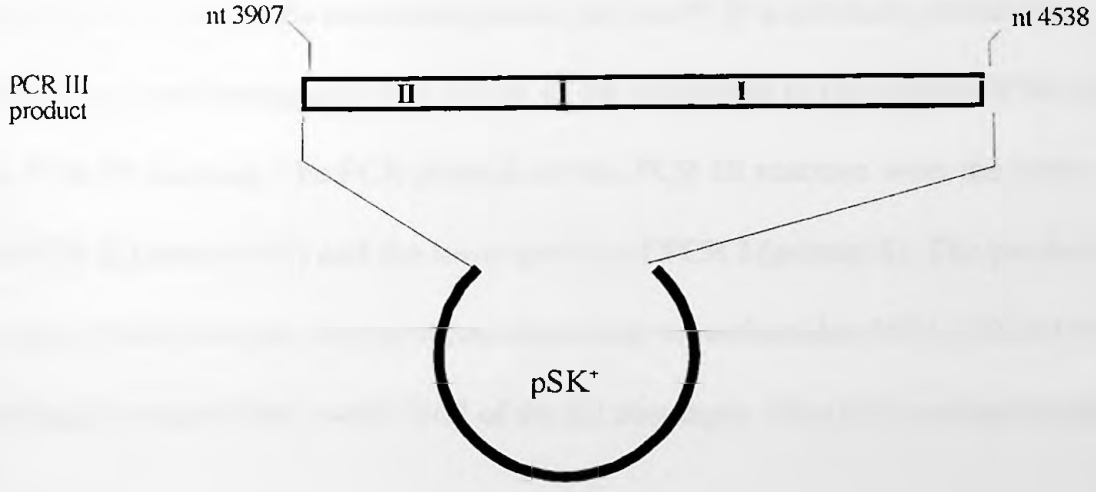


Figure 8. PCR was utilized to create the region which fuses domain I of the β2 chain to domains II-VI of the γ1 chain in the hybrid laminin chain $\gamma 1(\beta 2)_I$. Shown is a schematic of those three PCRs. The first PCR (PCR I) amplified the domain I of β2 (nucleotides 4401-5507) using the full-length β2 cDNA as the template. In this reaction the upper primer (primer H3) contained 21 nucleotides which annealed to nucleotides 4401-4421 of the γ1 cDNA plus 18 nucleotides at the 5' end (in normal font and angled off the box representing the cDNA), which corresponded to nucleotides 3886-3906 of the coding strand of the γ1 cDNA. The second PCR (PCR II) amplified the region from nucleotide 3670 through 3906 of γ1(corresponding to domain II). In this second PCR the lower primer (primer HD) contained 21 nucleotides that annealed to nucleotides 3906-3926 of the β2 cDNA (normal font) and 21 nucleotides at it 5' end (in italics and angled off the box representing the cDNA), which were complementary to nucleotides 4401-4421 of the coding strand of β2 cDNA. Therefore, the 45 nucleotides at the 5' end of the coding strand of the PCR I product (primer H3 sequences) were complementary to the 45 nucleotides at the 5' end of the non-coding strand of the PCR II product (primer HD sequences). This overlapping region is shown as the sequences in the middle of the figure under the PCR III heading. The PCR primers for the PCR III reaction were the upper primer of PCR II (primer HC) and the lower primer of PCR I (primer 4). The product of PCR III was a 1344 base pair fragment corresponding to nucleotides 3670-3906 of γ1 sequence fused to nucleotides 4401-5507 of the β2 sequence. This PCR product (bottom

of the page) was ligated into Sma I - digested pSK$^+$. Creation of the full-length hybrid chain is described in *Methods* section.

The second PCR utilized the full-length γ1 cDNA as a template with 5'- ctgattgaga tcgcctccagggagctcgagaaa-3' as the upper primer. This primer annealed at nucleotides 3670-3702 in the domain II of γ1. The lower primer sequence was 5'- *tgtggctgctgct ccactgca*ttctcctgccagggtcctcaa-3'. The italicized letters correspond to nucleotides 4421-4401 of the β2 sequence and the normal letters correspond to nucleotides 3906-3886 of the domain II of the γ1 sequence. Using Taq polymerase (Promega, Madison, WI) as the PCR enzyme, 20 cycles of 94° C for 1 minute, 62° C for 1 minute, 72° C for 2 minutes were run. This reaction created a 258 base pair product corresponding to a portion of the domain II of the γ1 chain that was able to anneal to the first PCR product.

For the third PCR the first two PCR products were utilized as the template. The first cycle of the PCR functioned to create the proper template. First, equimolar amounts of the PCR products were denatured at 95° C for 3 minutes, then allowed to anneal to one another by cooling to 25° C for 5 minutes. Where a single strand of one PCR product annealed to the complementary end of a strand from the second PCR product, the Pfu polymerase enzyme could synthesize the rest of the complementary strands in the 5' to 3' direction. This was done in the final step of the first cycle at 75° C for 7 minutes. The amplification of a complete double-stranded hybrid product consisting of domain II of the γ1 chain in-frame with domain I of the β2 chain was completed by addition of the upper primer of the second PCR reaction (5'- ctgattgagatcgcctccagggagctcgagaaa-3') and the lower primer of the first PCR reaction (5'-tgcttggagacaaggctaaaccctagggagtggtca-3'). The

final 20 cycles consisted of 95° C for 1 minute, 65° C for 1 minute, 75° C for 2 minutes. The resulting hybrid fragment was 1344 base pairs in length and named $\gamma 1(\beta 2)_I$-PCR. To facilitate subsequent subcloning of this PCR product, it was ligated into the pSK^+ plasmid digested with the restriction endonuclease Sma I, which leaves blunt ends. The plasmid containing the hybrid PCR product within the pSK^+ vector was called $\gamma 1(\beta 2)_I$-PCR/ SK^+.

β2(Δ IV - V) /cDNA3: *Domain IV-V deletion of Ln β2 chain in the CMV promoter-containing mammalian expression vector.*

The first step in creating this construct was to make a PCR fragment corresponding to domains I-III of the β2 chain cDNA. Using the β2 cDNA as a template and the upper primer 5'-ggtaccgaattcccctgccagtgtgac-3' and the lower primer 5'-atcgatgtc gacgctagccaagagctc-3', the region coded by nucleotides 2420-5553 was amplified. 30 cycles of 94° C for 1 minute, 63° C for 30 seconds, 72° C for 7 minutes were done. The product was gel purified, digested with EcoR I and Sal I enzymes and subcloned into the pSK^+ plasmid. The resulting construct was named β2I-III/ SK.

In the second step, the full-length cDNA of β2 was again used in a PCR to amplify the 5' untranslated region, the signal sequence, and domain VI of the β2 chain. The region from nucleotide 1- 917 was amplified using the upper primer 5'-gcgccccggggcggccgga gtgactgctggtc-3' and the lower primer 5'- gggcccgaattcgcggatgacaagttcatagagagc-3'. The DNA was amplified for 30 cycles at 94° C for 1 minute, 65° C for 1 minute, and 72° C

for 2 minutes. This PCR product was gel purified, digested with EcoR I and Not I, and subcloned into the pSK^+ plasmid. The resulting construct was named β2UT-ss-VI/ SK.

To join the two portions of the Ln chain, β2UT-ss-VI/ SK was digested with EcoR I and Not I enzymes, and the insert was separated from pSK^+ plasmid by electrophoresis and gel purification. The upper region, signal sequence and domain VI of β2 was then ligated into β2I-III/ SK plasmid, which had also been digested with EcoR I and Not I. The resulting construct, $\beta2_{(\triangle IV - V)}$ /SK was then digested with Not I and Sal I to remove the β2 sequence and subcloned into the pcDNA3 vector at the Not I and Xho I sites.

$\gamma1(\beta2)_I$-β2up-HA /cDNA1 and $\beta2(\gamma1)_I$-β2up-HA /cDNA1: *Epitope-tagged hybrid Ln chains in the CMV promoter-containing mammalian expression vector.*

The construction of these vectors is diagrammed in Figure 9. Two oligonucleotides, corresponding to a nine amino acid fragment of hemagglutinin (HA) protein recognizable by commercially available HA antibodies, were synthesized by the Marshall University School of Medicine DNA Core Facility. The sequence of the first was 5'-gatcctacccatacgac gtcccagactacgctgag atctccg-3', which corresponds to the nucleotide sequence coding for these nine amino acids plus 5 nucleotides corresponding to consensus site for the EcoR I restriction enzyme. This oligonucleotide was termed "HA-coding". The second oligonucleotide sequence was 5'-aattcgaagatctcagcgtagtctgggacgtcgtatgggtag-3', which corresponds to the complementary

Figure 9.

Construction of the Epitope Tag Vector and Tagged Hybrid Ln Chains

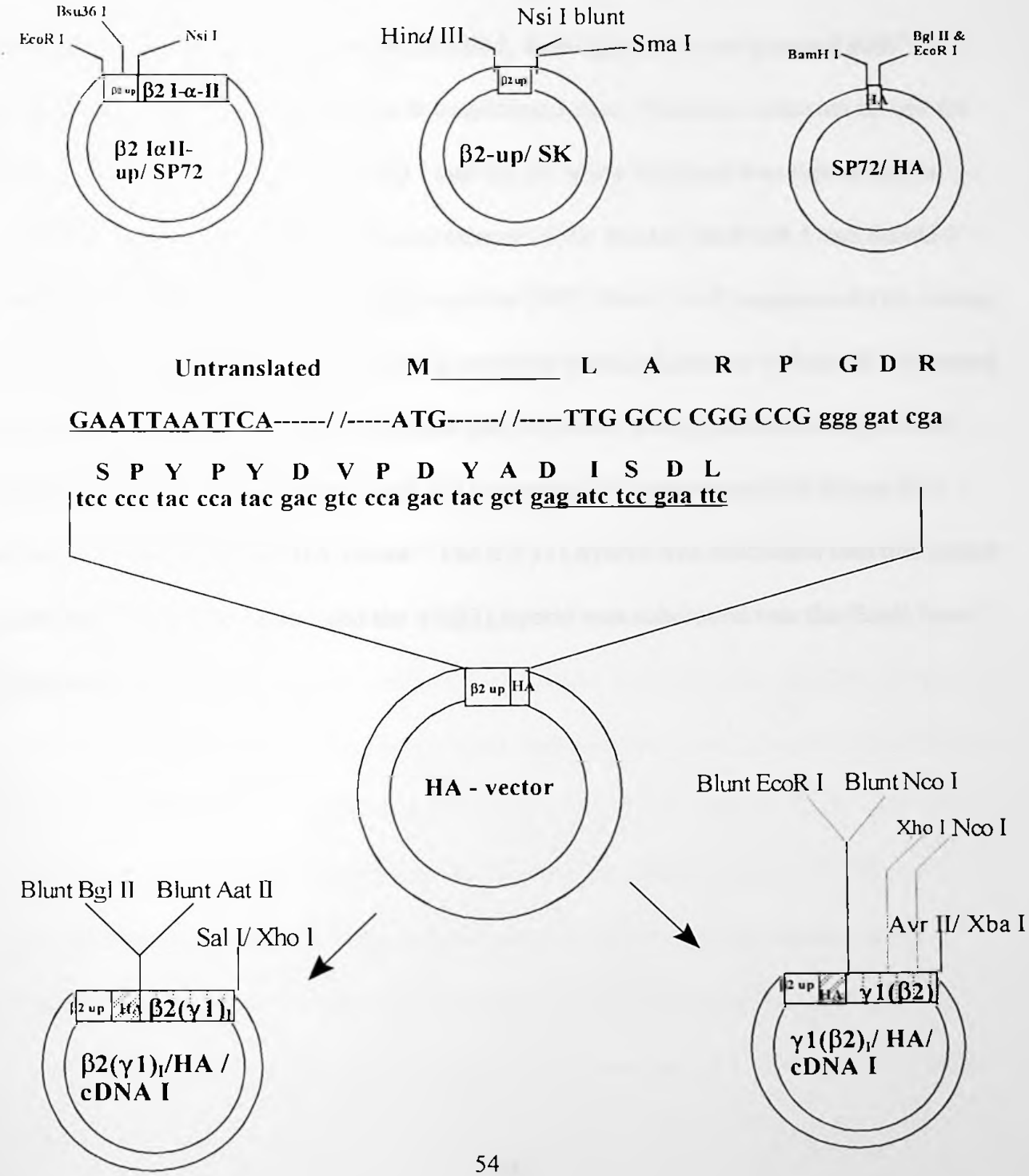


Figure 9. A schematic overview of the construction of the vector used to create epitope-tagged hybrid laminin chains. The β2 upper region was removed from the construct β2 I-α-II-up/ SP72 (not shown) by digestion with Nsi I (followed by blunt-ending with T_4 DNA polymerase) and EcoR I, then ligated into the plasmid pSK^+ (Stratagene, La Jolla, CA) at the EcoR I and Sma I sites. This new construct β2 up/ SK was digested with EcoR I and BamH I and the β2 upper fragment was ligated into a construct containing the HA epitope sequence (SP72/ HA) in the EcoR I and BamH I sites to create the construct named β2-up/ HA/ SP72. Next, the β2-upper and HA coding sequences were removed from the SP72 vector by partial digestion with EcoR I followed by digestion with Hin*d* III. This 254 base pair fragment was ligated into the pcDNAI/ Amp plasmid (Invitrogen, San Diego, CA) to create the construct called β2-up/ HA/ cDNA I, or alternatively "HA vector". The $\beta2(\gamma1)_1$ hybrid was subcloned into this vector at the Bgl II and Xho I sites, and the $\gamma1(\beta2)_1$ hybrid was subcloned into the EcoR I and Xba I sites.

bases of the HA coding region and also contains 5 nucleotides comprising the consensus site of the BamH I restriction enzyme. It was termed "HA complementary". To achieve a double stranded DNA capable of being ligated into a plasmid, the two oligonucleotides were annealed using the following conditions. Approximately 2 μmoles of HA coding and HA complementary were added to a 0.5 ml microcentrifuge tube containing 2.0 μl T_4 DNA ligase buffer [300 mM Tris HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP] (Promega, Madison, WI) and brought to a final volume of 20 μl with H_2O. This tube was capped and placed in a 9600 Thermocycler (Perkin Elmer- Cetus, Foster City, CA) and heated at 95^0 C for 2 minutes followed by cooling at a rate of 3^0 C per minute until a temperature of 50^0 C was reached. Once at 50^0 C the tube was removed and placed at room temperature for 2 hours and finally stored a 4^0 C for later use.

This double-stranded oligonucleotide was ligated into pSP72 vector (Promega, Madison, WI). EcoR I-digested pSP72 and the double-stranded HA oligonucleotide were incubated for approximately 4 hours at 16^0 C, and the T_4 DNA ligase was inactivated by heating to 70^0 C for 15 minutes. Next, the salt concentration was adjusted to 150 mM by addition of 1 µl of 1 M NaCl. Then 2 µl of BSA ($10\ ^{mg}/_{ml}$) and 1.0 µl BamH I enzyme were added and the DNA was digested for approximately 2 hours at 37^0 C. The reaction products were separated by electrophoresis on 0.7% agarose, which separated the linearized ligated species from unligated or concatamerized HA oligonucleotides. Linearized DNA was cut out from the gel and extracted from the agarose. This linearized,

extracted product was again ligated to yield circular plasmid DNA, which was transformed into competent XL-1 Blue bacteria. Colonies were selected on LB-ampicillin plates and 12 colonies were cultured for use in plasmid isolations. The plasmids were checked for presence of HA insert by digestion with Dde I, which has one site in the HA oligonucleotide sequence. This means that compared to pSP72 digested with Dde I, an extra band was seen in insert positive DNAs. In this case, the 686 base pair fragment (one of six seen in the pSP72 DNA digestion) is cleaved into two smaller fragments of 540 and 165 base pairs. Note that the smaller two fragments do not add up to 686 bp. This is due to the difference of 19 base pairs between the amount of DNA inserted (46 bp) and the amount of DNA cut out by the EcoR I- BamH I digestion (23 bp).

When the DNA from one of these positive colonies was sequenced, it contained a truncated HA coding region that had only 28 out the 51 bp that were HA sequence. For this reason all other putative HA DNAs were digested with XbaI and EcoRI, which cut out the multiple cloning site of pSP72 plus any inserted DNA. These digestion reactions were run on 6 % Tris-Borate-EDTA (TBE) polyacrylamide gel to detect several base pair differences in insert sizes. Compared to the known truncated positive, DNA from five clones appeared to be the proper size. This was confirmed by sequencing; two DNAs were intact full length HA in pSP72. The positive clones were termed pSP72/HA.

The next step involved placing the upper region of the β2 laminin chain (which contained the signal sequence) into the pSP72/HA construct. This was done before the

hybrid Ln chains were inserted because the enzymes used in this step would digest the hybrid Ln chains. Furthermore, a new construct containing the signal sequence of the β2 chain in-frame with the HA-tag sequence could be used repeatedly to epitope-tag genes of interest and allow them to be targeted to the endoplasmic reticulum for processing.

The upstream region of β2 was generated by digesting the plasmid β2 IαII-up/ SP72 (described above) with Nsi I, creating a blunt end using T_4 DNA polymerase, and digesting with EcoR I. The β2 upper region was separated by electrophoresis on 1.0% agarose, excised, and extracted from the agarose. This was ligated to SK^+ (Stratagene, La Jolla, CA), which had been digested with EcoR I and Sma I. The heat inactivated ligation reaction was transformed into XL- I Blue, and plasmid DNA was prepared from twelve colonies on LB-amp plates. These DNAs were screened for presence of insert by restriction digestion with Bsu36 I and visualization of DNA bands on a 1.0% agarose gel. One positive clone was sequenced and termed β2up/SK.

The upstream/signal sequence of β2 laminin was removed from the β2up/SK construct by digestion with EcoR I and BamH I. This 203 base pair fragment was separated from the 2.96 kilobase SK^+ plasmid, extracted from 1.0 % agarose, and ligated into the pSP72/HA DNA, which had also been digested with EcoR I and BamH I. Correctly ligated HA + β2 upper DNAs were determined by Bsu36 I digestion, which yielded a 3 kilobase linear DNA band in the case of insert-containing constructs. This new plasmid was called SP72/HA-β2up. Sequencing of this plasmid revealed that the HA

epitope portion was not in-frame with the signal sequence portion of the construct.

To place the upper region and HA epitope into the expression vector, the pSP72/HA-Sup plasmid was partially digested with EcoR I enzyme, then cleaved with Hin*d* III. A fragment of 254 base pairs in size, which corresponded to the size of the upper region and HA epitope, was removed from the gel and ligated into pcDNA I/ Amp (Invitrogen, San Diego, CA), which was also digested with EcoR I and Hin*d* III. DNAs from six colonies were tested for the presence of insert by digesting with Bsu 36 I, which yielded a 5 kilobase linear DNA for β2up-HA- positive constructs. The one DNA containing the insert was called β2up-HA/cDNA1.

Two changes were deemed necessary to keep the β2 signal sequence in-frame with the HA epitope fragment. First, β2up-HA/cDNA1 was digested with BamH I, ethanol precipitated, resuspended in TE, and subsequently treated with the Klenow fragment of DNA polymerase I (Promega, Madison, WI), which filled in the restriction site 5' overhangs. The Klenow enzyme was inactivated by heating at 75° C for 25 minutes. Next, 5 Units of T_4 DNA ligase (Promega, Madison, WI) plus ligase buffer were added and the ligation reaction was allowed to proceed overnight at 4° C. After initial transformation two plasmids that were not digested by BamH I enzyme were found and isolated. Next, this β2up-HA/cDNA1, with the filled-in BamH I site, was digested with Xmn I (a site which was created when the β2 upstream region ligated to the HA epitope-tag sequence), and filled in with the Klenow fragment enzyme. Religation yielded a circular plasmid,

and sequencing confirmed that the β2 upper region (containing the start site) was now in-frame with the HA coding region.

To facilitate cloning at the EcoR I site at the 3' end of the HA epitope sequence, a second EcoR I site at the 5' end of the upper region of the corrected β2up-HA/cDNA1 was removed. This was done by digesting the β2up /SK plasmid with EcoR I, heat inactivating, and blunt-ending with the Klenow fragment of DNA pol I (Gibco BRL, Gaithersburg, MD). This linear molecule was ligated into a circular plasmid using T_4 DNA ligase. DNAs which were not cleaved by EcoR I were isolated. The new plasmid, named β2up$^{\Delta Eco}$/SK was digested with Hin*d* III and Bsu36 I. A 131 base pair fragment was extracted and ligated into β2up-HA/cDNA1, also digested with Hin*d* III and Bsu 36 I. The result of this cloning strategy was an expression vector that contained the upper region and signal sequence of the laminin β2 chain attached to (and in-frame with) the HA epitope. The 3' end of the HA epitope sequence contained a Bgl II site and an EcoR I site, which allows for the cloning of cDNA in 2 of the 3 possible open reading frames.

The γ1(β2)$_1$ hybrid Ln chain cDNA was introduced into the β2up-HA/cDNA1 vector. γ1(β2)I/SK (described earlier) was digested with Nco I, and the 5' overhang was filled-in with the Klenow fragment of DNA polymerase I (MBI Fermentas, Amherst, NY). The β2up-HA/cDNA1 vector was digested with EcoR I and filled in with the Klenow fragment of DNA polymerase I. The two linear DNAs were mixed and ligated by T_4 DNA ligase in an overnight incubation at 4° C. After heat inactivation, transformation

of the ligation reaction and plating of transformed bacteria on selective medium, DNA from 20 colonies was digested with Xmn I to yield fragments of 3329, 1898, 1400, 1283, 848 and 166 base pairs that were the correct sizes for insert positive DNA. Sequencing of one positive clone revealed that the cDNA for the $\gamma 1(\beta 2)_I$ chimera was in-frame with the signal sequence and HA epitope tag, but the Nco I digestion removed the 3' most 677 nucleotides of β2 I domain. To complete the full-length hybrid DNA, $\gamma 1(\beta 2)_I$ /SK was digested with Xho I and Avr II, which removed a 1,292 bp fragment corresponding to nucleotides 3694-3905 of the γ1 sequence and nucleotides 4400-5481 of the β2 domain I sequence. This fragment was ligated to Xho I - Xba I digested $\gamma 1(\beta 2)_I$ /cDNA1/ β2up-HA to create the full-length hybrid. Transformation, selection and culture of bacteria were done as before. Twelve transformed colonies were screened by plasmid isolation and Bgl II restriction digestion, which generated three fragments (5.4 kilobases, 3.6 kilobases and 1, 028 base pairs) for insert-containing DNAs. Five DNA clones were putatively positive and named $\gamma 1(\beta 2)_I$ /cDNA1/ β2up-HA, or $\gamma 1(\beta 2)_I$-HA for short. Sequencing the C-terminal end of the molecule revealed that the Avr II site had indeed annealed to the Xba I site and that the sequence of the β2I inserted, including the stop codon, was correct.

The introduction of $\beta 2(\gamma 1)_I$ hybrid LN chain cDNA was achieved by digesting the $\beta 2(\gamma 1)_I$/SK construct (described elsewhere) with Aat II, removing the 3' overhang of the Aat II site with T_4 DNA polymerase, and digesting with Sal I. This separated the $\beta 2(\gamma 1)_I$ cDNA, excepting the β2 upper region, from the SK^+ vector. The β2up-HA/cDNA1

construct was digested with Bgl II, treated with Klenow fragment of DNA Pol I to fill-in the 5' overhang, and digested with Xho I, which forms a complementary 5' overhang with Sal I -digested DNA. To ligate the Aat II- SalI -digested $\beta2(\gamma1)_I$ to Bgl II-Xho I -digested β2up-HA/cDNA1, the two DNAs were mixed with 2.0 μl of 10X ligase buffer (Promega, Madison, WI), 0.5 μl of 50 mM Hexamine Cobalt Chloride, and 1.1 μl (6 U) of T_4 DNA Polymerase enzyme (Promega, Madison, WI). To aid in the annealing of the adhesive ends, the reaction was placed at 4° C for 15 minutes, then incubated at room temperature (approximately 20° C) for 5 hours. Heat inactivation was achieved by heating to 75° C for 15 minutes. Plasmid extraction and restriction digestion with Xba I enzyme was carried out on 10 clones. Three of these clones generated restriction fragments of 6.6 , 3.0 and 1.0 kilobases which indicated the presence of inserted $\beta2\ (\gamma1)_I$ DNA. One of these clones was sequenced at the 5' and 3' ends and found to be both in-frame with the β2 upper-HA epitope sequence and full-length hybrid chain. This clone will be referred to as $\beta2(\gamma1)_I$-β2 up-HA/cDNA1 or just $\beta2(\gamma1)_I$-HA.

Yeast transformation

The yeast strain used was SFY526, which was provided in the Clontech Matchmaker™ kit. For all transformations, one 2-3 mm colony from a YPD (20 $^g/_L$ Difco peptone, 10 $^{mg}/_L$ yeast extract, 2 % dextrose) plate was used to inoculate 20 ml of YPD medium. This culture was incubated at 30° C overnight with vigorous shaking to attain a

static culture. From this culture an aliquot was added to 300 ml of YPD medium in a 1 L Erlenmeyer flask to achieve an OD_{600} of between 0.2 and 0.3, which generally required about 15 ml of the overnight culture. This culture was incubated with vigorous shaking at 30°C for 3 hours. Cells were then pelleted by centrifugation for 5 minutes at approximately 1,500×*g*. The medium was decanted and the cells were resuspended in 50 ml of reagent grade water. The cells were spun again for five minutes at 1,500×*g* and the supernatant removed. Cells were then resuspended in 1.5 ml of freshly prepared 1× TE/LiAc (0.01M Tris-HCl, 1mM EDTA, 0.1M lithium acetate, pH 7.5).

For the transformation, 100 ng of each construct (one activation and one binding domain hybrid) and 100 ng of carrier DNA (herring testis DNA; Clontech Palo Alto, CA) were added to a 1.5 ml microcentrifuge tube along with 100 µl of competent cells. Next, 600 µl of freshly prepared PEG/LiAc solution (40% polyethylene glycol [M.W. 3,350] in TE/LiAc) were added to each transformation tube and the contents mixed by inversion. The tubes were then incubated at 30°C for 30 minutes with shaking. After incubation, 70 µl of 100% DMSO were added and the tubes were incubated at 42° C for 15 minutes. The cells were briefly placed on ice, then pelleted by centrifugation at 14,000 rpm in a microcentrifuge. After removal of supernatant liquid, the cells were resuspended in 0.5 ml 1× TE buffer (10 mM Tris-HCl, 1 mM EDTA). Finally, 250 µl of transformed cells were plated onto a synthetic dextrose (SD) [6.7 $^{g}/_{L}$ Difco yeast nitrogen base without amino acids, 2% dextrose, 30 $^{mg}/_{L}$ L-isoleucine, 150 $^{mg}/_{L}$ L-valine, 20 $^{mg}/_{L}$ adenine hemisulfate

salt, 20 $^{mg}/_{L}$ L-arginine HCl, 20 $^{mg}/_{L}$ L-histidine HCl monohydrate, 30 $^{mg}/_{L}$ L-lysine HCl, 20 $^{mg}/_{L}$ L-methionine, 50 $^{mg}/_{L}$ L-phenylalanine , 200 $^{mg}/_{L}$ L-threonine, 30 $^{mg}/_{L}$ L-tyrosine, 20 $^{mg}/_{L}$ L-uracil] plate lacking both tryptophan and leucine (-leu, -trp) to select for yeast that were transformed with both vectors. These plates were incubated 3-6 days at 30°C.

Beta-galactosidase assay

A single colony of transformed yeast, 2-3 mm in diameter (or several smaller colonies which comprised the same mass), from SD (-leu, -trp) plates was used to inoculate 10 ml of SD (-leu, -trp) medium and grown overnight at 30° C to yield a static culture. The next day 2 ml of this culture was added to 8 ml of YPD medium and grown for approximately 3 hours to achieve a culture in logarithmic growth. The OD_{600} for this culture was measured and recorded. Next, 1.5 ml of the culture were placed in a microcentrifuge tube, then pelleted, washed once with 0.5 ml of Z buffer and resuspended in 0.3ml of Z buffer (63mM Na_2HPO_4, 10mM KCl, 2mM $MgSO_4$; pH 7.0). This aliquot was divided into 3 tubes with 100 µl each and the assays were performed in triplicate for each transformant. Cells were next frozen for 1.5 minutes in liquid nitrogen, then thawed quickly at 37°C to lyse the yeast. Subsequently, 600 µl of Z buffer + β-mercaptoethanol (0.27%) were added. At this point a timer was started and immediately 160 µl of o-nitrophenylpyranogalactoside (ONPG) [Sigma, St. Louis, MO) at a concentration of 4 $^{mg}/_{ml}$ in Z-buffer were pipetted into the tubes. Tubes were gently inverted to mix and then

incubated at 30° C for 1.5 - 6 hours until a yellow color developed. In some cases reactions were allowed to proceed overnight to allow possible weak interactions to occur. Reactions were stopped by adding 0.4 ml of 1 M $NaHCO_3$ (Fisher Scientific, St. Louis, MO), and the tubes were centrifuged for 10 minutes to remove cellular debris. The supernatant was then transferred to a cuvette and the OD_{420} was determined on a spectrophotometer. Beta-galactosidase activity was then determined using the formula: β-gal units = $1000 \times OD_{420} / (t \times v \times OD_{600})$, where t = time in minutes, v = 0.1 × concentration factor (usually 5). Values were then normalized for daily differences in the assay by dividing by a positive control interaction TD1 + VA3. The data is represented as a percentage of this control.

In one experiment yeast were lysed via the glass bead disruption method (Dunne and Wobbe 1988) to determine if the freeze-thaw disruption method was yielding sufficient enzyme for assay. For these lysates, a liquid β-galactosidase assay was also performed on 100 µl of sample extract from each transformant using the above protocol. The number of β-galactosidase units was calculated using the equation: specific activity = $(OD_{420} \times 1.36) \div (0.0045 \times \text{protein conc.} \times \text{extract vol.} \times \text{time})$ [Rose et al.1990]. For this equation, 1.36 is a correction factor for the volume of the reaction and 0.0045 is the OD_{420} of a 1 $^{nmole}/_{ml}$ solution of o-nitrophenol. The values for this assay were divided by the VA3+TD1 positive control value to give a relative value to compare among different assays. The relative values among transformants for assays of the glass bead disruption

extracts showed no difference from those of the freeze-thaw method outlined above. This observation assured us that the extracts used in detection could indeed give an accurate representation of hybrid interaction as well as hybrid protein expression.

Statistical Analysis

To assess differences among β-galactosidase assay results from a set of samples, a one-way analysis of variance was performed. Statistical comparison between pairs within a set of samples was performed by a pairwise Student-Neuman-Keuls *post-hoc* test. All analyses were performed using SigmaStat™ statistical software from Jandel Scientific. Statistical significance was determined at the $p < 0.05$ level.

Detection of Hybrid Expression

One colony of approximately 2-3 mm in size or an equivalent mass of smaller colonies of transformed yeast from selective (-leu, -trp) plates was inoculated in 5 ml of SD(-leu, -trp) medium, vortexed briefly to eliminate clumps, and grown overnight at 30° C with vigorous shaking. The next day 2 ml of this culture were added to 8 ml of YPD medium in 50 ml conical tubes and incubated at 30° C for 3 hours. At the end of the incubation 1 ml was removed and the OD_{600} was measured to allow a relative comparison of cell density among the cultures. The remaining 9 ml were pelleted by centrifugation at approximately 1,500 ×*g* in a Beckman GS-6R centrifuge. The medium was aspirated and

the pelleted cells were resuspended in 100 µl of glass bead disruption buffer (20 mM Tris-HCl, pH 7.9; 10 mM $MgCl_2$; 1 mM EDTA; 5% glycerol; 1 mM DTT; 0.3 M ammonium sulfate; 1 mM PMSF; 2 $^{\mu g}/_{ml}$ aprotinin; 1 $^{\mu g}/_{ml}$ pepstatin A; 0.5 $^{\mu g}/_{ml}$ leupeptin). This yeast cell suspension was then frozen overnight a -20° C. After thawing, 200 µl of glass bead disruption buffer and 400 mg of 0.45 mm acid-washed glass beads (Sigma, St. Louis, Mo) were added to the tube. To lyse the yeast, this mixture was vortexed vigorously 10 - 12 times for 30 seconds each with at least 2 minutes incubation on ice between vortexing. Approximately 10 µl from each of two of the sample lysates were checked for cell breakage under a light microscope. This technique yielded about 90 % cell breakage. After the last vortex step, the glass beads were allowed to settle out of the buffer for approximately 5 minutes. Next, the supernatant lysate was carefully removed from the beads, 200 µl of glass bead disruption buffer was added to the left-over beads, and the tube was gently inverted several times to remove residual lysate. Once the beads had settled, the resulting supernatant was again removed and combined with the first supernatant. This lysate was centrifuged at approximately 14,000 ×*g* to remove cellular debris. The resulting supernatant was removed and this cleared lysate was used for the protein assay and western blot.

Protein concentration of each of the transformed yeast cell extracts was determined using the Bradford reagent (Bio-Rad, Richmond, CA). For each sample, 2 µl of extract was removed, placed into a microcentrifuge tube with 10 µl of 0.1 M NaOH and allowed

to stand at room temperature for 5 minutes. Next, 790 µl of reagent grade H_2O and 200 µl of Bradford reagent were added to each sample tube and again allowed to stand for 5 minutes. The full contents of each tube were subsequently added to separate 2 ml disposable spectrophotometric cuvettes and the OD_{595} was measured. A standard curve for the assay was calculated using known concentrations of bovine IgG from 1 $^{\mu g}/_{\mu l}$ - 20 $^{\mu g}/_{\mu l}$. From the graph of the standard curve, a linear regression was calculated using SigmaStat© statistical software, which allowed protein concentrations of samples to be extrapolated. Alternatively, a Beckman model DU650 spectrophotometer was used, and preloaded software calculated these values. Expression of hybrid proteins was determined by western blot using equal amounts of protein (~ 100 µg) from each transformant extract.

Mammalian Cell Transfection

The human embryonic kidney cell line HEK 293 (ATCC, Rockville, MD) was used to create both stably and transiently expressed Ln chains. No Ln chains were detected in western blots of these cells using available antibodies that recognize the Ln trimer or specific Ln chains. Other laboratories have shown the same results (Yurchenco et al. 1997).

The HEK 293 cells and the stable-transfected cell lines derived from the HEK 293 cells initially showed poor adhesion in the low serum Opti-MEM® medium and therefore the following protocol was adopted. The day before transfection the medium from a 10

cm dish of confluent, or nearly confluent, HEK 293 (or derived cell lines) cells was removed and replaced with 12 ml of fresh 293 medium (DMEM + 10 % heat inactivated horse serum), then the cells were detached from the plate by trituration of the medium, using a glass tissue culture pipette. A 2.5 ml volume of the removed cells was aliquoted onto 60 mm tissue culture plates coated with collagen type I (rat tail collagen; Collaborative Research, Bedford, MA). To coat the plates, 80 µl of a 100 µg/ ml solution of rat tail collagen in sterile water was added to the dish and spread using the plastic pipette tip. Excess solution was removed. This solution was allowed to dry, then the plate was washed twice with 0.7 ml of 1× PBS and allowed to dry again. Cells suspended in medium were added to this plate. After transfer to 60 mm dishes the cells were replaced in the tissue culture incubator overnight.

For both stable and transient transfections the Lipofectin® cationic lipid reagent (Life Technologies, Grand Island, NY) was used as per manufacturer's directions with modifications made empirically to optimize conditions. For most transfections, 6 µg of plasmid DNA were added to 200 µl of Opti-MEM® medium pre-warmed to 37° C in a 3 ml plastic test tube. Into a second test tube, 15 µl of Lipofectin® reagent and 200 µl of 37° C Opti-MEM® medium were added and allowed to stand for 30 minutes. The entire contents of the first tube were added drop-wise to the second tube, and the mixture was stirred gently by tapping the side of the tube. This mixture was then allowed to incubate in the tissue culture hood at room temperature for 15 minutes. Subsequently, 1 ml of

warm Opti-MEM® was added to the mixture tube, and the entire contents were pipetted onto a 60 mm tissue culture dish containing an approximately 80 % confluent growth of HEK 293 cells. Prior to adding the transfection mixture, the plate had already been rinsed with 0.5 ml of Opti-MEM®, which was removed, and 1 ml of prewarmed Opti-MEM® medium was added. This plate with the transfection mixture was then placed overnight in a tissue culture incubator at 37° C, 5 % CO_2 and 95 % humidity. The next afternoon (about 16 hours later), the Opti-MEM® transfection medium was removed and replaced with standard 293 medium (DMEM + 10 % heat inactivated horse serum). For most transient transfections, this plate was incubated in the tissue culture incubator for 48 hours.

Stable Transfection and Characterization of Laminin Chain Expressing Cell Lines.

To create cell lines which could constitutively express both the β2 and γ1 Ln chains, HEK 293 cells were transfected with the β2 /cDNA1 and γ1 /cDNA1 expression vectors, using the transfection protocol above. As a selectable marker for the transfected cells the SV2-Neo vector, which conferred Neomycin (or the synthetic analog G418) resistance, was added to transfected DNAs. The following amounts of the three DNA plasmid vectors were used: 25.6 μg, 24.7 μg and 1.73 μg of β2 /cDNA1, γ1 /cDNA1 and SV2-Neo, respectively, which represented a 5:5:1 molar ratio.

After incubating overnight, the Opti-MEM® medium with DNA was removed and

replaced with 3 ml of fresh 293 medium, and this plate was placed back into the incubator for three days. At this time the medium was replaced with 2 ml of fresh 293 medium, and the cells were removed from the plate by trituration using a plastic pipette tip. The resuspended cells were then aliquoted, 400 µl each, into five 150 mm tissue culture plastic dishes, each containing 15 ml 293 medium plus 500 $^{\mu g}/_{ml}$ G418. This achieved a low density of cells that could then be isolated as clones. These plates were incubated in the tissue culture incubator for 28 days with medium refreshed after the first two weeks, then weekly. After 28 days, 12 colonies were removed from the five plates. This was achieved by removing the medium from the 150 mm plate, then soaking a small autoclaved circular piece of filter paper (the size of a hole punch) in 0.05% trypsin- 0.53 mM EDTA (Gibco BRL, Gaithersburg, MD) and placing it on top of a previously identified and marked clone. The filter papers were quickly removed in a wiping motion with sterilized tweezers, and each was placed in one well of a 24 well tissue culture dish containing 293 medium plus 500 $^{\mu g}/_{ml}$ G418. Three days later this procedure was used to remove twelve more clones. Shortly thereafter, the cells of the 150 mm plates were too numerous to isolate as individual clones, so the remaining cells were removed as a population and frozen down for storage in liquid nitrogen.

After placement into the 24 well plates, the cloned cells were cultured for one to two weeks with medium changes every three or four days. When an individual clone in its well became confluent as judged by light microscopy, the cells were removed from the

well by trituration in fresh medium and transferred to a 60 mm tissue culture dish containing 2.5 ml of 293 medium plus 500 μg/ ml G418. Plates also contained several circular glass coverslips coated with rat tail collagen, which were used later for immunofluorescent detection. Expression levels of Ln β2 within individual clones of transfected HEK 293 cells were determined using either the C4 or D19 monoclonal antibody. These antibodies were raised against the β2 chain and they recognize epitopes in domain I or domain III, respectively. Expression of the γ1 chain was detected by the polyclonal serum YY13 (gift of Dr. H. Kleinman, NIH). Visualization of these antibodies was achieved by addition of a 1:200 dilution of fluorescein-conjugated goat, anti-mouse IgG secondary antibody (Sigma, St. Louis, Mo) or rhodamine-conjugated goat, anti-rabbit IgG secondary antibody (Boeringer-Mannheim, Indianapolis, IN), respectively. Therefore, cells which expressed both Ln chains would emit a red (590 nm) signal when a 570 nm incident light was used and a green (518 nm) signal for a 494 nm incident light, when visualized by a fluorescent microscope. Transfer to 24 well plates and the low adhesion of these cells plus the added time of exposure to G418 caused the demise of about half of the 24 original clones. Thus, less than 10 went on to be effectively stained. From this group, six (A-1, A-2, B-4, C-2, D-2 and D-4) showed good enough expression of one or both chains to be immunoblotted. Western blot results showed that two of the cell lines (A-1 and C-2) had high levels of both β2 and γ1 chains, one cell line (A-2) contained only β2, and a fourth cell line (D-4) expressed only γ1. These four stable cell

lines were utilized in subsequent transfection and immunoprecipitation experiments.

Mammalian Cell Lysis

Cultured 293 cells were removed from the incubator and placed on ice. The medium was removed and the cells were rinsed twice with cold 1X PBS (1 ml for 10 cm plates and 0.5 ml for 60 mm plates). After removal of the PBS the cells were lysed in cold RIPA lysis buffer (50 mM Tris, 150 mM NaCl, 10 mM EDTA, 0.5 % Na Desoxycholate, 1.0 % NP-40, 0.1 % Na dodecyl sulfate, 0.1 $^{mg}/_{ml}$ PMSF). A 300 µl volume was added to 60 mm plates and 900 µl was added to 10 cm plates. After a 2 -3 minute incubation on ice, cells were removed using a plastic Falcon® cell scraper (Becton-Dickinson, Lincoln Park, NJ), and the lysate was transferred to a 1.5 ml centrifuge tube. 100 µl of RIPA were added to the plate to remove any residual cells, which were then pooled with the original lysate. The protease inhibitors aprotinin (2 $^{\mu g}/_{ml}$) and leupeptin (0.5 $^{\mu g}/_{ml}$) were added to the cell lysate tube on ice and then gently mixed. Next, the cellular debris was pelleted by a 4 minute centrifugation at approximately 12,000×*g* in a microcentrifuge at room temperature. The cleared supernatant was the cell lysate used in subsequent western blots and immunoprecipitations. These cell lysates were often frozen at -20° C for later use.

Western Blot

This protocol is based on that of Laemmli (1970). Cells were lysed and protein concentrations were determined as previously described. A common volume of lysate or protein sample was 40 µl. Prior to loading the sample into a well, 10 µl of 5× sample buffer (50 % glycerol, 25 % 2-mercaptoethanol, 0.6 M Tris, 0.6 M sodium dodecyl sulfate, 0.1% bromophenol blue) were added, then the sample was boiled for 3 - 5 minutes and centrifuged for 10 seconds in a bench-top microcentrifuge. Polyacrylamide gel electrophoresis was carried out using a vertical electrophoresis apparatus, at a current setting of approximately 10 mV, and allowed to run overnight (approximately 16 hours). The next morning the current was increased to approximately 30 mV and run an additional hour after the bromophenol blue (BPB) tracking dye ran out the bottom of the gel.

All acrylamide gels were made as described by Harlow and Lane (1988). The acrylamide concentration differed according to which recombinant molecules or cell lysates were examined, but generally a 6 % acrylamide gel (29:1 ratio of acrylamide to N,N-methylene bis-acrylamide) was used when separating full length laminin chain proteins, and a 10 % gel was utilized for blots of yeast lysates containing the two-hybrid fusion proteins. For all denaturing protein gels, a 5% stacking gel was poured atop the running gel with a comb inserted to create sample wells.

After the samples were separated by electrophoresis, the gel, after marking to

denote the molecular weight marker lanes, was equilibrated in glycine transfer buffer (12.5 mM Tris-base, 100 mM glycine, 20% methanol). A sandwich of nitrocellulose membrane and the polyacrylamide gel between two layers of blotting paper was placed into the transfer apparatus containing glycine transfer buffer. The proteins were transferred to nitrocellulose membrane for 1.4 hours at 0.4 amperes.

The cassette was removed from the transfer apparatus, and the nitrocellulose membrane was separated from the gel and blotting paper. Next, the molecular weight marker lane was cut off from the rest of the membrane using a clean razor blade. This portion of the membrane was stained with 0.5 % Ponceau Red protein stain. The rest of the membrane containing the protein samples was placed into a Seal-A-Meal plastic bag (Rival, Clinton, MO) with 5 % non-fat dry milk in Tween-Tris-Saline [T-T-S] (15 mM NaCl, 200 mM Tris, 200 mM EDTA [pH 8.0], 0.05% Tween-20) as a blocking reagent. The membrane was incubated in this solution for 30 minutes. Membranes that were to be stained with the polyclonal antibody YY15 also had normal mouse serum (NMS) at 1:200 dilution added to this blocking step to reduce background levels during the detection step.

After the blocking step, the milk was discarded and the primary antibody was added in 2.5 % non-fat dry milk. In the case of the monoclonal antibody (mAb) D5, the dilution was either 1:1,000 from ascites or 1:2 from D5 culture medium. For the mAb to hemagglutinin, anti-HA (BAbCo, Berkeley, CA), the polyclonal antisera R49 (anti-Ln $\beta 2$), YY15 and YY13 (the latter two being anti-Ln $\gamma 1$), the optimal dilution was 1:3,000

in 5 % non-fat dry milk. The anti-GAL4 binding domain (anti-GAL-DBD) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was used at a dilution of 1:2,000 in 5 % non-fat dry milk. The membrane was incubated in the primary antibody solution in one of three incubation conditions: one hour at 37° C, 2.5 hours at room temperature, or overnight at 4° C. All incubation conditions produced approximately equal outcomes. At the end of the incubation time, the membrane was removed from the primary antibody solution and washed three times with T-T-S for 10 minutes each.

The secondary antibodies were conjugated to horseradish peroxidase enzyme (HRP), which permitted detection using chemiluminescent substrate for the HRP enzyme. When the primary antibody was a monoclonal antibody the secondary antibody was rabbit anti-mouse IgG (HRP-Rb-anti-MoIgG)[Santa Cruz Biotechnologies, Santa Cruz, CA]. If the primary antibody was a polyclonal antibody, the secondary antibody was goat anti-Rb IgG (HRP-G-anti-RbIgG)[Sigma Co., St. Louis, Mo]. The dilution for the HRP-Rb-αMo -IgG antibody was 1:3,000 in 5 % non-fat dry milk. For the HRP-G-αRb-IgG antibody the dilution was either 1:4,000 or 1:5,000 in 5 % non-fat dry milk. These concentrations were empirically found to yield strong signal and minimize background during detection.

The detection of secondary antibody utilized the ECL chemiluminescent system (Amersham, Arlington Hts., IL), following manufacturer's instructions. Equal volumes of solutions 1 and 2 were mixed shortly before use, then poured evenly over the protein-blotted side of the nitrocellulose membrane. This solution was allowed to stand for one

minute then removed, and the membrane was wrapped in cellophane or enclosed in a Seal-A-Meal® bag. The wrapped membrane was then placed in a cardboard film cassette, protein side up, and a piece of X-ray film was placed atop the membrane. The film was held in place with a piece of tape and the position of the film relative the membrane was marked to allow the measurement of protein migration for comparison to later antibody detections. The first film was generally exposed for two minutes, then quickly removed and placed in an X-ray film developer. Additional films, if needed, were exposed for more or less time based on the outcome of the first film.

For most samples two or more proteins recognized by different antibodies were detected on the same nitrocellulose membrane. To do this the primary and secondary antibody were removed before beginning another antibody detection by incubating the membrane in antibody stripping buffer (2% SDS, 37.5mM Tris [pH 6.8], 0.7% β-mercaptoethanol) in a Seal-A-Meal® bag at 50° C for 30 - 60 minutes.

Immunoprecipitation

Immunoprecipitation was carried out essentially as described by Green et al. (1992) with modifications made in our laboratory to optimize conditions. TachiSorb-M or TachiSorb-R immunosorbent precipitation reagent (Calbiochem, San Diego, CA) was used to precipitate complexes of mouse mAb or rabbit polyclonal Ab, respectively. For most experiments equivalent amounts of protein (as determined by Bradford assay) were

precipitated. To clear the cell lysate of proteins which nonspecifically bound to immunoglobulin, 15 µl of NMS or normal rabbit serum were added to 100 µl of TachiSorb solution and incubated for one hour at 4° C, with shaking. The normal serum-bound TachiSorb matrix was then pelleted and washed three times with 100 µl of cold RIPA buffer. After the washes 300 - 400 µl cell lysate (~1 mg of total protein) were added to the pellet and the matrix resuspended. This mixture was allowed to incubate for one hour at 4° C, with shaking. At the end of this incubation the TachiSorb matrix was again pelleted, and the resulting supernatant was termed the "cleared lysate".

During the first incubation, the primary antibody was added to 350 µl of TachiSorb solution and incubated for one hour at 4° C, with shaking. The primary antibodies (and amounts used) were D19 (3 µl), which recognized the Ln β2 chain; YY13 (2 µl) which recognized the γ1 chain; α-HA (4 µl), which recognized HA epitope-tagged hybrid Ln chains. After the incubation, the TachiSorb matrix with the primary antibody adsorbed was pelleted and washed three times with 500 µl of RIPA buffer. The primary antibody-TachiSorb pellet was then resuspended in the cleared lysate and allowed to incubate for two hours at 4° C.

After the incubation, the primary antibody-TachiSorb matrix was pelleted and washed three times in RIPA buffer. This pellet was resuspended in 80 µl of immunoprecipitation sample buffer [50 mM Tris (pH 6.8), 2 % SDS, 0.1 % BPB, 10 % glycerol and 100 mM DTT (added just prior to use)]. The resuspended pellet was boiled

for 5 minutes and centrifuged at 14,000 $\times g$ for 3 minutes. The supernatant was removed and placed in one well of a polyacrylamide gel for electrophoresis and western blotting.

Results

Yeast Two-Hybrid Assay

Recombinant fragments of the α, β and γ chains have been utilized in the study of Ln chain interaction (Nomizu et al. 1994; Utani et al. 1994 & 1995; Kammerer et al. 1995) Most of these studies, given their biophysical nature, were performed in a test tube, not in a cellular context. Intracellular studies of Ln assembly have focused on full-length chains and their ability to be secreted (Matsui et al. 1995; Yurchenco et al. 1997). Niimi et al. (1997) have tested whether fragments of the β1 and γ1 Ln chains could assemble intracellularly with full-length Ln chains to form β- γ dimers or the α- β- γ trimer. They reported that the carboxyl terminal 200 amino acids of β1 and γ1 Ln chains were both able to form dimers.

The studies described do detect Ln chain interactions but they only tell whether or not dimers (or trimers) can form. The yeast two-hybrid assay links protein-protein interaction to a quantifiable enzyme assay, thereby allowing not only detection of dimer formation but also the assessment of levels of dimeric interaction in pairs of chain fragments. These two-hybrid assay experiments were performed to assess the relative levels of interactions between pairs of Ln chain long fragments and then determine the minimal essential regions responsible for these interactions.

The cDNA fragments of the Ln chains were created by PCR (or restriction digestion in the case of the $\beta 2_{75}$ fragment) [Figures 4 and 5] and inserted into the

activation domain containing vector (GAD424) and the binding domain containing vector (GBT9). This created hybrid constructs which encode fusion proteins; e.g., β2*I* /GAD424 = $\beta 2I_{act}$ and β2*I* / GBT9 = $\beta 2I_{bind}$. Yeast strain SFY526 was transformed with paired activation and binding domain constructs, plated on appropriate medium, and single colonies were cultured for use in a spectrophotometric assay to determine the β-galactosidase activity, which served as a measurement of Ln domain interaction.

The two-hybrid assay by design detects the interaction of two separate proteins (or protein fragments); one fused to the GAL4 activation domain and one fused to the GAL4 binding domain. The proximity of the two interacting proteins then allows the two GAL4 subunits to function as a transcriptional activator. This design is short-circuited, however, if one of the fusion proteins acts as a transcriptional activator on its own. Therefore, to ensure that the presence of measurable β-galactosidase activity was due only to the protein-protein interaction of the two-fusions within the yeast, and not a single fusion acting alone to activate transcription, control interactions were performed. These control transformations combined each activation hybrid construct with empty GBT9 plasmid or each binding domain hybrid construct with empty GAD424 plasmid. Only one of the 14 fusion proteins created was able to activate transcription in these controls. This construct $\gamma 1_{75}$ will be discussed later, but these control transformations found that the remaining two-hybrid fusions were not activating transcription of the β-galactosidase reporter gene (data not shown).

To rule out the possibility that differences in β-galactosidase activity for a given assay were due to differences in protein expression levels, western blots of yeast transformant lysates were done to detect fusion proteins and visualize relative levels of expression. Binding domain fusions were detected using the anti-GAL4-DBD antibody, and the β2 containing fusions, except $\beta2_{38}$, could be detected with the R49 antibody. Expression levels of these proteins, as determined by visualization, could not explain the differences in β-galactosidase activity seen in the assays for a particular group of transformants. For instance, in Figure 12A levels of binding domain fusion proteins for all transformant lysates are similar. In several of these groups ($\gamma1I_{act}$+ $\alpha1I_{bind}$ and $\alpha1I_{act}$+ $\beta2I_{bind}$) no enzyme activity was seen with average protein levels. Contrasting this to to the group $\gamma1I_{act}$+ $\beta2I_{bind}$ with its average protein expression but high levels of β-galactosidase activity, it appears that levels of fusion protein expression do not correspond to activity levels.

Furthermore, the expression levels of the $\beta2I_{act}$ fusion (Figure 12B, white arrows) are consistent in the groups $\beta2I_{act}$+ $\beta2I_{bind}$, $\beta2I_{act}$+ $\gamma1I_{bind}$ and $\beta2I_{act}$+ $\alpha1I_{bind}$, which all had very different levels of activity in enzyme assays (Figures 10 and 11). Another example of the discontinuity between expression levels of fusion proteins and β-galactosidase activity is the Ln domain II fusions. The activity of these transformants is not above background and expression of these proteins is low in western blots, but when compared to the expression level of the VA3 binding fusion (which is half of the positive control fusion

pair) the levels are quite similar. The activity level for the TD1+VA3 transformants is orders of magnitude above the domain II transformants' activity. The results of the western blots implicate protein-protein interaction, and effectively rule out differences of fusion expression levels, in determining the activity of a given yeast transformant in these two-hybrid assays.

Fusion proteins containing domain I of β2 interact most strongly with fusion proteins containing domain I of γ1.

Ln domain I hybrid constructs were introduced into yeast in pairs and interaction of fusion proteins was assessed by β-galactosidase activity assay. Examination of the data for domain I fusions showed the strongest interactions occurred between a β2*I* fusion and a γ1*I* fusion. Weaker interactions were seen for the $\beta 2I_{act}$ + $\beta 2I_{bind}$ pair and the $\beta 2I_{act}$ + $\alpha 1I_{bind}$ pair. Heterodimeric pairings (that is transformants which paired β2 + γ1, β2 + α1 or γ1 + α1) were compared separately from homodimeric pairings (β2 + β2, γ1 + γ1, and α1 + α1) because heterodimeric interactions are the ones seen *in vivo*, whereas homodimeric interactions are only found in *in vitro* experiments that utilize fragments of Ln chains.

The β-galactosidase activity in yeast transformed with $\beta 2I_{act}$ + $\gamma 1I_{bind}$ was significantly greater than all other groups (Figure 10). Activity of the reciprocal pair, $\gamma 1I_{act}$ + $\beta 2I_{bind}$, was significantly weaker than $\beta 2I_{act}$ + $\gamma 1I_{bind}$, but still stronger than all

other heterodimer pairs. Pairwise comparison determined that, among other heterodimeric interactions, only the $\beta 2I_{act} + \alpha 1I_{bind}$ had β-galactosidase activity significantly greater than the negative control group TD1+LAM5'. Since β-galactosidase activity is directly proportional to the strength of interaction, the intracellular interaction of the fusion protein $\beta 2I_{act}$ with fusion protein $\gamma 1I_{bind}$ is stronger than the interaction of fusion proteins $\gamma 1I_{act} + \beta 2I_{bind}$. Except for $\beta 2I_{act} + \alpha 1I_{bind}$, no other domain I heterodimer pair showed statistically significant interactions above background.

Figure 10.

The Strongest Heterodimeric Interaction of Domain I Fusions is β2*I* with γ1*I*

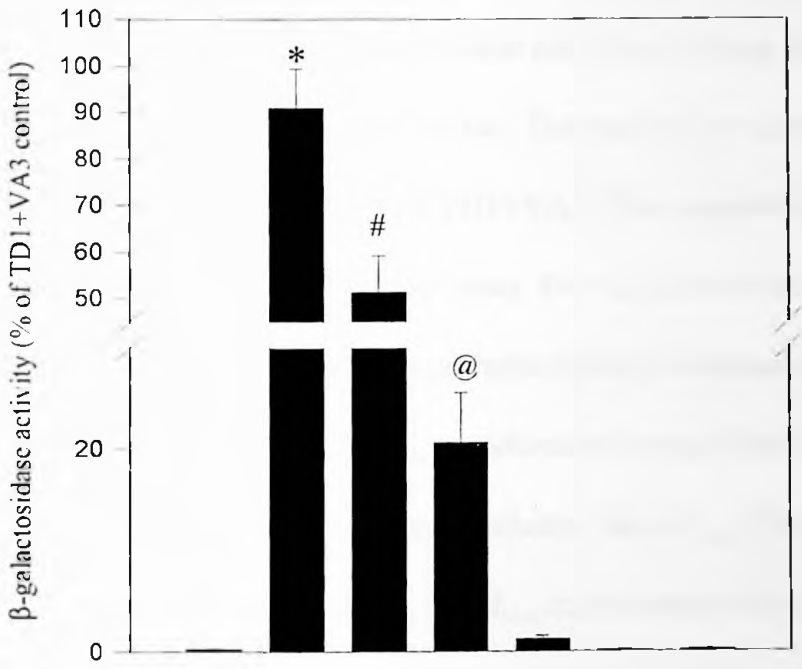


Figure 10. Heterodimeric interactions of the domain I fragments of the α1, β2, and γ1 Ln chains were compared using the yeast two-hybrid assay. Yeast strain SFY526 was transformed with pairs of one activation domain construct and one binding domain construct, which generated activation fusions or binding fusions with the domain I Ln chain fragments. Pairs of activation and binding fusions are listed in rows at bottom of the graph. Interactions of the paired fragments drive expression of the reporter gene, β-galactosidase. This enzyme activity is then assayed as described in *Materials and Methods*. The *y*- axis represents the β-galactosidase activity of lysates from yeast transformed with the pairs indicated below the bar. This activity is reported as a percentage of the positive control interaction TD1+VA3. The negative control interaction TD1+LAM5' is shown as background for this assay. For the data shown a one-way analysis of variance was done followed by a pairwise Student-Neuman-Keuls *post-hoc* test. * indicates the activity of $\beta 2I_{act} + \gamma 1I_{bind}$ transformant is significantly greater than all other transformants in pairwise comparisons. # indicates the $\gamma 1I_{act} + \beta 2I_{bind}$ transformant has significantly less activity than the $\beta 2I_{act} + \gamma 1I_{bind}$ transformant but significantly greater activity than all other pairs. @ signifies that the $\beta 2I_{act} + \alpha 1I_{bind}$ transformant has activity significantly above the four transformants TD1+LAM5', $\alpha 1I_{act} + \beta 2I_{bind}$, $\gamma 1I_{act} + \alpha 1I_{bind}$, and $\alpha 1I_{act} + \gamma 1I_{bind}$, but less than the $\beta 2I_{act} + \gamma 1I_{bind}$ and $\gamma 1I_{act} + \beta 2I_{bind}$ transformants. Bars without symbols indicate that the activity for these transformants is not above background (TD1+LAM5') levels. Statistical significance was determined at

the $p< 0.05$ level. The "n" number of assays for each transformant (in parentheses) are as follows: TD1+LAM5' (n=9), $\beta 2I_{act} + \gamma 1I_{bind}$ and $\gamma 1I_{act} + \beta 2I_{bind}$ (n=7) , $\beta 2I_{act} + \alpha 1I_{bind}$ and $\alpha 1I_{act} + \beta 2I_{bind}$ (n=5), $\gamma 1I_{act} + \alpha 1I_{bind}$ and $\alpha 1I_{act} + \gamma 1I_{bind}$ (n=6). Note the chart break which deleted the region from 25-45 on the vertical axis in order to see smaller bars more clearly.

For the homodimeric interactions (Figure 11), only β2*I* had β-galactosidase activity levels significantly above background; i.e., β-galactosidase units for TD1+LAM5'. No β-galactosidase activity could be measured for either the γ1*I* or the α1*I* homodimers. Thus, while only the β2 domain I is capable of homodimeric interactions in this assay, it is important to note that this interaction is much weaker than the heterodimeric interactions of β2 domain I with γ1 domain I. These results indicate that intracellular conditions are most favorable for formation of γ1- β2 dimers, but homodimeric interactions of β2 can occur.

Western blots detected similar expression levels for all binding domain fusion proteins in all transformants (Figure 12A). Also, the $\beta 2I_{act}$ fusion protein was detected in similar amounts for the three transformants with this construct (Figure 12B). Therefore, the amount of β-galactosidase activity measured does not correlate to fusion expression levels. This indicates that the ability to activate transcription is due to interaction of the two fusion proteins and not to greater protein expression in those transformants that show activity.

Figure 11.

Domain I Fusions of β2 but not γ1 or α1 Show Homodimeric Interactions

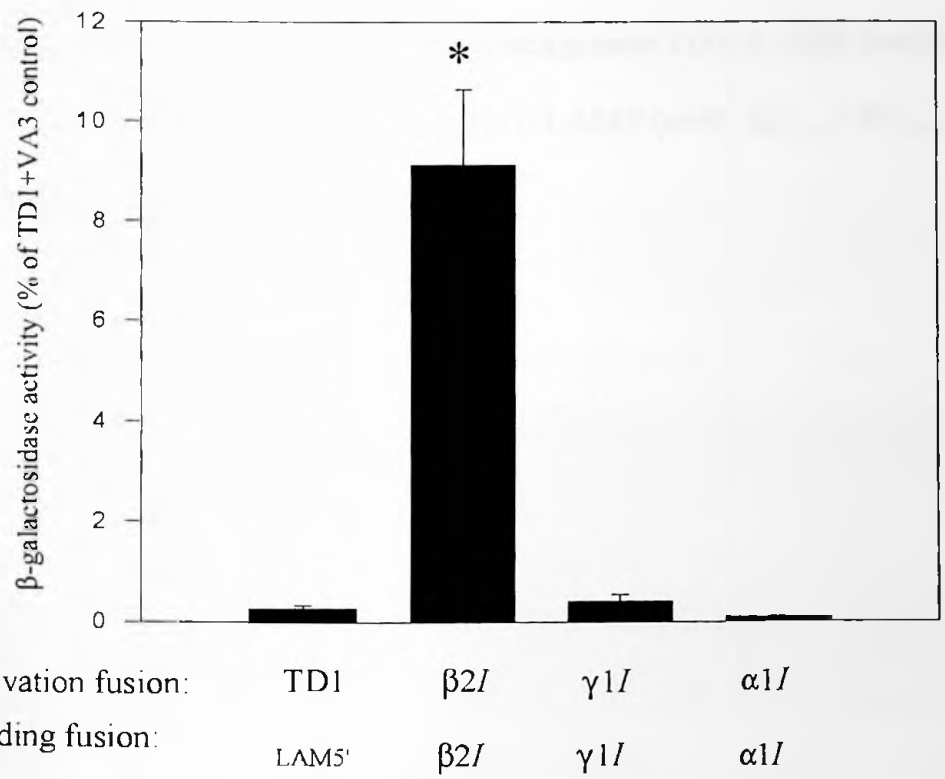


Figure 11. Homodimeric interactions of the Ln domain I fragments were assessed by the yeast two-hybrid assay. Pairs of an activation domain construct with a binding domain construct were transformed into yeast and fusions created by these constructs are noted in the rows at bottom of the graph. The β-galactosidase activity of the transformed yeast presented as the percentage of the positive control transformant TD1+VA3, is plotted as vertical bars. * indicates the activity of the $\beta 2I_{act}$ + $\beta 2I_{bind}$ transformant is significantly greater ($p<0.05$) than the other three transformants. The activity of $\gamma 1I_{act}$ + $\gamma 1I_{bind}$ and $\alpha 1I_{act}$ + $\alpha 1I_{bind}$ was not significantly above background TD1+LAM5' levels. The number of assays "n" for each transformant was TD1+LAM5' (n=9), $\beta 2I_{act}$ + $\beta 2I_{bind}$ and $\gamma 1I_{act}$ + $\gamma 1I_{bind}$ (n=7), and $\alpha 1I_{act}$ + $\alpha 1I_{bind}$ (n=4).

Figure 16.
$\beta 2_{75}$, β_{38}, and $\gamma 1_{75}$ Fusions are Expressed in Yeast

Act.	TD1	GAD	GAD	$\gamma 1_{75}$	GAD	$\beta 2_{38}$	$\gamma 1_{75}$	$\gamma 1_{75}$	$\beta 2_{75}$			$\beta 2_{75}$	$\gamma 1_{75}$	$\beta 2_{38}$
Bind.	LAM	GBT	$\gamma 1_{75}$	GBT	$\beta 2_{38}$	GBT	$\gamma 1_{75}$	$\beta 2_{75}$	$\gamma 1_{75}$			$\beta 2_{75}$	$\beta 2_{38}$	$\gamma 1_{75}$

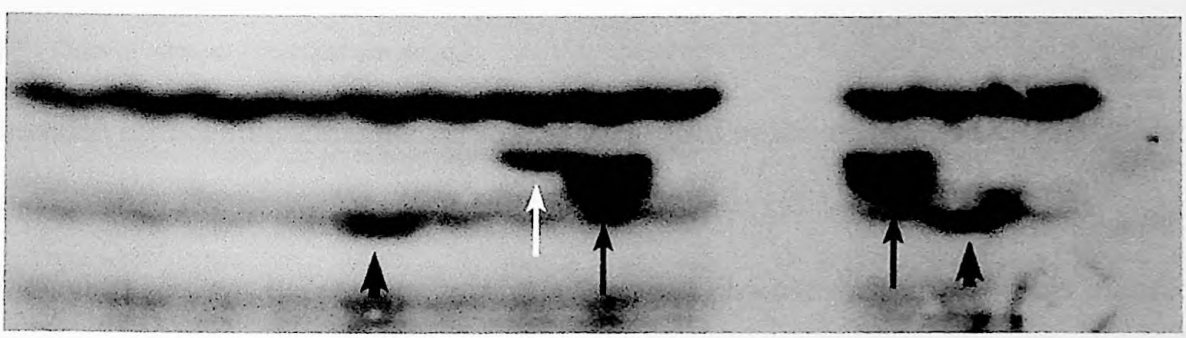


Figure 12. Western blots were performed to determine the expression levels of the domain I fragment fusion proteins. The Ln chain fragments of the activation domain (Act.) and binding domain (Bind.) fusions are named in rows above the respective sample lanes. The lane labeled "---" for both Act. and Bind. rows is a cell lysate from HEK 293 cells transfected with a truncated β2 chain, which is a positive control for the anti-β2 antibody R49. The secondary antibody was detected via the ECL chemiluminescent system (Amersham, Arlington Hts., IL) and exposure to x-ray film.

(A) A representative immunoblot with the anti-GAL4 binding domain antibody (anti-GAL-DBD). The antibody was diluted 1:1,000 in 5% non-fat dry milk (NFDM) and incubated for 2.5 hrs at room temperature. The secondary antibody was goat anti-rabbit IgG conjugated to horseradish peroxidase enzyme (G-anti-RbIgG-HRP), diluted 1:4,000 in 5% NFDM. Arrows mark the bands corresponding to $\alpha 1I_{bind}$ and $\gamma 1I_{bind}$ (upper arrow) or $\beta 2I_{bind}$ (lower arrow) fusion proteins.

(B) The same nitrocellulose membrane in A was stripped of the anti-GAL-DBD antibody and immunoblotted with anti- β2 antibody R49 diluted 1:3,000 in 5 % NFDM. The secondary antibody was the same as in A. Black arrows point to the $\beta 2I_{bind}$ protein in three of the lanes and white arrows point to $\beta 2I_{act}$ fusion protein.

The C-terminal 75 amino acids of β2 form heterodimers with domain I of γ1 but not homodimers with domain I of β2.

Other investigators have shown that a ten amino acid sequence 10 - 20 residues from the C-terminus of the γ1 chain is essential for dimerization with the β2 chain (Utani et al. 1994), and much of the study of Ln chain interaction has focused on the C-terminal 100 - 200 amino acids of domain I in each chain. To evaluate the strength of interaction of smaller portions of domain I, hybrids were constructed which fused the last 75 amino acids of β2 with either the activation domain or binding domain of GAL4 ($\beta2_{75\text{-act}}$ and $\beta2_{75\text{-bind}}$, respectively). The interaction of these fusions with domain I fusions of β2 or γ1 chains was evaluated by the two-hybrid assay as described above.

β-galactosidase activity for colonies transformed with a $\beta2_{75}$ construct together with a $\gamma1I$ construct was significantly above background and of similar magnitude to the activity of colonies transformed with $\gamma1I + \beta2I$ pairs (Figure 13). Statistically, the $\gamma1I_{act} + \beta2_{75\text{-bind}}$ transformants had significantly greater β-galactosidase activity than the three other heterodimeric pairs $\beta2_{75\text{-act}} + \gamma1I_{bind}$, $\gamma1I_{act} + \beta2I_{bind}$ and $\beta2I_{act} + \gamma1I_{bind}$. The β-galactosidase activity of the $\beta2_{75\text{-act}} + \gamma1I_{bind}$ transformants was not statistically different from the activity of $\gamma1I_{act} + \beta2I_{bind}$ transformed colonies, but was less than the $\gamma1I_{act} + \beta2_{75\text{-bind}}$ and $\beta2I_{act} + \gamma1I_{bind}$ transformants. The activity data for $\beta2I_{act} + \gamma1I_{bind}$ and $\gamma1I_{act} + \beta2I_{bind}$ transformants is the same as for the first experiment. These results indicate that fusions containing only the C-terminal 75 amino acids of β2 are not different from

fusions containing the entire domain I of β2 in their ability to interact with domain I fusions of γ1.

In order to compare the homodimeric interaction potential of the C-terminal 75 amino acids of the β2 chain, the constructs containing $\beta2_{75}$ were paired with $\beta2I$ constructs or transformed together to test for interaction in the two-hybrid system The results are seen in Figure 14. They show that transformants containing $\beta2_{75\text{-act}} + \beta2_{75\text{-bind}}$ did not have activity significantly above background, differing from the data shown previously for the $\beta2I_{act} + \beta2I_{bind}$ transformants. Furthermore, the $\beta2_{75}$ fusions paired with either $\beta2I_{act}$ or $\beta2I_{bind}$ did not show significant activity above background. These experiments indicate that fusions of the last 75 amino acids of β2 ($\beta2_{75\text{-act}}$ or $\beta2_{75\text{-bind}}$) did not form homodimeric interactions, whether with all of domain I of β2 or with another $\beta2_{75}$ fusion. These results suggest that the structure of the C-terminal 75 amino acids allows for heterodimeric but not homodimeric interactions. Western blots showed similar expression levels of the fusions in these experiments.

Figure 13.

$\beta 2_{75}$ Fusions are Capable of Forming Heterodimers with $\gamma 1I$ Fusions Which are as Stable as Those of $\beta 2I$ Fusions

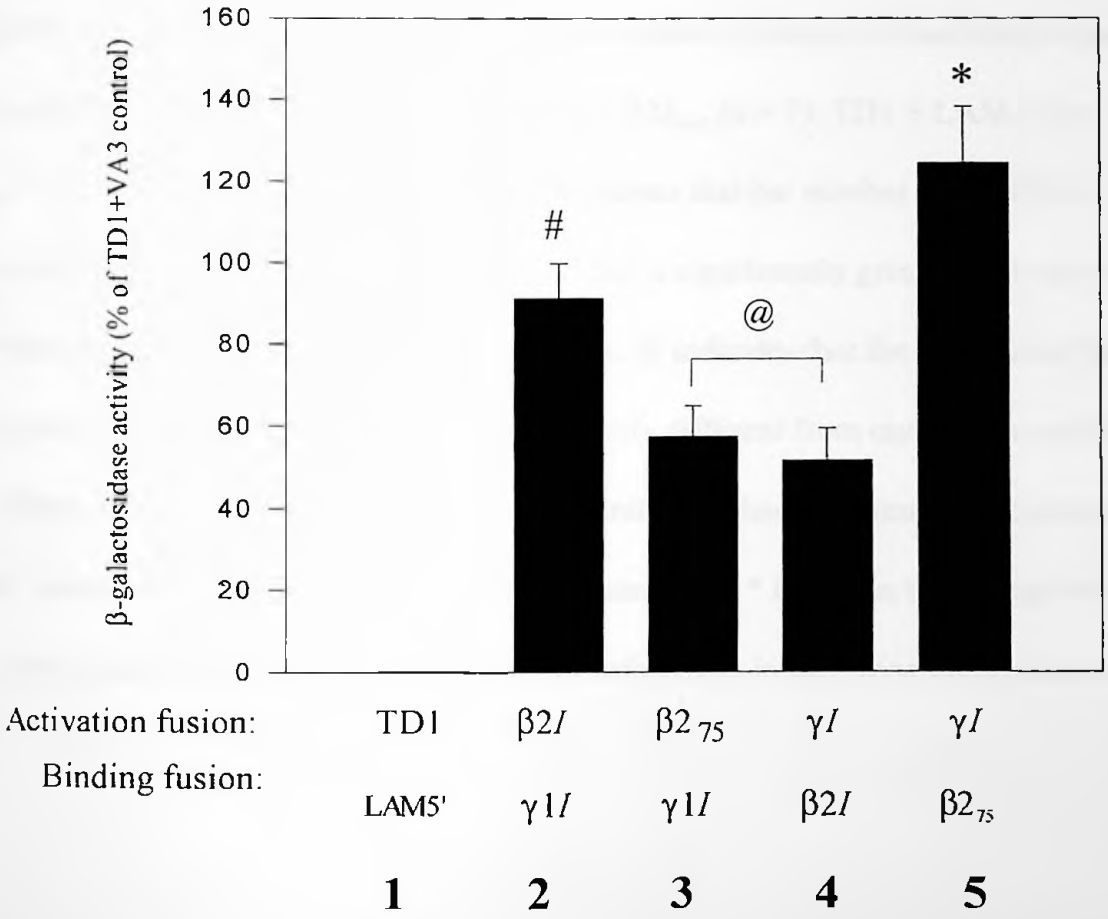
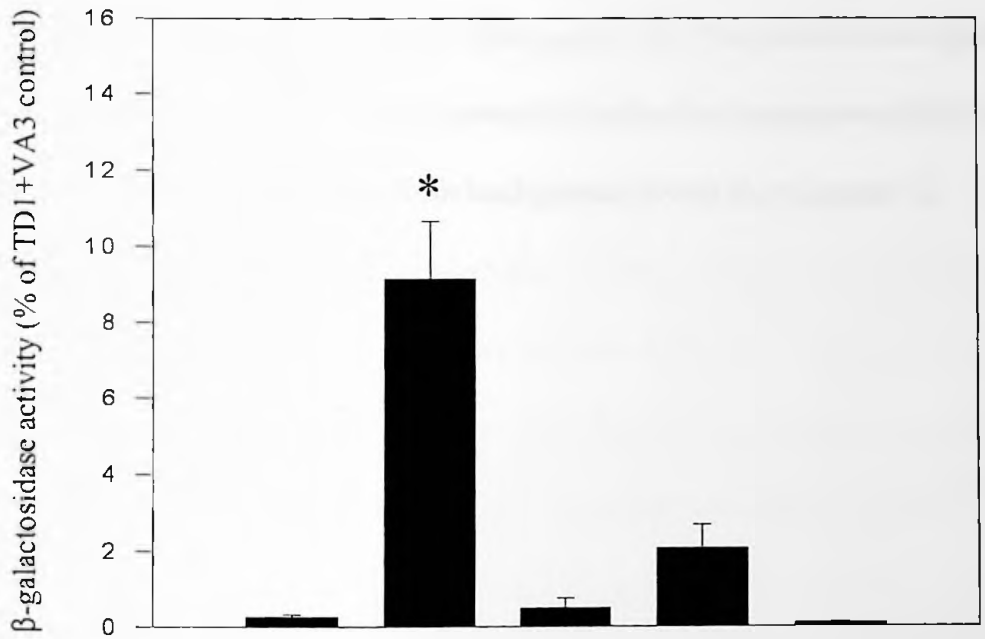


Figure 13. Yeast were transformed with activation domain- Ln fragment fusion + binding domain- Ln fragment fusion pairs as listed below the graph. $\beta 2I$ represents the domain I fragment of Ln β2 chain, $\beta 2_{75}$ represents the C-terminal 75 amino acids of the Ln β2 chain, and $\gamma 1I$ represents the domain I of the Ln γ1 chain. The TD1 + LAM 5' transformant is a negative control as these fusions do not interact. Numbers are given below pairs of fusion proteins to easily identify transformants discussed. Bars represent the mean β-galactosidase activity ± the standard error reported as a percentage of the positive control interaction, TD1+VA3. The number of assays for each fusion pair transformant (n) is $\beta 2I_{act} + \gamma 1I_{bind}$ and $\gamma 1I_{act} + \beta 2I_{bind}$ (n = 7); TD1 + LAM 5' (n = 9); $\beta 2_{75\text{-}act} + \gamma 1I_{bind}$ and $\gamma 1I_{act} + \beta 2_{75\text{-}bind}$ (n = 6). # indicates that bar number 2 activity is significantly less than that of bar number 5 but is significantly greater than activity of all other transformants in pair-wise comparisons. @ indicates that the activities of bar number 3 and bar number 4 are not significantly different from one another and that both of these transformants have activity significantly less than bar number 2. Both number 3 & number 4 are also significantly less than number 5. * indicates that group bar number 5 was significantly greater than all other transformants in pair-wise comparisons.

Figure 14.

Fusions of the C-terminal 75 Amino Acids of β2 Do Not Form Homodimers, Unlike Fusions of Domain I of β2



Activation fusion:	TD1	β2*I*	$\beta2_{75}$	β2*I*	$\beta2_{75}$
Binding fusion:	LAM5'	β2*I*	$\beta2_{75}$	$\beta2_{75}$	β2*I*
	1	**2**	**3**	**4**	**5**

Figure 14. $\beta 2_{75}$ - activation and binding domain constructs were transformed into yeast together or with the complementary $\beta 2I$ construct to assess homodimeric interactions of this shortened chain fragment. Pairs of transformed constructs are given below the graph and numbers are used to denote these pairs. Bars represent mean β-galactosidase activity ± standard error reported as a percentage of the positive control paired transformant (TD1 + VA3). The TD1 + LAM 5' transformant is a negative control as these fusions do not interact. The number of assays for each transformant (n)is $\beta 2I_{act}$+ $\beta 2I_{bind}$ (n = 7); $\beta 2I_{act}$ + $\beta 2_{75\text{-}bind}$ and $\beta 2_{75\text{-}act}$ + $\beta 2I_{bind}$ (n = 4), $\beta 2_{75\text{-}act}$ + $\beta 2_{75\text{-}bind}$ (n = 6). * represents the significant difference of bar number 2 from all other groups by pair-wise comparison. Bar number 3, 4 and 5 are not significantly different from background levels (bar number 1).

The C-terminal 38 amino acids of β2 do not form dimers with γ1, nor do they combine to form homodimers.

To further narrow down the region of laminin chain dimer interaction, fusions were created which combined the GAL4 activation and binding domains with the C-terminal most 38 amino acids of the β2 chain. These fusions were named $\beta2_{38\text{-act}}$ and $\beta2_{38\text{-bind}}$, respectively. The $\beta2_{38\text{-act}}$ fusion was paired with the $\gamma1I_{bind}$ fusion in two-hybrid assays to determine their affinity for domain I of the γ1 chain. Also, the $\beta2_{38\text{-act}}$ and $\beta2_{38\text{-bind}}$ fusions were transformed together into yeast to detect the presence of homodimeric interaction.

Repeated β-galactosidase assays showed no statistically significant activity for the $\beta2_{38\text{-act}}$ + $\beta2_{38\text{-bind}}$ transformants, unlike that of $\beta2I_{act}$+ $\beta2I_{bind}$ transformants (Figure 15). Transformants containing the $\beta2_{38\text{-act}}$ fusion paired with the $\gamma1I_{bind}$ fusion had no activity above background either (Figure 15). Western blots for these assays did not detect any protein due to technical problems with the anti-GAL4-DBD antibody and the anti-β2 antibody R49. Lysates from other assays showed detectable levels of the $\beta2_{38\text{-bind}}$ fusion (Figure 16). These assays did not have appreciable β-galactosidase activity either. This indicates that the construct created can generate a fusion protein, and that absence of activity is not likely a result of the lack of fusion protein. These results suggest that the C-terminal 38 amino acids of the β2 chain can not form stable interactions with the γ1 chain, nor can it form stable homodimeric structures.

Figure 15.

$\beta 2_{38}$ Fusions Do Not Interact with $\gamma 1I$ Fusions Nor Do They Form Homodimers

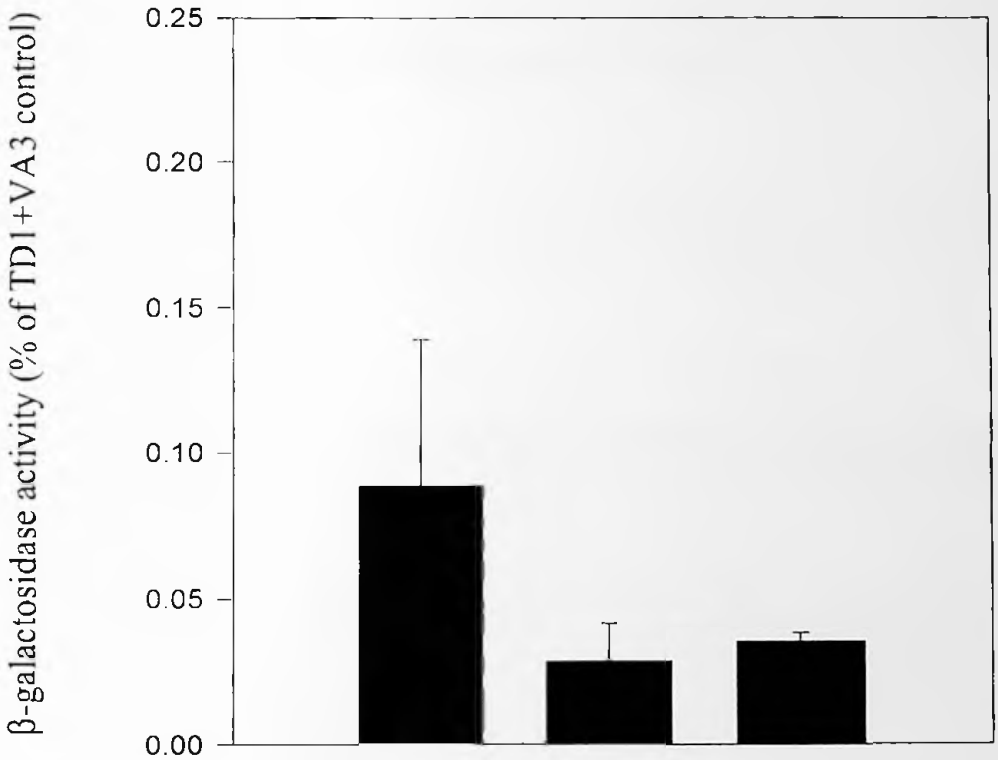


Figure 15. Fusions of the C-terminal 38 amino acids of Ln β2 chain were created and transformed into yeast in pairs as shown below the graph. Bars represent mean β-galactosidase activity ± standard error reported as a % of the positive control, TD1+VA3. Three assays were performed for each transformant. Neither of the transformants showed significant difference from the negative control transformant TD1 + LAM5'. Note the extremely small numerical values on the y - axis.

Figure 16.

$\beta 2_{75}$, β_{38}, and $\gamma 1_{75}$ Fusions are Expressed in Yeast

Act. TD1 GAD GAD $\gamma 1_{75}$ GAD $\beta 2_{38}$ $\gamma 1_{75}$ $\gamma 1_{75}$ $\beta 2_{75}$ $\beta 2_{75}$ $\gamma 1_{75}$ $\beta 2_{38}$

Bind. LAM GBT $\gamma 1_{75}$ GBT $\beta 2_{38}$ GBT $\gamma 1_{75}$ $\beta 2_{75}$ $\gamma 1_{75}$ $\beta 2_{75}$ $\beta 2_{38}$ $\gamma 1_{75}$

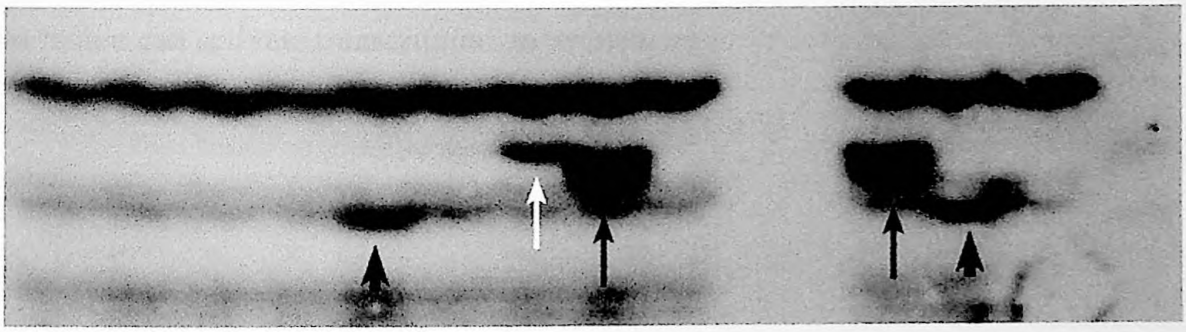


Figure 16. A representative western blot showing expression levels of the $\beta 2_{75\text{-bind}}$, $\beta 2_{38\text{-bind}}$ and $\gamma 1_{75\text{-bind}}$ fusion proteins. Equal amounts of protein from each sample were loaded onto the gel. Bands of the appropriate sizes are recognized by the anti-GAL4-DBD antibody and marked with arrows. These bands are $\beta 2_{38\text{-bind}}$ fusion (black arrows), $\beta 2_{75\text{-bind}}$ (black arrowheads) and $\gamma 1_{75\text{-bind}}$ (white arrows). Note the absence of the $\gamma 1_{75\text{-bind}}$ band in the lane labeled $\beta 2_{75}$ for activation fusion and $\gamma 1_{75}$ for the binding domain fusion. The band is also absent in the lane labeled $\beta 2_{38}$ for activation fusion and $\gamma 1_{75}$ for the binding domain fusion. The $\gamma 1_{75\text{-bind}}$ fusion protein is detected in the lane for $\gamma 1_{75\text{-act}} + \gamma 1_{75\text{-bind}}$. For these three lanes only the lack of fusion protein corresponds to the lack of activity for that assay. In the one lane with the $\gamma 1_{75\text{-bind}}$ fusion protein present, this assay did have measurable β-galactosidase activity. This indicates that for the $\gamma 1_{75\text{-bind}}$ fusion only, the amount of activity does correspond to the amount of protein. This is likely due to the fact that this fusion can activate transcription on its own.

Two-hybrid assays using the $\beta 2_{38}$ fusions with constructs of the C-terminal 75 amino acids of $\gamma 1$ were also done. These showed no activity in the case of $\gamma 1_{75\text{-act}} + \beta 2_{38\text{-bind}}$, but the reciprocal pair, $\beta 2_{38\text{-act}} + \gamma 1_{75\text{-bind}}$, did sporadically show activity, albeit a small amount. These results were not compared to the others due to the confounding activity of the $\gamma 1_{75\text{-bind}}$, as discussed below.

Fusion proteins containing the C-terminal 75 amino acids of γ1 have mild transcriptional activity.

To examine the regions of the $\gamma 1$ chain involved in dimer interactions with the $\beta 2$ chain, a fragment corresponding to the C-terminal 75 amino acids of $\gamma 1$ was created by PCR and ligated into both the GAD424 and GBT9 vectors. As before, these constructs were transformed into yeast and fusion protein interaction was determined using the β-galactosidase activity. The activity seen in these assays was not replicable from one assay to the next. The only activity seen for any of the transformants were those which contained the $\gamma 1_{75\text{-bind}}$ construct. In fact, one of the control transformant (GAD424 + $\gamma 1_{75\text{-bind}}$) assays did show a low level of activity.

These observations led to testing the transcriptional activity of the $\gamma 1_{75\text{-bind}}$ fusion alone. The $\gamma 1_{75}$/GBT9 construct was transfected by itself into competent yeast and plated on selective medium lacking tryptophan. Colonies containing this fusion alone were assayed for activity as for other transformed yeast. Three repeated assays all showed a

low level of β-galactosidase activity, confirming the ability of this fusion protein to act as a transcriptional activator in this assay. This transcriptional activity is not sufficient to explain the activity seen in the double transformed pairs such as $\gamma 1_{75\text{-act}} + \gamma 1_{75\text{-bind}}$, $\beta 2_{75\text{-act}} + \gamma 1_{75\text{-bind}}$, and $\beta 2_{38\text{-act}} + \gamma 1_{75\text{-bind}}$. What may explain the differences are expression levels of the $\gamma 1_{75\text{-bind}}$. In one immunoblot (Figure 16) expression of the $\gamma 1_{75\text{-bind}}$ fusion is seen for the transformant which had activity ($\gamma 1_{75\text{-act}} + \gamma 1_{75\text{-bind}}$), but no expression of the fusion protein is detectable in two lanes which did not show activity ($\beta 2_{75\text{-act}} + \gamma 1_{75\text{-bind}}$ and $\beta 2_{38\text{-act}} + \gamma 1_{75\text{-bind}}$). Because of the ability of the $\gamma 1_{75\text{-bind}}$ fusion to activate transcription of the reporter gene on its own and because the expression levels affected activity of the transformants, these fusion proteins were not used in this assay.

Substitution of the C-terminal cysteine of β2 Domain I does not ablate interactions of β2 with γ1.

It is known that the β and γ chains form a disulfide cross-bridge between their respective C-terminal cysteine residues (Cys_{1765} in β2 and Cys_{1563} in γ1) [reviewed in Beck et al. 1990; see I. Hunter et al. 1992]. Antonsson et al. (1995) reported that disruption of disulfide bonding by alkylation of the Ln chains did not alter the α-helical nature of the Ln chains nor did it prohibit dimer formation of β1 chain fragments with γ1 chain fragments. To determine if this covalent bond was necessary for the domain I interaction to occur in our system, a domain I fragment of the β2 chain cDNA was created

with a mutation at nucleotide 5466. The single base pair mutation resulted in a cysteine to serine switch at residue 1765 of the mature protein. This mutated cDNA fragment was used to create hybrids in the GAD424 and GBT9 vectors. The fusion products were called $^{C\rightarrow S}\beta 2I_{act}$ and $^{C\rightarrow S}\beta 2I_{bind}$, respectively.

In repeated two-hybrid β-galactosidase assays, the mutated β2*I* fusions showed differences from wild-type β2*I* fusions in both homodimeric and heterodimeric interactions. The differences from the wild-type were not seen in the reciprocal pair, however. In fact, the same statistically significant difference of the $\beta 2I_{act} + \gamma 1I_{bind}$ interaction being stronger than the reciprocal pair $\gamma 1I_{act} + \beta 2I_{bind}$ was seen for the cys → ser mutants as well (Figure 17). That is, the pairing of $^{C\rightarrow S}\beta 2I_{act} + \gamma 1I_{bind}$ had significantly greater activity than the pairing of $\gamma 1I_{act} + ^{C\rightarrow S}\beta 2I_{bind}$ in β-galactosidase assays. For other heterodimer pairs (Figure 17), the activity of $\beta 2I_{act} + \gamma 1I_{bind}$ transformants was not significantly different from the activity of the $^{C\rightarrow S}\beta 2I_{act} + \gamma 1I_{bind}$ transformants in these assays. The β-galactosidase activity of the $\gamma 1I_{act} + \beta 2I_{bind}$ pair was, however, significantly different from the $\gamma 1I_{act} + ^{C\rightarrow S}\beta 2I_{bind}$ pair, and the $\gamma 1I_{act} + ^{C\rightarrow S}\beta 2I_{bind}$ pair was reduced to levels not statistically above background.

For homodimeric pairs we saw similar results between mutant and wild-type fusions as well (shown in Figure 18). All pairs tested, except $\beta 2I_{act} + ^{C\rightarrow S}\beta 2I_{bind}$, had activity significantly above background levels in β-galactosidase assays. The activity of mutant $^{C\rightarrow S}\beta 2I$ fusions together ($^{C\rightarrow S}\beta 2I_{act} + ^{C\rightarrow S}\beta 2I_{bind}$) was not significantly different

Figure 17.

Dimer Formation of β2 Domain I C to S Mutation with γ1

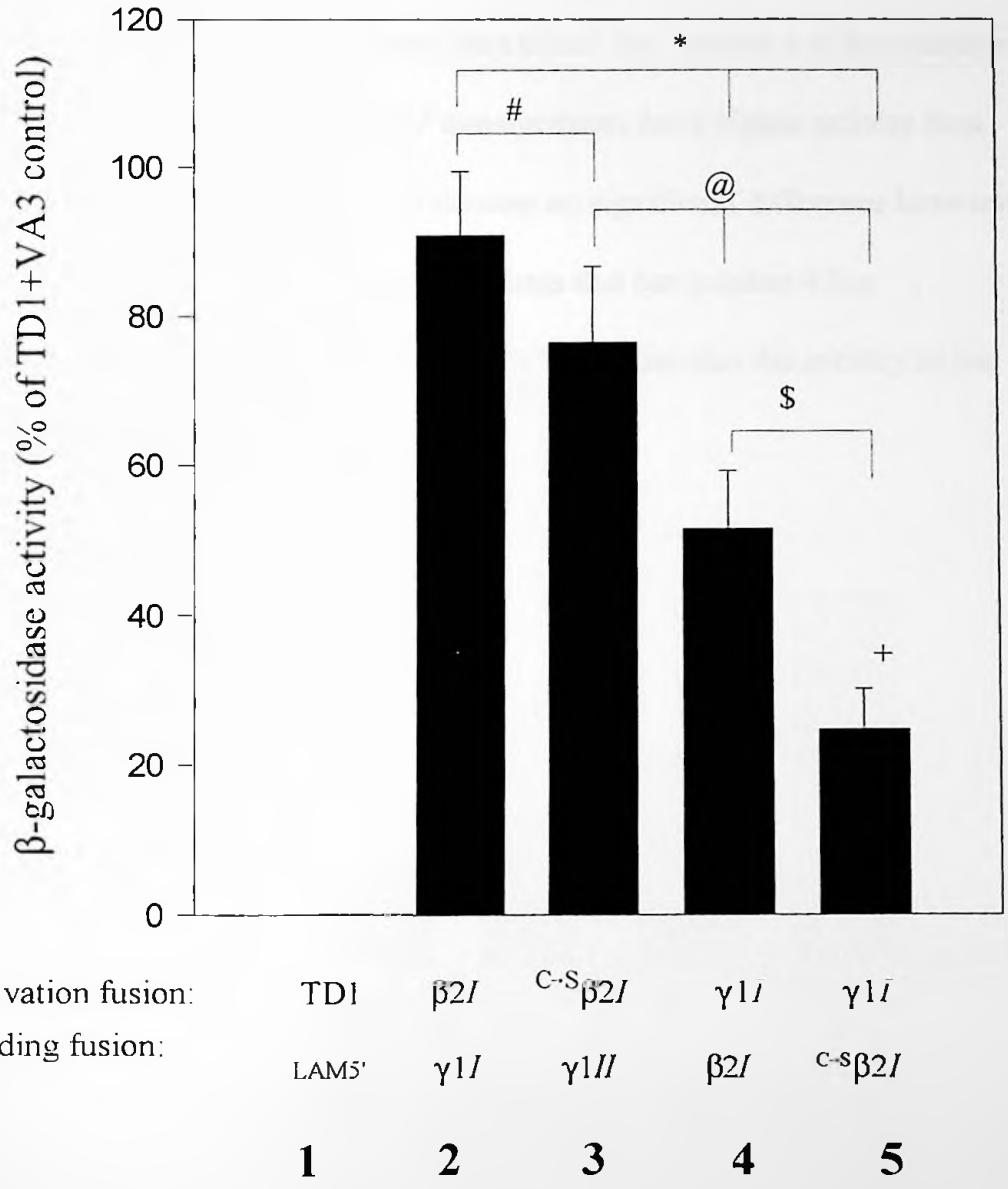


Figure 17. β2*I* fusions that substituted ser for cys at amino acid 1765 were created and compared to wild-type β2*I* fusions for their ability to interact with γ1*I* fusions in the two-hybrid assay. Yeast were transformed with the pairs of activation and binding domain fusions indicated below the graph of β-galactosidase activity. Symbols show statistical differences between transformants in pair-wise comparisons. *indicates that bar number1 transformants have a statistically higher activity than either bar number 4 or bar number 5 transformants. @ indicates that bar number 2 transformants have higher activity than either bar number 4 or bar number 5 as well. # denotes no significant difference between bar number 2 and bar number 3 transformants. $ denotes that bar number 4 has significantly greater activity than bar number 5 and "+" indicates that the activity of bar number 5 is not significantly above background.

Figure 18.

Homodimer Formation of β2 Domain I Cys To Ser Mutation

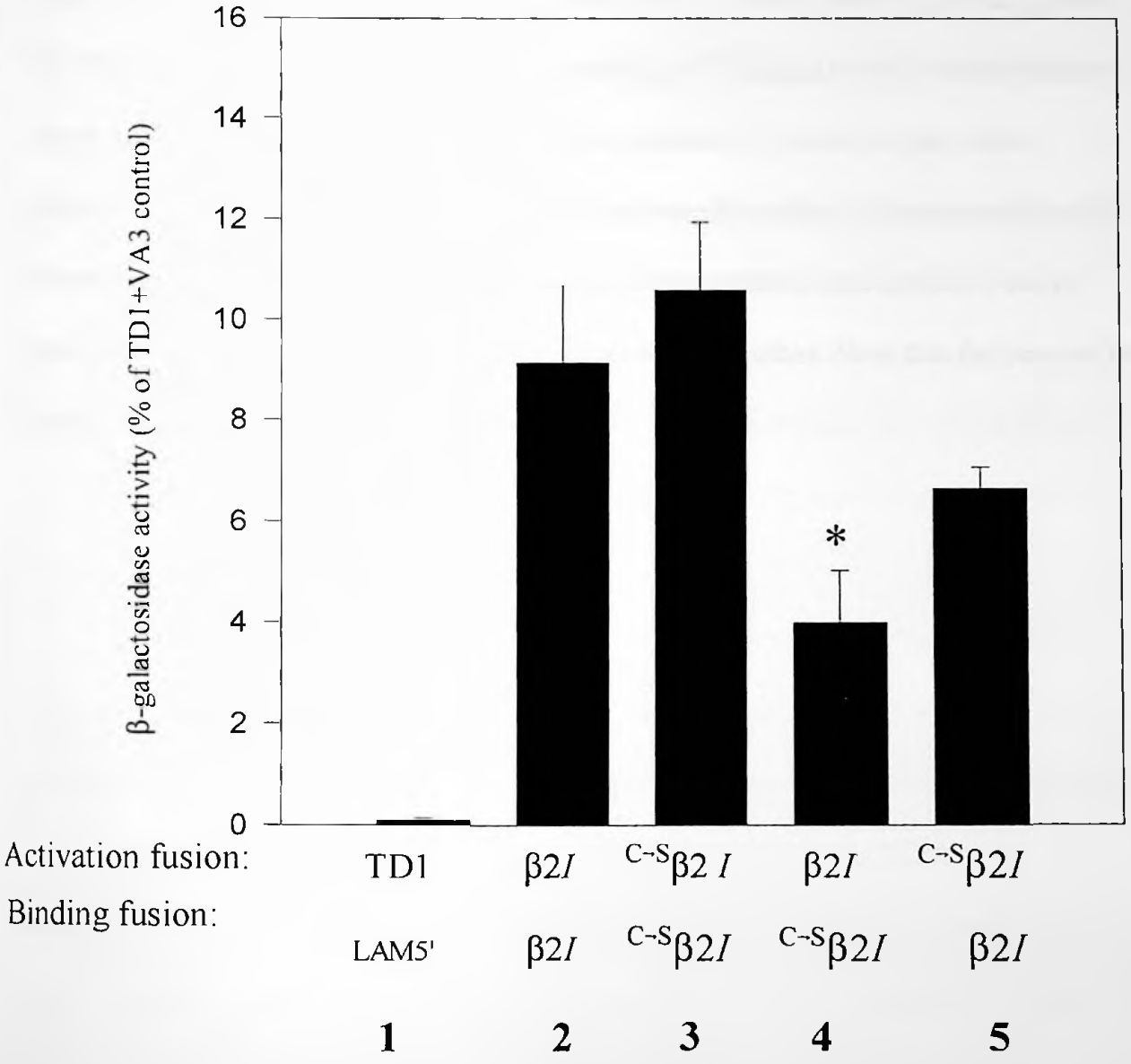


Figure 18. Cys →ser mutated β2*I* constructs were transformed together or with the complementary wild-type β2*I* construct to assess homodimer formation in the absence of disulfide bonding. The bar graph is the results of assays performed on those transformants. Individual bars represent mean β-galactosidase activity ± standard error reported as a percentage of the positive control transformant, TD1 + VA3. The number of assays performed for each transformant (n) was: TD1 + LAM5' and $\beta 2I_{act} + \beta 2I_{bind}$ (n= 7); $^{C\rightarrow S}\beta 2I_{act} + ^{C\rightarrow S}\beta 2I_{bind}$, $^{C\rightarrow S}\beta 2I_{act} + \beta 2I_{bind}$ and $\beta 2I_{act} + ^{C\rightarrow S}\beta 2I_{bind}$ (n = 6). * indicates that transformant number 4 is significantly less than numbers 2, 3 and 5 in pair-wise comparisons, and is not significantly above transformant number 1 (background levels). Transformants bar numbers 2, 3 and 5 are greater than transformant number 1 but in individual pair-wise comparisons none is greater than any other. Note that the scale of the vertical axis is less than that of Figure 17.

from that of the wild-type pair, $\beta 2I_{act} + \beta 2I_{bind}$. Similarly, $^{C\rightarrow S}\beta 2I_{act} + \beta 2I_{bind}$ transformants had activity which was not different from $^{C\rightarrow S}\beta 2I_{act} + {}^{C\rightarrow S}\beta 2I_{bind}$ or $\beta 2I_{act} + \beta 2I_{bind}$ transformants. The $\beta 2I_{act} + {}^{C\rightarrow S}\beta 2I_{bind}$ pair had the lowest activity of the heterodimeric pairs and was significantly less than the $\beta 2I_{act} + \beta 2I_{bind}$, $^{C\rightarrow S}\beta 2I_{act} + {}^{C\rightarrow S}\beta 2I_{bind}$ and $^{C\rightarrow S}\beta 2I_{act} + \beta 2I_{bind}$ pairs. Taken together with the heterodimeric interactions of domain I, these results show some differences between the $^{C\rightarrow S}\beta 2I$ fusions and the wild-type $\beta 2I$ fusions. Because a significant difference is seen between the wild type and the mutant $\beta 2I_{bind}$ fusions in their ability to interact with the $\gamma 1I_{act}$ fusions, it is postulated that the cysteine 1765 residue does play a role in dimer formation. But since transformants which contained $^{C\rightarrow S}\beta 2I_{act}$ or $^{C\rightarrow S}\beta 2I_{bind}$ always showed activity when paired with the complementary $\gamma 1I$ fusion, this mutation does not ablate β2-γ1 interaction. This indicates the Cys_{1765} residue, by reason of its ability to form disulfide bonds, may be a factor in interactions of $\beta 2I$ with $\gamma 1I$, but is not absolutely required for these interactions in this system.

Unfortunately, the $^{C\rightarrow S}\beta 2I$ fusions were not detect in a western blot. Based on knowledge of the system the lack of signal for this procedure is likely due to antibody binding difficulties and not lack of protein expression. Also, since the mutant $\beta 2I$ fusions were so similar in their interaction patterns compared to wild-type $\beta 2I$ fusions it is reasonable to assume that expression levels of the fusion proteins are not responsible for the differences in β-galactosidase activity seen in these assays. These results, though,

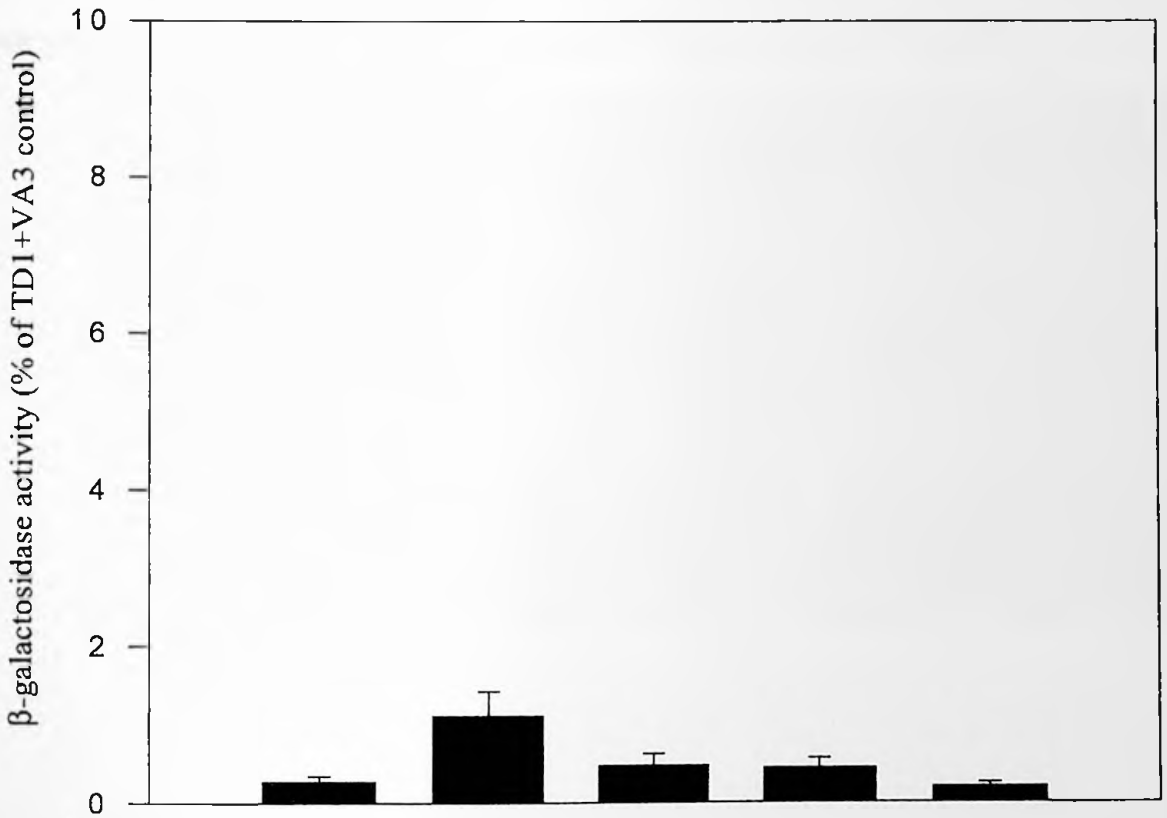
must come with the caveat that protein expression is not known.

Constructs of Domain II of β2 and γ1, separate from Domain I, do not interact

The domain II region of each of the Ln chains is part of the long arm of the trimeric molecule and presumably involved in chain assembly, but little work has been done to study the role of domain II in interactions between the chains. To determine the contribution of domain II to the β2-γ1 dimer formation apart from domain I, we created activation and binding fusions which contained domain II of β2 or γ1 for use in the two-hybrid assay. As shown in Figure 19, none of the two-hybrid transformants had β-galactosidase activity above background levels as determined by statistical analysis. The absence of interaction is not due to lack of fusion protein, as shown in a western blot with the anti-GAL4-DBD antibody (Figure 20). Unlike the domain I heterodimeric interactions, yeast colonies transformed with a β2*II* fusion together with a γ1*II* fusion did not have activity levels above background. These results indicate that interactions of domain II, apart from domain I, are transient or non-existent.

Figure 19.

Domain II Fusions of β2 or γ1 Do Not Interaction in Any Pairings



ıctivation fusion:	TD1	β2*II*	β2*II*	γ1*II*	γ1*II*
3inding fusion:	LAM5'	β2*II*	γ1*II*	β2*II*	γ1*II*

Figure 19. Constructs of the domain II of the β2 and γ1 chains were created and transformed into yeast in complementary (activation and binding domain) pairs for detection of their interactions by the two-hybrid assay. Pairs of β2*II* and γ1*II* fusions are indicated below the graph. Bars represent mean β-galactosidase activity ± standard error reported as a percentage of the positive control TD1 + VA3. Pair-wise comparisons showed none of the transformants had significantly greater activity than any other and none was greater than background (TD1+LAM5'). For each transformant, three assays were performed.

Figure 20.

Domain II Hybrids are Expressed in the Two-Hybrid System

Act. : TD1 β2*II* β2*II* γ1*II* γ1*II* α1*I*

Bind.: VA3 β2*II* γ1*II* β2*II* γ1*II* α1*I*

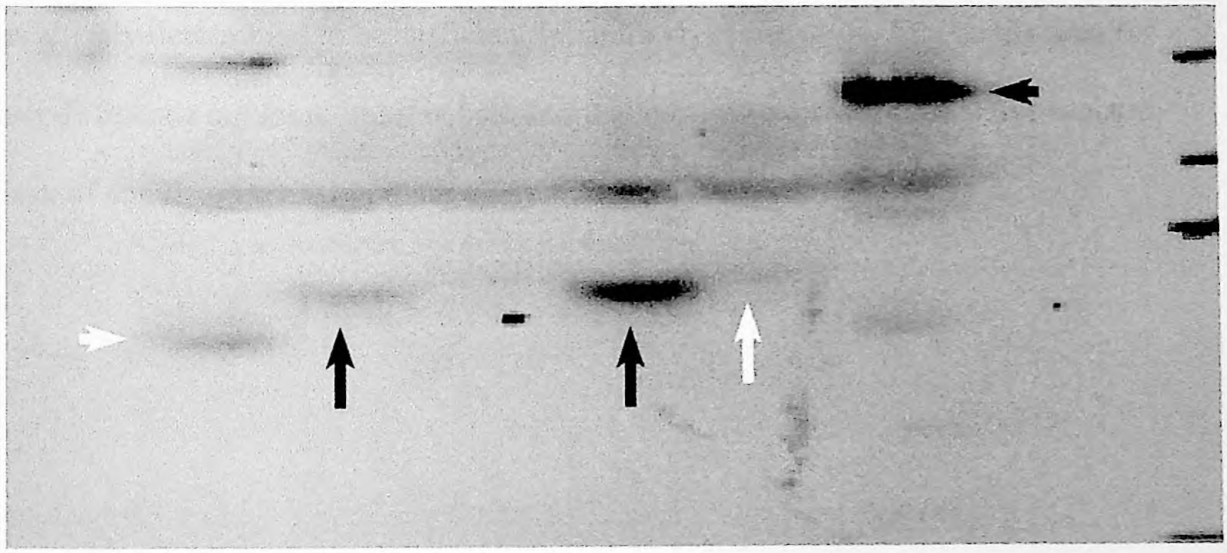


Figure 20. A representative blot of lysates from transformants used in domain II β-galactosidase assays. Equal amounts of protein from each sample were separated on denaturing 10 % polyacrylamide gel. The proteins were then blotted onto nitrocellulose membrane that was probed with the anti-GAL4-DBD antibody to detect binding domain fusions. The size of the molecular weight markers is given in kilodaltons. Specific bands of the appropriate size for VA3 (white horizontal arrowhead), $\beta 2II_{bind}$ (black vertical arrows) and $\gamma 1II_{bind}$ (white vertical arrow) fusion proteins are noted. The $\alpha 1I_{bind}$ (black horizontal arrow) protein was run on the same gel as a control because its expression level was already determined to be sufficient for antibody recognition. The bands seen for the domain II fusions are faint, but it is believed that this low expression does not account for the lack of activity detected in these assays.

Two-Hybrid Summary

The two-hybrid assay provided a good system for measuring the relative strength of dimer interaction of long arm domains and domain fragments. The results of these two-hybrid assays are summarized as follows:

1) The strongest interactions in this system are those of domain I of β2 with γ1 domain I.

2) Homodimers of β2*I* fusions (but not α1*I* or γ1*I*) did occur.

3) Dimeric interactions of the α chain with γ1*I* are not seen; interactions with β2*I* are weak in one pair, non-existent in the other.

4) Mutation studies showed that replacement of Cysteine 1765 in β2*I* did alter, but did not ablate, the ability of these fusions to interact with either γ1*I* or β2*I* fusions.

5) The most C-terminal 75 amino acids of β2 are sufficient to generate heterodimeric interactions with γ1, but homodimeric interactions of this fragment are not seen.

6) Fusions of the last 38 amino acids have no affinity for γ1*I*, nor do they form homodimers.

7) Lastly, domain II, while part of the "long arm", does not seem to be involved in β2 -γ1 dimer formation.

Mammalian Cell Culture and Immunoprecipitation

The question of which regions or amino acids of the Ln chains are responsible for dimer and trimer formation has until now been studied using either fragments of the Ln chains mixed in a test tube or by deleted segments of recombinant chains in intracellular assembly studies. What these fragments lack is the context of the full-length glycoprotein. Deleting a portion of the internal structure of the chain does remove a potential interaction site, but also leaves the remaining coiled-coil domains out of alignment. By replacing domain I of the Ln γ1 chain with the same domain of the β2 chain it is possible to study the role of domain I as part of a full-length molecule. If this region is responsible for specific β-γ dimer formation as postulated, this hybrid chain would interact with full-length γ1 chain but not with full-length β2 chain. Likewise, a hybrid which replaced domain I of Ln β2 with domain I of γ1 would be expected to interact via domain I with full-length β2 chain but not with the γ1 chain. Will domain I of β2 interact with domain I of γ1, regardless of the primary structure of the rest of the Ln chain to which either is attached?

To test this hypothesis, hybrid Ln chains, which had their domain I region replaced with another Ln domain I, were created by PCR and subcloning. These were then used in immunoprecipitation experiments that would detect dimerization with a wild-type recombinant Ln β2 or γ1 chain. To assure that adequate amounts of the wild-type protein were expressed in transformed cells, stably transfected cell lines were created which

constitutively expressed either recombinant Ln β2 chain, or Ln γ1 chain, or both β2 and γ1 chains. One other cell line, which expressed a partially deleted β2 chain, was also used. It contained the long arm domains, and because it was smaller than the full-length β2 chain, it was easily differentiated from the hybrid Ln chains after polyacrylamide gel electrophoresis and western blotting.

The mammalian cell culture experiments involved transient transfection of these stably transfected cell lines with epitope-tagged hybrid laminin chain cDNAs. An immunoprecipitation was performed to precipitate the hybrid Ln chain and any proteins associated with it. This precipitated material was then separated by polyacrylamide gel electrophoresis and the Ln chain(s) associated with the hybrid were detected by blotting with specific antibodies to the β2 or γ1 chain. The wild-type chains were differentiated from the hybrid chains by antibody specificity and size differences. The size differences were determined by marking the position of the nitrocellulose membrane relative to the film during the chemiluminescent detection step. In this manner bands which appeared when blotting with one antibody could be differentiated from bands that appeared on subsequent blots with a second (or third) antibody.

Stable transformation yielded several cell lines that constitutively express recombinant laminin chains.

Several daughter cell lines were generated from HEK 293 cells by transfection with the cDNA for β2 and γ1 in separate expression vectors together with a plasmid containing a selectable marker (neomycin resistance). Two G418-resistant cell lines, (**C2-293$^{\beta-\gamma}$** and **A1-293$^{\beta-\gamma}$**), showed high levels of constitutive expression of both Ln chains in western blots. Two other cell lines were characterized which expressed only one Ln chain. The **A2-293$^{\beta}$** cell line expressed only the β2 chain in western blots, and the **D4-293$^{\gamma}$** cell line expressed only γ1 chain. It is believed that the **D4-293$^{\gamma}$** cell line is unique in its expression of only the γ1 chain, as another laboratory has noted their inability to create such a cell line (Yurchenco et al. 1997). These single chain expressing clones were not unexpected since the cDNA for the chains were on separate vectors, allowing a percentage of transfected cells to incorporate only the β2 or γ1 chain cDNA into their genome. Each of these cell lines were used to study the assembly of full-length Ln molecules within the cell.

Another cell line created by transfection of HEK 293 cells, called **β2 (Δ IV-V)-293,** was also utilized in Ln assembly studies. These cells express a β2 Ln chain which has the domains IV and V deleted. The cDNA for this recombinant molecule was incorporated into the vector pcDNA3, which has a CMV promoter and the selectable marker gene, Neo^{R}. This allows a stable cell line to be generated in the presence of G418.

The recombinant Ln chain of these cells is expressed at high levels and is approximately 55 kilodaltons smaller in size than the full-length β2 chain, but it is still recognized by the anti-β2 antibody R49 (Figure 21). For ease of reference a table is provided to show which cell lines express which Ln chains (Table 4).

The **C2-293**$^{\beta\text{-}\gamma}$ cells were lysed in RIPA buffer, and this lysate was immunoprecipitated with the D19 anti-β2 mAb, which precipitates the β2 chain and any proteins in a complex with it. After precipitation and separation on 4.5 % polyacrylamide gel, a western blot was performed using the anti-Ln Ab (Polysciences, Warrington, PA) to detect the presence of γ1 protein. The results did show the γ1 chain was co-precipitated with the β2 chain in these cells which express both chains (Figure 22). This indicates that the β2 and γ1 chains "assembled" into a stable dimer.

Figure 21. Polyacrylamide gel electrophoresis (PAGE) of lysates from cell lines A-$1^{\beta\text{-}\gamma}$ 293 and β2 (ΔIV-V) 293 blotted with the anti-β2 Ab, R49. One band is seen for each lysate (arrows), which is the β2 chain expressed constitutively by that particular cell line. In A-$1^{\beta\text{-}\gamma}$ 293, the chain is full-length β2 (approx. 190 kD); in β2 (ΔIV-V) 293 the β2 chain is deleted for domains IV and V and is therefore approximately 55 kD smaller in size than the full-length β2 chain.

Table 4. Stably Transfected Cell Lines

Cell Line	Recombinant Laminin Chain Expressed
C2-293$^{\beta-\gamma}$	full-length β2 and full-length γ1
A1-293$^{\beta-\gamma}$	full-length β2 and full-length γ1
A2-293$^{\beta}$	full-length β2
D4-293$^{\gamma}$	full-length γ1
β2 (Δ IV-V)-293	β2 deleted in domains IV and V

Figure 22. The Ln γ1 Chain is Co-precipitated with the Ln β2 Chain in Stable Transfected Cells

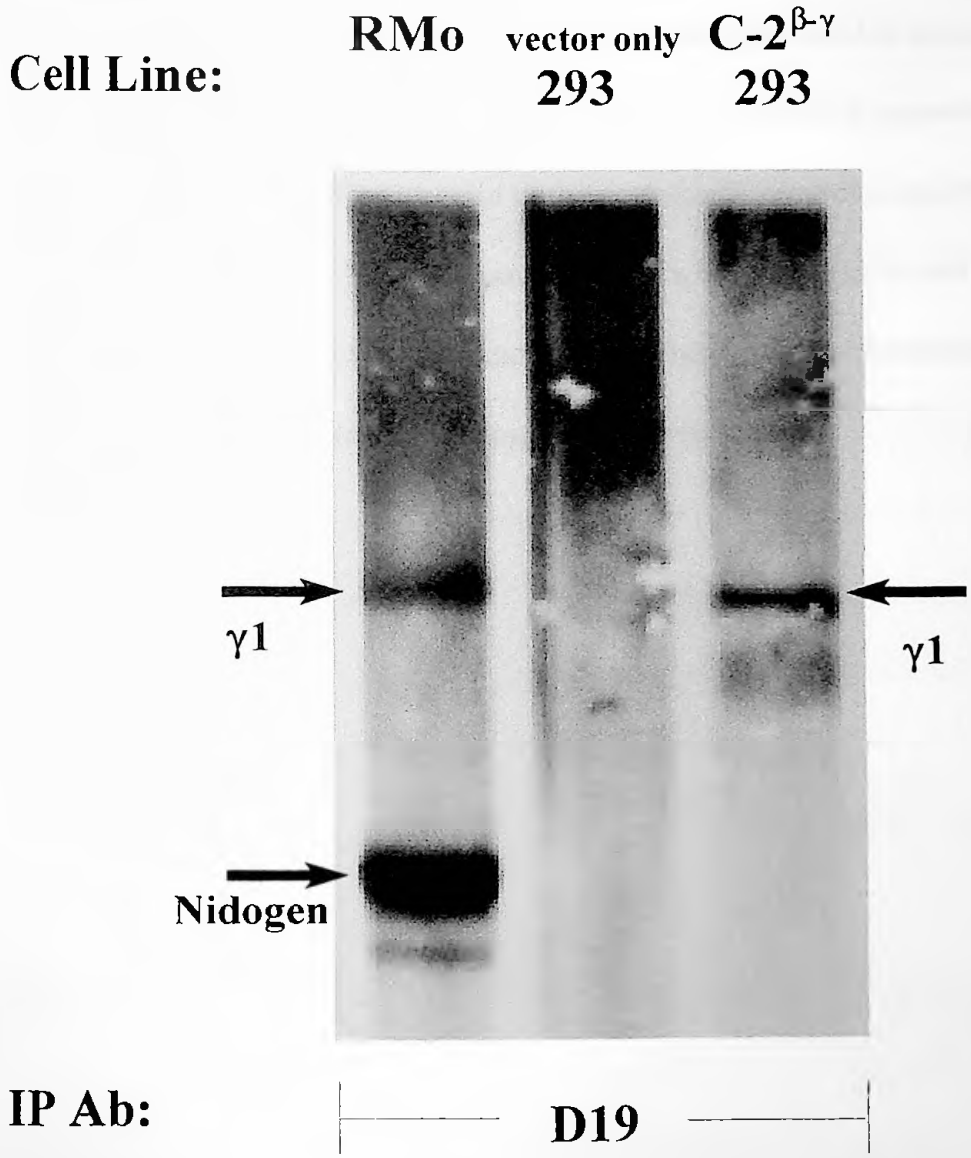


Figure 22. PAGE of immunoprecipitated lysates from 3 cell lines - rat muscle cells (RMo), HEK 293 cells, and A-1$^{\beta\text{-}\gamma}$ 293 cells. The precipitation antibody was the anti-β2 antibody D19. The blotting antibody was anti-Ln antibody that recognizes the β1, γ1 and α1 Ln chains, but not the β2 chain, on a western blot. Also, because it was raised against native, partially purified Ln, it recognizes nidogen /entactin, which is tightly attached to Ln in basement membrane extracts. Note that this nidogen band is seen in the RMo cell line but not in the A-1$^{\beta\text{-}\gamma}$ 293, indicating the difference in matrix protein expression between these cell lines. The plasmid in the vector only control is pcDNA I/ Amp. A band of the correct size for γ1 (labeled arrow) is seen in both RMo cells and A-1$^{\beta\text{-}\gamma}$ 293 cells. No band is seen in the vector only control lane. These results demonstrate that recombinant β2 and γ1 are assembling into a dimer.

Epitope-tagged hybrid laminin chains can be expressed and recognized by selective antibodies.

Since the recombinant Ln chains were constitutively expressed at significant levels and that these recombinant molecules could assemble, these cell lines were used to study the role of domain I in chain assembly. Is only domain I of β2 required for correct assembly or are other sequences involved as well?

To test this, two hybrid Ln chains were made- **$\beta2(\gamma1)_I$-HA**, which combined domain I of γ1 with domains α - VI of β2, and **$\gamma1(\beta2)_I$-HA**, which combined domain I of β2 with domains II - VI of γ1 chain (diagrammed in Figures 7 and 8). The "HA" in the name of these hybrids denotes that they are also epitope-tagged with a nine amino acid peptide of the *H. influenza* hemagglutinin protein (diagrammed in Figure 9). This peptide facilitated the identification and immunoprecipitation of these hybrids because commercially available antibodies could selectively recognize the tagged hybrids in the presence of both γ1 and β2 full-length Ln chains, which antibodies selective for one or the other full-length chain could not do.

A commercial monoclonal antibody to a nine amino acid HA peptide (anti-HA mAb) was used to precipitate the tagged hybrid laminins from cell lysates and to recognize these chains on western blots. The expression level of both hybrids, **$\beta2(\gamma1)_I$-HA** and **$\gamma1(\beta2)_I$-HA**, was tested by transiently transfecting HEK 293 cells with the $\beta2(\gamma1)_I$-HA/cDNA1 and $\gamma1(\beta2)_I$-HA/cDNA1 constructs, then performing western blots

after harvesting the cells. Initial blots showed both the **$\gamma1(\beta2)_I$-HA** and the **$\beta2(\gamma1)_I$-HA** hybrid chains were expressed and recognized by the anti-HA mAb (Figure 23 A & B). In addition to the anti-HA mAb, the anti-β2 antibodies, D5 and R49, as well as the anti-γ1 antibody, YY15, were tested for their ability to recognize the hybrid chain proteins on western blots. D5 and R49 recognized **$\gamma1(\beta2)_I$-HA** but not **$\beta2(\gamma1)_I$-HA**. On the other hand, YY15 had good affinity for **$\beta2(\gamma1)_I$-HA**, but also had a low affinity for **$\gamma1(\beta2)_I$-HA**. Next it was determined if the HA epitope-tagged proteins could be precipitated by the anti-HA mAb. In the immunoprecipitation protocol, this antibody did precipitate a protein from HEK 293 cell lysates transfected with either the $\beta2(\gamma1)_I$-HA/cDNA1 or the $\gamma1(\beta2)_I$-HA/cDNA1 construct. This protein could be detected by the anti-HA mAb upon western blotting. However, the band for the $\beta2(\gamma1)_I$-HA/cDNA1 hybrid was faint. These proteins were of the size expected for the hybrid laminin chains (between 200 and 160 kilodaltons) and were recognized by the same antibodies previously shown to bind the hybrids in western blots. After correcting for low expression of the **$\beta2(\gamma1)_I$-HA** protein by increasing the amount of plasmid DNA used (8 μg up from 6 μg) in later transfections this hybrid also yielded strong bands in immunoprecipitation experiments.

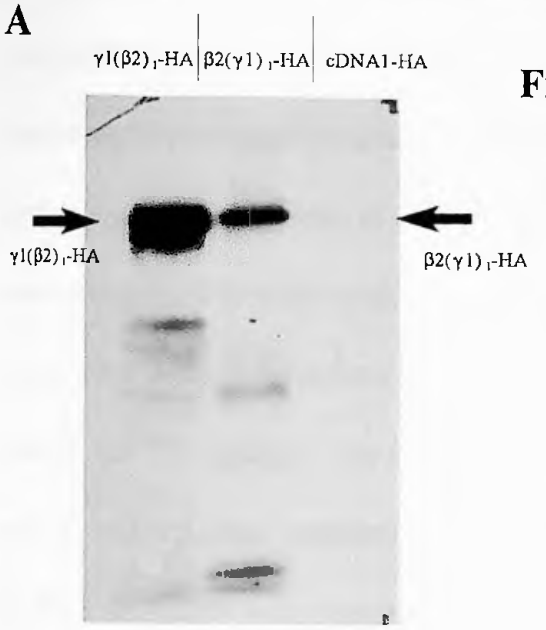


Figure 23. Western Blot and Immunoprecipitation of Hybrid Ln Chains

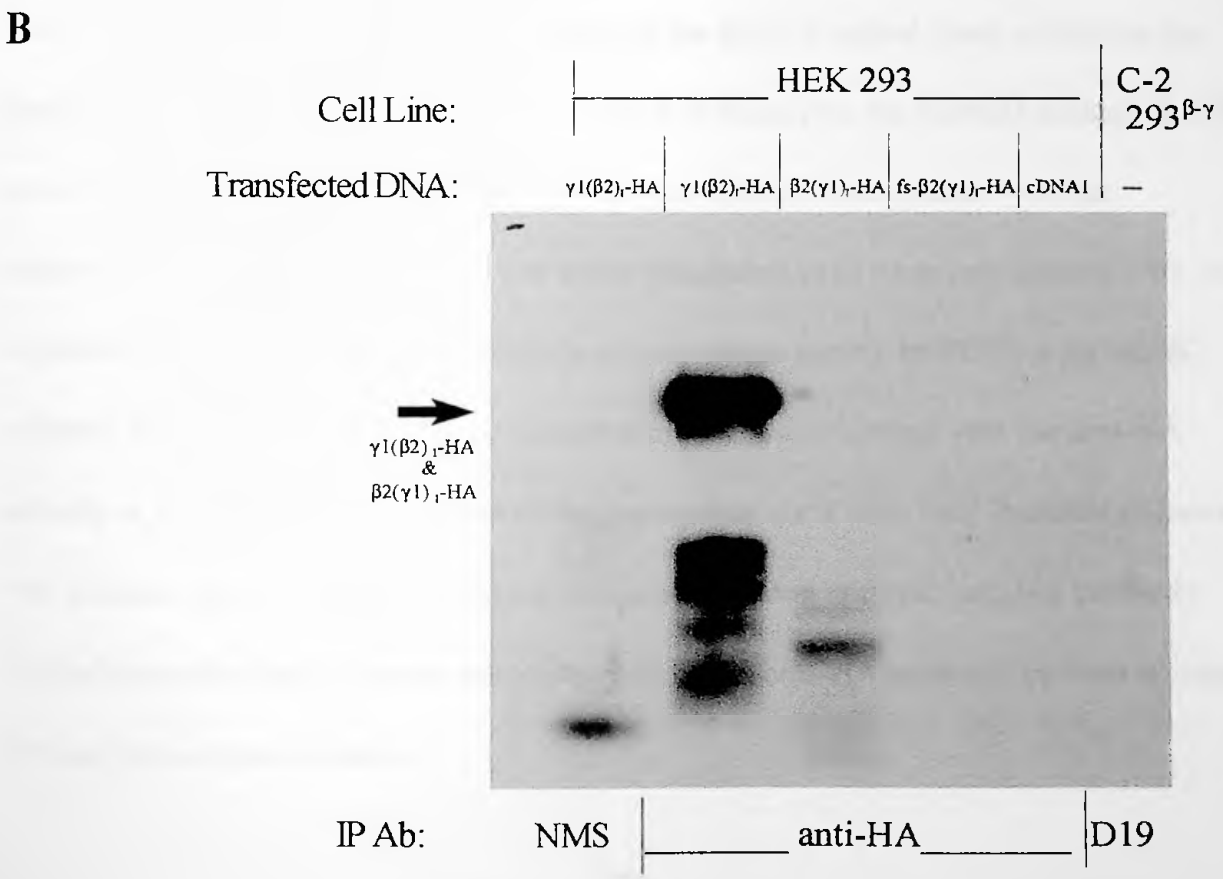


Figure 23. Hybrid Ln chains were created and epitope-tagged by subcloning in-frame into the β2-up/ HA/ cDNA I vector. A) PAGE of HEK 293 cell lysates transiently transfected with 6 μg DNA each of $\beta2(\gamma1)_I$, $\gamma1(\beta2)_I$ or β2-up/ HA/ cDNA I and probed with the anti-HA antibody at 1:1,000 dilution. Proteins of the expected sizes (~ 190 kD) were recognized in cells transformed with a hybrid construct but not those with the vector alone. This demonstrates that these cells express the hybrid protein, that the protein does contain the HA epitope, and the anti-HA antibody recognizes specifically the tagged protein and not other proteins in HEK 293 cells.

B) HEK 293 cells were transiently transfected with 6 μg each of the DNA construct listed above the lanes. All "-HA" names indicate that hybrid is in the β2-up/ HA/ cDNA I vector. "F.S. $\beta2(\gamma1)_I$" is a frame-shift mutant of the $\beta2(\gamma1)_I$ hybrid chain which has the signal sequence and the epitope tag region fused in frame plus the hybrid Ln chain fused out of frame. HA/cDNA I is the empty β2-up/ HA/ cDNA I vector and ---- is the untransfected C-2 $^{\beta-\gamma}$293 cell lysate. The lysed transfected cells were precipitated with the antibodies listed below the figure. NMS is normal mouse serum, and D19 is an anti-β2 antibody. Precipitated proteins were subjected to PAGE and blotted with the anti-HA antibody at 1:1000 dilution. Proteins of the appropriate sizes were only detected in lanes with in-frame epitope-tagged hybrid Ln chains precipitated with the anti-HA antibody. This indicates that these proteins are recognized by the anti-HA antibody by both western blot and immunoprecipitation.

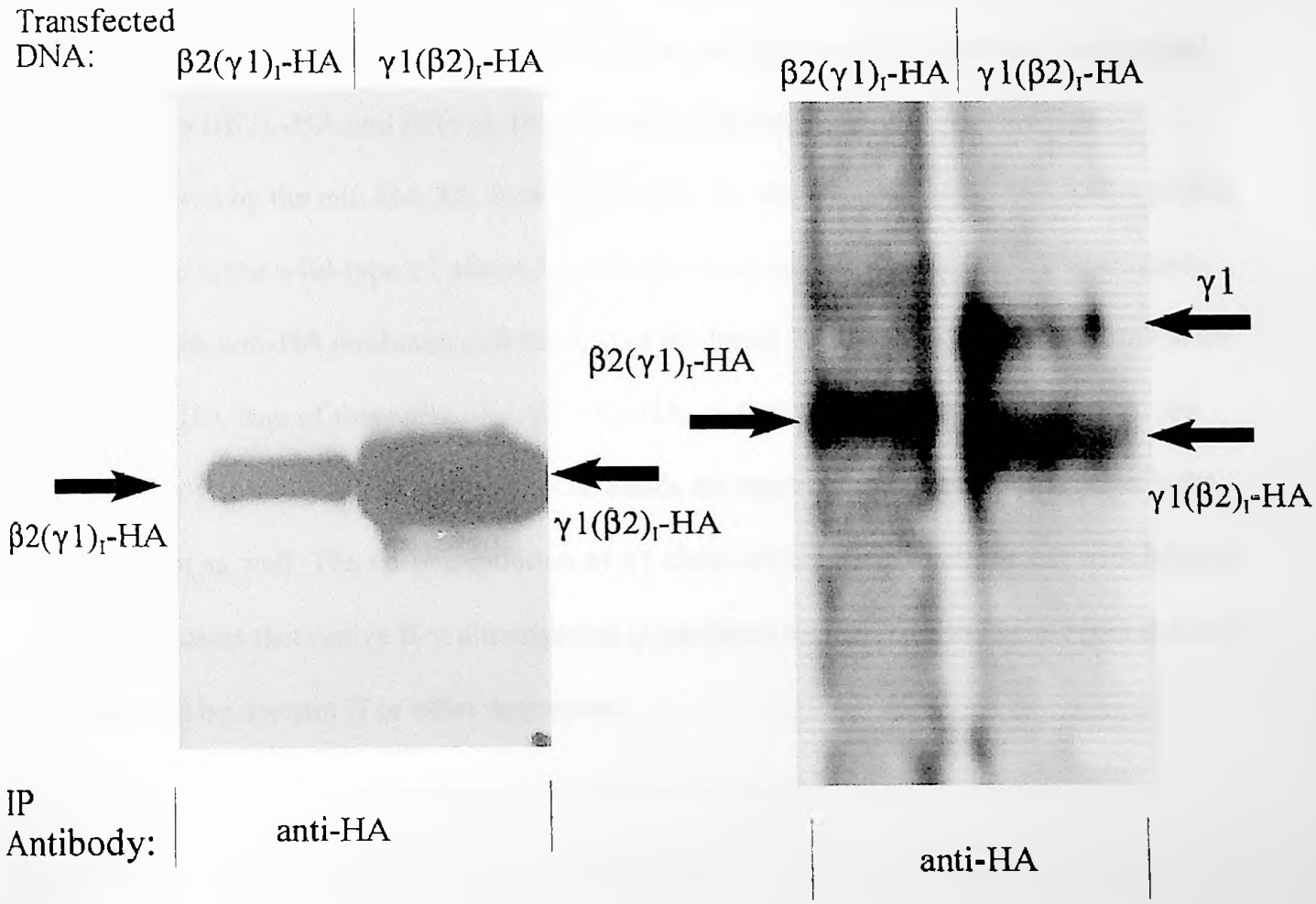
Domain I of the hybrid laminin chains determines interaction specificity.

Once the hybrids were shown to be expressed and were able to be precipitated by anti-HA antibody, these hybrid laminin chains were then transiently transfected into the **C2-293$^{\beta-\gamma}$**, **A2-293$^{\beta}$**, **D4-293$^{\gamma}$** and **β2 (Δ IV-V)-293** cell lines to determine if they could assemble with wild-type β2 or γ1 chains or the truncated β2. Cell lines were transiently transfected with one of three DNA plasmid constructs, β2(γ1)$_I$-HA/cDNA1, γ1(β2)$_I$-HA/cDNA1 or the empty HA/ cDNA1 vector. After an overnight incubation with the DNA, the medium was changed to 293 medium + 200 $^{\mu g}/_{ml}$ G418 and the cells were incubated for 48 - 72 hours. Immunoprecipitation followed by western blot detected the presence of chains which co-precipitated with the HA-tagged hybrid chain.

In the **D4-293$^{\gamma}$** cell line, which constitutively express only γ1 chain protein, the γ1 chain co-precipitates with the **γ1(β2)$_I$-HA** hybrid Ln chain but not with the **β2(γ1)$_I$-HA** hybrid, when precipitated with anti-HA mAb. No γ1 chain protein was detected in the empty HA vector control lane (not shown). On one western blot utilizing the YY15 Ab, a doublet can be seen in the lane containing transfected **γ1(β2)$_I$-HA** hybrid immunoprecipitated by the anti-HA mAb (Figure 24). As mentioned before, the YY15 antiserum did cross-react slightly with **γ1(β2)$_I$-HA** chain. Careful comparison with the anti-HA mAb blot of the same membrane showed that the lower band was the **γ1(β2)$_I$-HA** hybrid and the upper band was the full-length γ1 chain (Figure 24). The wild-type γ1 chain then, was co-precipitated with **γ1(β2)$_I$-HA** by the anti-HA mAb.

Figure 24.

D-4$^{\gamma}$-293 Cell Line Transfections and Immunoprecipitations



Western Blot Antibody : Anti-HA YY15

Figure 24. D-4$^{\gamma}$- 293 cells, which express the γ1 chain, were transiently transfected with 8 μg of HA epitope-tagged β2(γ1)$_I$/HA/cDNA I or 6 μg of HA epitope-tagged γ1(β2)$_I$/HA/cDNA I. The cells were lysed and protein concentrations were measured. Equal amounts of protein from each cell lysate were precipitated by anti-HA antibody. Immunoprecipitated proteins were separated by electrophoresis on a 4.5 % polyacrylamide gel, and transferred to nitrocellulose membrane for detection by YY15 (anti-γ1) antibody (right blot). This same membrane was stripped of the first Ab and reprobed with anti-HA antibody (left blot). The left blot (anti-HA) shows a strong signal for both γ1(β2)$_I$-HA and β2(γ1)$_I$-HA protein, indicating that both proteins were precipitated by the anti-HA Ab. In the right blot, the larger of the two bands in the γ1(β2)$_I$ -HA lane is the wild-type γ1 chain, based both on its recognition by the YY15 antibody, but not the anti-HA antibody, and the size of the band. Wild-type γ1 is not detected in the β2(γ1)$_I$-HA lane of this same blot. β2(γ1)$_I$-HA, and to a lesser extent γ1(β2)$_I$-HA, are both recognized by YY15 antibody. The bands for these hybrid chains are labeled in the right blot as well. The co-precipitation of γ1 chain with γ1(β2)$_I$-HA but not with β2(γ1)$_I$-HA indicates that native β-γ dimerization is mediated by the domain I sequences and not inhibited by domain II or other sequences.

Transfection of the **A2-293$^{\beta}$** cell line with the **$\gamma1(\beta2)_I$-HA** and the **$\beta2(\gamma1)_I$-HA** hybrids showed more evidence for the specificity of domain I in Ln chain interactions. The full-length β2 constitutively expressed in these cells was co-precipitated with the transiently expressed **$\beta2(\gamma1)_I$-HA** hybrid by using the anti-HA mAb (Figure 25). However, the same was not true with the **$\gamma1(\beta2)_I$-HA** hybrid; full-length β2 chain was not co-precipitated with this hybrid (Figure 25). Precipitation with the anti-HA mAb of the empty HA vector transfected cells did not show a recognizable band upon immunoblotting with R49 or D5 antisera (not shown). Thus, the hybrid chain containing domain I of the γ1 chain, but not the hybrid containing domain I of the β2 chain, interacted with the full-length β2, indicating that heterodimeric (β - γ) chain interaction is due to domain I sequences.

Using the **β2 (Δ IV-V)-293** cell line, the interaction of β2 with the $\beta2(\gamma1)_I$-HA hybrid seen in the **A2-293$^{\beta}$** line was confirmed. The constitutively expressed truncated β2 chain co-precipitated with the **$\beta2(\gamma1)_I$-HA** hybrid, but not with the **$\gamma1(\beta2)_I$-HA** hybrid, in immunoprecipitations with the anti-HA mAb. No bands were seen in lysates of cells transfected with the empty HA vector, precipitated by the anti-HA mAb, and immunoblotted with anti-HA, anti-β2, or anti-γ1 (not shown). As seen in Figure 26, domain I of γ1 in the hybrid protein showed affinity for the IV-V deleted β2 chain, while domain I of β2 in the hybrid did not. This again implicates domain I sequences in forming β - γ interactions. Since this truncated β2 chain was capable of forming heterodimers

Figure 25. A-2$^{\beta}$-293 Cell Line Transfections and Immunoprecipitations

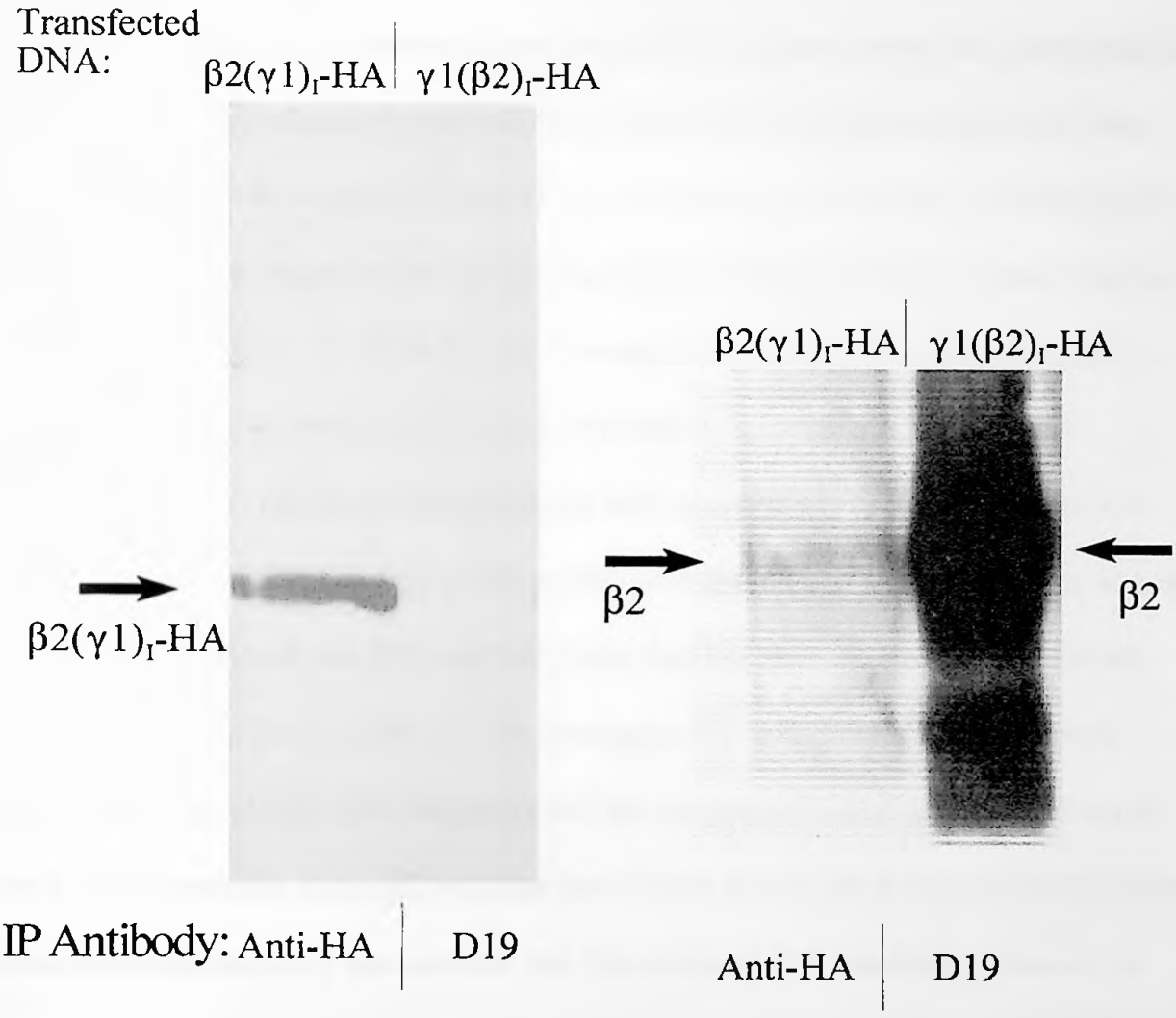


Figure 25. The A-2$^{\beta}$-293 cell line, which constitutively expresses the β2 chain, was transiently transfected with 8 μg of HA epitope-tagged β2(γ1)$_I$/HA/cDNA I or 6 μg of HA epitope-tagged γ1(β2)$_I$/HA/cDNA I. The cells were lysed and protein concentrations were measured. Equal amounts of protein from each of the cell lysates were precipitated by anti-HA antibody or D19 Ab as indicated below the blots. Immunoprecipitated proteins were separated by electrophoresis on a 4.5 % polyacrylamide gel, and transferred to nitrocellulose membrane for detection by R49 (anti-β2) antibody (right blot). This same membrane was stripped of the first Ab and reprobed with anti-HA antibody (left blot). The D19 Ab recognizes an epitope in domain III of the β2 chain; thus it does not recognize the γ1(β2)$_I$-HA chain but does recognize full-length β2 expressed in these cells. In the left blot, the band detected by the anti-HA antibody is β2(γ1)$_I$-HA, demonstrating that the immunoprecipitation with anti-HA Ab worked. No protein is recognized by anti-HA antibody in the γ1(β2)$_I$-HA lane. Therefore, the γ1(β2)$_I$-HA did not co-precipitate with the full-length β2 chain that was precipitated by the D19 Ab.

In the right blot the R49 Ab does recognize the full-length β2 chain in cells transfected with γ1(β2)$_I$-HA indicating that the precipitation with D19 Ab did occur. The band of the same size in the β2(γ1)$_I$-HA lane is also full-length β2 chain, but since the immunoprecipitation Ab was anti-HA Ab, this demonstrates that the β2 chain is co-precipitated with β2(γ1)$_I$-HA chain. Thus, β2(γ1)$_I$-HA does associate with the β2 chain, but the γ1(β2)$_I$-HA hybrid does not.

Figure 26. β2(△VI-V)-293 Cell Line Transfections and Immunoprecipitations

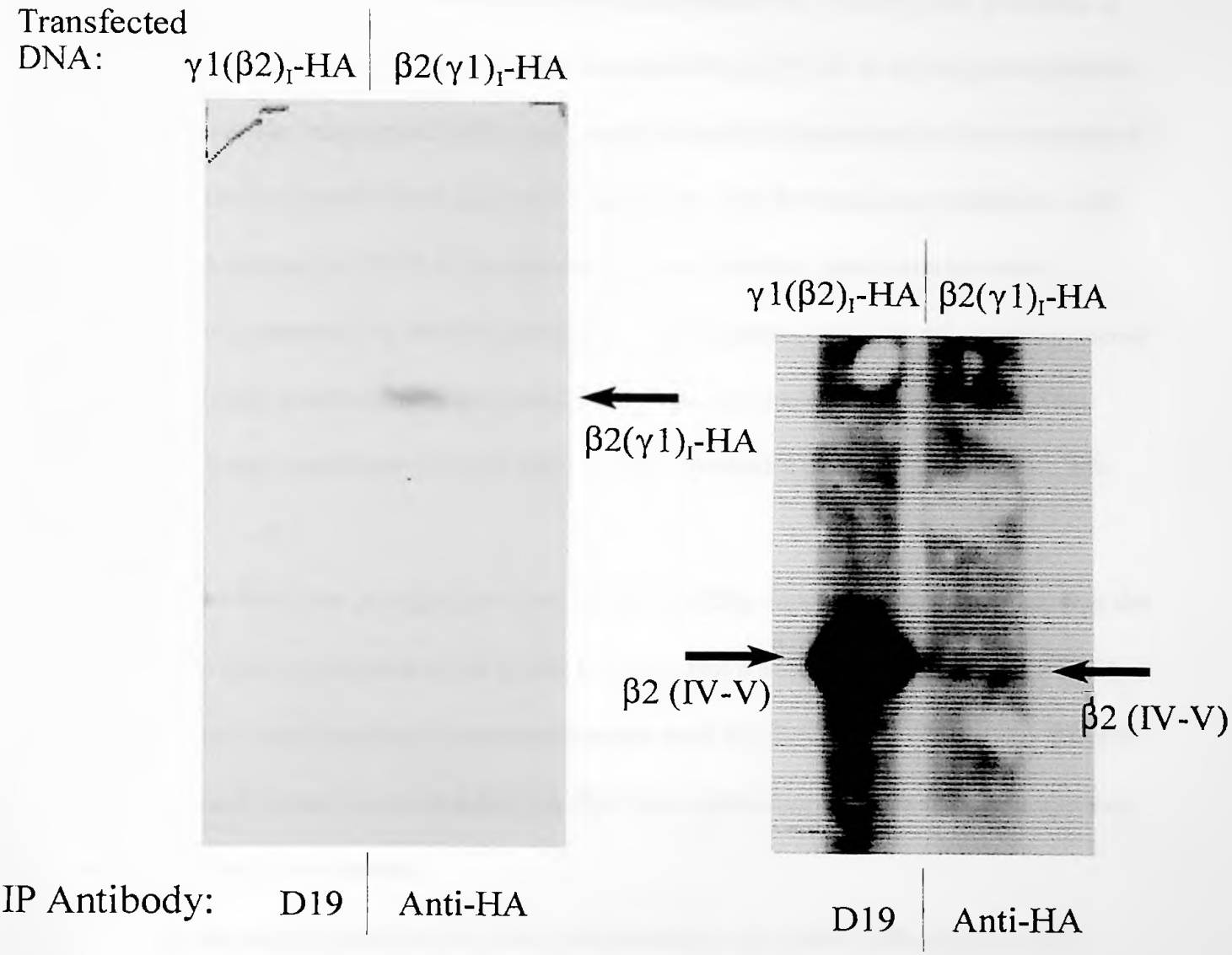


Figure 26. A second β2 chain expressing cell line, β2(ΔIV-V)-293, was transiently transfected with the $\beta2(\gamma1)_I$-HA and $\gamma1(\beta2)_I$-HA hybrids. This cell line constitutively expresses a β2 chain which has domains IV and V deleted and is therefore approximately 55 kD smaller than full-length β2; however, it is still recognized by the R49 Ab (Figure 21). Since it still contains the entire long arm plus domain III, it should still assemble in the same manner as full-length β2 and be recognized by D19 Ab in immunoprecipitation.

Transiently transfected cells were lysed and protein concentrations were measured. Equal amounts of protein from each cell lysate were used in immunoprecipitations with the anti-HA antibody or D19 Ab as indicated below the blots. Immunoprecipitated proteins were separated by electrophoresis on a 4.5 % polyacrylamide gel, and transferred to nitrocellulose membrane for detection by R49 (anti-β2) antibody (right blot). This same membrane was stripped of the first Ab and reprobed with anti-HA antibody (left blot).

In the R49 blot (at right), the lane labeled $\gamma1(\beta2)_I$-HA has a band showing that the β2(ΔIV-V) chain was precipitated by the D19 Ab. But a band corresponding to $\gamma1(\beta2)_I$-HA is absent, demonstrating no co-precipitation with the β2(ΔIV-V) chain. The β2(ΔIV-V) chain band is also seen in the $\beta2(\gamma1)_I$-HA lane, indicating that it was co-precipitated with the $\beta2(\gamma1)_I$-HA hybrid.

In the anti-HA blot (at left) the band marked by the arrow is the $\beta2(\gamma1)_I$-HA hybrid protein. No band was detected in the $\gamma1(\beta2)_I$-HA lane, indicating that it did not

co-precipitate with β2(ΔIV-V). These results, as with the results from the D-4$^{\gamma}$- 293 and A-2$^{\beta}$-293 cell lines, shown in Figures 24 and 25, implicate domain I in dimer formation of γ1 and β2. They also show that domain IV and V sequences of β2 are not necessary for chain assembly.

with γ1 like the full-length chain, it also indicates that domains IV and V are not involved in dimer formation.

Unlike the yeast two-hybrid system, which showed dimer formation of two β2 fragments, the β2 chain (full-length or IV-V deleted) did not interact with the **$\gamma 1(\beta 2)_I$-HA** hybrid. This may be do to the weak nature of these interactions, which are unstable in a full-length dimer. Another possibility is that they have formed but make up such a small percentage of the total number of chains that they are not detectable by blotting.

In transient transfections of the **C2-293$^{\beta-\gamma}$** cell line, which express both full-length β2 and γ1, full-length β2 did not co-precipitate with the **$\beta 2(\gamma 1)_I$-HA** hybrid nor did full-length γ1 chain co-precipitate with the **$\gamma 1(\beta 2)_I$-HA** hybrid when the precipitation antibody was anti-HA mAb. Occasionally the full-length γ1 chain co-precipitated with the full-length β2 chain when the precipitation antibody was the anti-β2 mAb D19. These results do not necessarily refute those found with the other cell lines, it may only indicate that the conditions for interaction are different in cells which constitutively express both β2 and γ1 versus those cells which express either β2 or γ1 alone.

Summary of the mammalian cell transfection and immunoprecipitation experiments

In order to determine the role of domain I of the β2 and γ1 chains within the context of the full-length protein, hybrid laminin chains were created and utilized in immunoprecipitation experiments to determine their ability to assemble with the wild-

type β2 or γ1 chain. The results of the mammalian cell culture expression of wild-type and hybrid laminin chains and immunoprecipitation experiments are:

1) The epitope-tagged hybrid laminin chains **β2(γ1)$_I$-HA** and **γ1(β2)$_I$-HA** are expressed at high levels when their respective vectors are transiently transfected into HEK 293 cells or the derived cell lines **C2-293$^{β-γ}$**, **A2-293^{β}**, **D4-293^{γ}** and **β2(Δ IV-V)-293**.

2) These hybrid chains are recognized and precipitated by the antibody to HA.

3) Full-length or truncated Ln β2 chains co-precipitate with the **β2(γ1)$_I$-HA** but not with the **γ1(β2)$_I$-HA** hybrid.

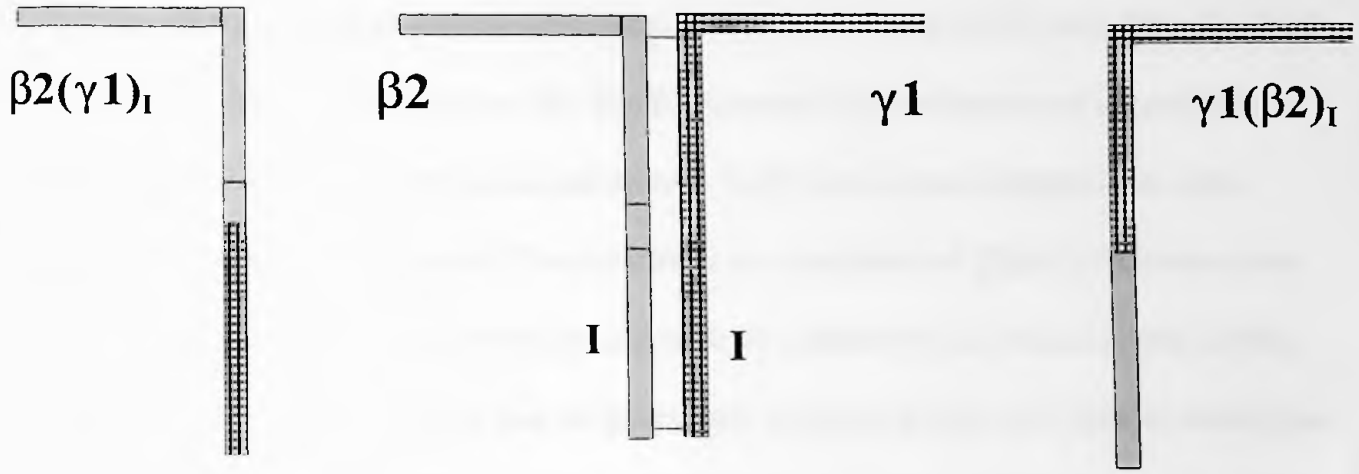
4) The Ln γ1 chain co-precipitates with the **γ1(β2)$_I$-HA** but not with the **β2(γ1)$_I$-HA** hybrid.

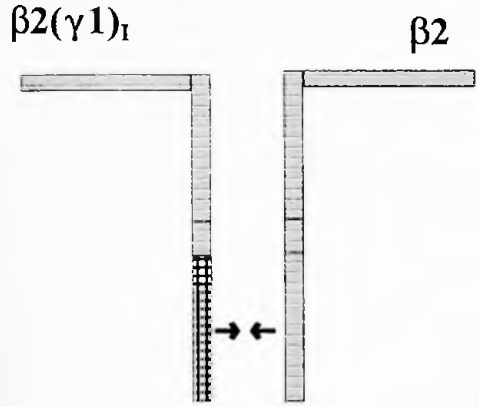
5) Neither hybrid Ln chain can co-precipitate a wild-type β2 or γ1 chain in the cell line **C2-293$^{β-γ}$**, which constitutively expresses both the β2 and γ1 chains.

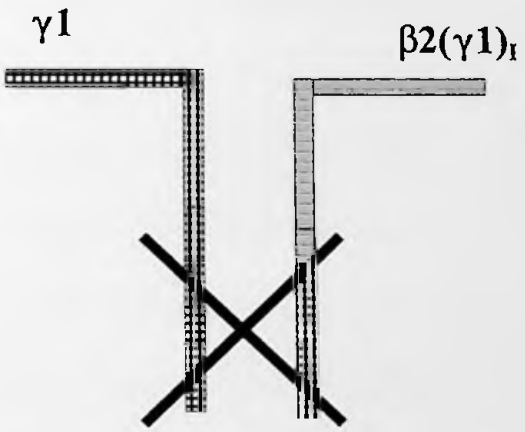
6) The empty HA vector does not produce proteins that are recognized or precipitated by the anti-HA mAb.

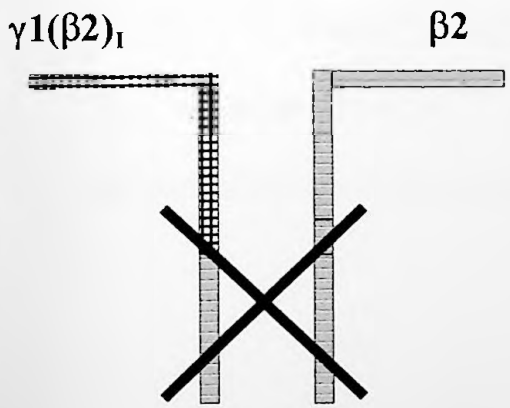
These results (schematically diagrammed in Figure 27) support the hypothesis that domain I sequences, and only domain I sequences, are involved in the interaction of the β and γ chains.

Figure 27. Interactions of Hybrid Laminin Chains in HEK 293 Cells









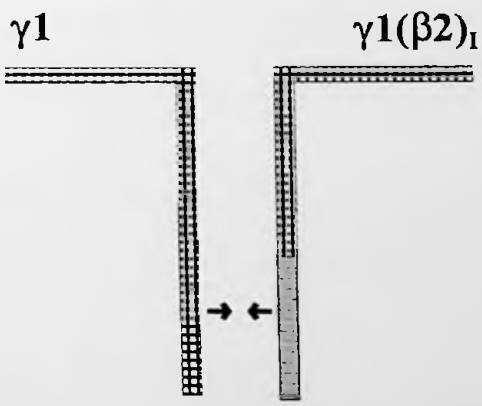


Figure 27. Schematic representation of the results of immunoprecipitation studies using the hybrid laminin chains. In the top row, the hybrid chains $\beta2(\gamma1)_I$-HA and $\gamma1(\beta2)_I$-HA are diagrammed on either side of the β2-γ1 dimer, which was shown to form in the C-2 $^{\beta}$-293 cell line (Figure 22). The β2 portions (stippled bars) and γ1 portions (checked bars) of the hybrid chains are shown. In the second row, interactions of the $\beta2(\gamma1)_I$ hybrid with full-length β2 or γ1 chains are diagrammed. $\beta2(\gamma1)_I$ -HA associates with β2 (arrows pointing together) but not with γ1 (shown by Xs). Results with $\gamma1(\beta2)_I$-HA (bottom row) demonstrate lack of interaction with β2 (at left), and dimeric interaction with γ1 (at right). These results indicate that β2-γ1 dimers are formed by interactions of the domain I of the hybrids.

Discussion

In order to determine the role of domain I in ordered specific assembly of the laminin chains and the regions within it that are responsible for this role, Ln chain interaction was assayed using both the yeast two-hybrid system and immunoprecipitation of full-length laminin molecules in mammalian cells. The immunprecipitation results indicate that domain I, and not other parts of the glycoprotein, is responsible for mediating specific β-γ dimer formation. The two-hybrid assay showed that dimers of domain I can occur in the pair β2 with γ1, but not in the pairs β2 with α1 or γ1 with α1. Dimers of β2-β2 also occur, but are weaker. The region from 38-75 amino acids from the C-terminus of the β2 chain is implicated in the mediation of the interaction with the γ1 chain. Finally, β-γ dimer formation is not dependent on disulfide bonding, based on the ability of the $^{C \rightarrow S}$ β2*I* fusion proteins to form dimers with γ1.

The long arm of the laminin trimer, which contains domain I and II, has been studied by both biochemical and biophysical methods. This has led to the discovery of its alpha-helical secondary structure and its dominant role in coiled-coil formation (reviewed in Beck et al. 1990). In conjunction with biochemical experiments of the reassembled E8 fragment, which first must form a dimer, then a trimer, an order of Ln chain assembly was postulated (I. Hunter et al. 1990). First the β-γ dimer forms followed by the addition of the α chain to complete the secreted trimeric glycoprotein. These studies have focused predominantly on the first step of this assembly, dimer formation.

Earlier experiments have focused on the very C-terminal region of domain I. Biophysical studies using small recombinant peptides of the Ln-2 domain I in *in vitro* assembly experiments determined that several 10-20 amino acid sites in the last 100 amino acids of the γ1 chain were responsible for dimer or trimer formation. Also, deletion of the C-terminal 17 amino acids of the β1 chain was sufficient to ablate its binding to a 217 amino acid C-terminal fragment of the γ1 chain (Utani et al. 1994). In this dissertation, the yeast two-hybrid system was used to study intracellular interactions of the entire 358 amino acids of domain I of the β2 chain as well as two fragments of it. Based on these results (Figures 13, 14 and 15), the C-terminal 75 amino acids are sufficient for strong interactions with the domain I of γ1, but the C-terminal 38 amino acids do not form dimers with γ1. Furthermore, both the 75 amino acid and the 38 amino acid fragment did not form homodimers, as did the full domain I fragment. This shows that the 37 amino acids from 1691- 1728 of the β2 chain may be involved in dimer formation with the γ1 chain. Because $\beta2_{75}$ interacts with γ1 but not with another β2 fragment, this region may impart specificity to Ln chain interactions by favoring interactions with only the γ1 chain.

The presumptive dimerization region found here for the β2 chain is more distal from the C-terminus than the one described by Utani et al. (1994), but in that work it was noted that trimer formation was abolished by deletion of the C-terminal 40 amino acids of the β1 chain. This may indicate that dimer structures are more stable intracellularly than

in vitro. Alternatively, it may simply show a difference in assembly regions for these two β chain isoforms. The results of the two-hybrid assays reported here do support the contention that the region of domain I that guides β-γ dimer formation is very near the C-terminus of these chains.

Cystine disulfide bridges have been shown to stabilize the coiled-coil structure of the Ln long arm and are found to occur in β-γ dimers (Antonsson et al. 1995; Kammerer et al. 1995; Niimi et al. 1997). Similarly, a mutation of the Cys_{1765} residue of the β2 chain to Ser (which prevents cystine disulfide bonding with other chains) does decrease, but does not ablate, domain I β-γ dimer formation in the two-hybrid system. This indicates that the C-terminal cysteine is not required for dimers to form, but may stabilize them.

One of two transformants containing the mutant $^{C\rightarrow S}\beta 2I$ and wild-type $\gamma 1I$ has decreased activity compared to transformants containing both wild-type $\beta 2I$ and wild-type $\gamma 1I$; however, no difference in homodimer formation is seen between $^{C\rightarrow S}\beta 2I$ and wild-type $\beta 2I$ fusions (Figure 17). Of the three Ln chain types the β chains have a conformation that is most likely to form coiled-coils. Homodimer formation of the β2 C-terminus may therefore occur as a default assembly of two proteins with a strong capability to form these structures. The fact that the $^{C\rightarrow S}\beta 2I$ mutant fusions still form homodimers indicates that the α-helical nature of this fragment is not disrupted by the amino acid substitution. These domain I mutants also behave like wild type in that there is a significant difference between the $^{C\rightarrow S}\beta 2I_{act} + \gamma 1I_{bind}$ transformants and the $\gamma 1I_{act} +$

$^{C\rightarrow S}\beta 2I_{bind}$ transformants. The reason for the increased activity in transformants containing the $\beta 2I_{act}$ (either wild-type or mutant) fusion was not able to be determined in this system. One possible explanation is that the β2 domain I fragment, when fused to the activation domain, takes on a conformation that is better able to form a coiled-coil with the γ1*I* fragment. This would make the proximity of the activation and binding domains more like the native conformation and therefore better able to activate transcription.

Besides domain I (and domain α of β2), the long arm of Ln also includes domain II (Figure 2). This domain, separate from domain I, has not been studied as to its role in chain assembly, since domain I fragments are able to form dimers and trimers in the same manner as the entire protein (I. Hunter et al. 1990 & 1992; Nomizu et al. 1994 & 1996; Utani et al. 1994; Kammerer et al. 1995; Niimi et al. 1997). The α-helical nature of this domain suggests that it may mediate chain interactions (reviewed in Beck et al 1990). Results of two-hybrid assays revealed that domain II fragments of β2 and γ1 formed neither heterodimers nor homodimers. It may be that domain II plays a passive role in chain assembly and is simply a filler domain giving length to the Ln glycoprotein to allow proper spacing of its numerous cell and ECM-binding sites (reviewed in Beck et al. 1990). Alternatively, domain II interactions may require domains I and α to place them in proper register.

One intriguing and confounding observation also came from studies of domain I fragments in the two hybrid assay. The C-terminal 75 amino acids of γ1 in a fusion

protein with the GAL4 binding domain ($\gamma 1_{75\text{-bind}}$) was able to activate transcription on its own; i.e., apart from an activation domain fusion. One possible explanation is that this 75 amino acid fragment is able to interact with another transcriptional activator via non-specific α-helical interactions. Another explanation, given observations that the Ln β2 and γ1 proteins are found in nuclear fractions of transfected cell lines (Cui and Green, unpublished observations), is that the γ1 chain does have some transcriptional regulatory capability. Also, $\gamma 1_{75\text{-bind}}$ was the only fusion tested that showed a correlation between expression levels of the fusion protein and β-galactosidase activity. This differs from the other two-hybrid fusions, which required interaction to activate transcription. The amount of activity in these fusion proteins is related to the strength of interaction and not on amounts of protein.

The two-hybrid assay was an informative tool for studying the interactions of the Ln chains. It can be used to characterize further the individual amino acids responsible for guiding the specific interactions of Ln dimer formation. For instance, charged amino acids in the "a" and "d" positions of the heptad repeats of the β2 and γ1 chains could be substituted with apolar residues. Also, characterization of the differences between the β1 and β2 chains could be studied. As mentioned earlier there seems to be a discrepancy between the region responsible for dimer formation in β1(Utani et al. 1994) and β2 chains. With this system it would be possible to use the same fragments studied in biophysical recombinant peptide studies, such as the C-terminal 200 amino acids of β1

chain and its deletions, in order to detect differences between intracellular and *in vitro* interactions of Ln chain fragments.

As mentioned in the *Materials and Methods* section, sequencing revealed a single base pair difference between the γ1 cDNA, used to construct both the C-terminal 75 amino acid fusion and the mammalian expression vectors, and the published sequence at nucleotide 4958 (Durkin et al. 1988). This mutation changes amino acid 1589 from a leucine to a methionine residue. This amino acid was assigned to the "c" position in the heptad repeat structure of the γ1 chain by Beck et al. (1993). It is not a critical position for chain interaction because neither is it part of the hydrophobic face nor is it one of the charged amino acid positions, which interact to impart specificity to coiled-coil interactions. Furthermore, while methionine residues do not occur at this position with high frequency in coiled-coil proteins, they are not unusually rare (Lupas et al. 1991). For these reasons this mutation should not effect the protein structure and subsequent interactions in the recombinant proteins.

In order to achieve high yield of Ln chain proteins for study, cell lines were created that expressed either recombinant rat β2 chain (**A2-293$^{\beta}$**), recombinant mouse γ1 chain (**D4-293$^{\gamma}$**), or both together (**C2-293$^{\beta\text{-}\gamma}$** and **A1-293$^{\beta\text{-}\gamma}$**). Another cell line that expressed a truncated recombinant rat β2 chain (**β2(Δ IV-V)-293**) was also created. With these cell lines the intracellular interaction of full-length (or truncated) Ln chains was studied. *In vivo* immunohistochemical labeling and *in vitro* cell culture studies have

shown the presence of native (non-recombinant) β-γ dimers in cells that were not part of a trimer (Sorokin and Ekblom 1992; Matsui et al. 1995). Subsequent studies using larger or full-length recombinant Ln chains expressed in mammalian cells have shown that recombinant Ln chains can be assembled and secreted as trimers, and that disulfide bonded β-γ dimers do form (Matsui et al. 1995; Yurchenco et al. 1997). The long arm portion of these proteins was found to be essential for these interactions, as fragments corresponding to amino acids 1540-1765 of the β1 chain were seen to form both dimers with γ1 and trimers with α1(Niimi et al. 1997). In this work, recombinant β2 could form dimers with γ1 in HEK 293 cells.

The newly created stably transfected cell lines will be a useful model system for further studies of Ln chain assembly. With the addition of a full-length cDNA for an α chain, and antibodies which recognize that chain, it will be possible to examine trimer assembly as well. Furthermore, these cell lines are capable of post-translationally modifying the native Ln chains, making the recombinant molecule similar to the wild-type glycoprotein. Western blots and immunoprecipitations indicate that high levels of the transformed Ln chains are expressed in these cells. Using biochemical methods such as affinity chromotography, these cells could be harvested to provide purified Ln chains in native (or near native) conformation for use in biophysical or biochemical characterization of the Ln structure. Similarly, these purified chains could provide material for studies such as cell-binding or matrix formation properties like those

performed by Cheng et al. (1997), who studied polymerization of Ln trimers into a matrix *in vitro*. These studies would normally require purifying Ln isoforms that may be produced only in small quantities from organs such as kidney.

To assess the role of domain I in determining Ln chain interactions, it was studied in the context of a full-length molecule by creating two different hybrid Ln chains. The first hybrid, **$\gamma 1(\beta 2)_I$**, fused domain I of β2 to domains II-VI of γ1, and the second hybrid, **$\beta 2(\gamma 1)_I$**, fused domain I of γ1 with domains α-VI of β2. Subsequently, HA-epitope tagged versions of these hybrids (**$\gamma 1(\beta 2)_I$-HA** and **$\beta 2(\gamma 1)_I$-HA**, respectively) were created that retained the 5' untranslated region of the β2 cDNA as well as the β2 signal sequence (cDNA nucleotides 69-173 as reported in D. Hunter et al. 1989a), and which placed the HA epitope at the N-terminus of the protein. Placing the HA epitope at the N-terminus should avoid possible disruption of the interactions to be examined, which occur in the C-terminal portion of the protein. Also, using the authentic Ln signal sequence should make the processing of the recombinant glycoprotein more like the processing of the native protein.

The hybrid proteins were transfected into the Ln chain expressing cell lines to determine the role of domain I in interaction specificity. The **$\gamma 1(\beta 2)_I$-HA** hybrid interacted with full-length γ1 chain but not with full-length or truncated β2 chains, and the **$\beta 2(\gamma 1)_I$-HA** hybrid interacted with full-length and truncated β2 chains but did not form dimers with full-length γ1 chain. This indicates that the domain I of β2 or γ1 could

mediate dimer formation of full-length glycoprotein similarly to native Ln chain assembly. Thus, domain I alone can guide specific assembly of Ln chains into dimers.

The fact that the hybrid containing β2 domain I did not interact with the full-length β2 chain appears contradictory to the results of the two-hybrid system which showed interactions of two β2 domain I fragments. Remembering that the strength of these two-hybrid interactions was weaker than that of heterodimer interactions, it is probable that in the mammalian cell these interactions are too weak to sustain the dimerization of the whole molecule. Furthermore, in the HEK 293 cell the presence of chaperones may also play a role in specific interactions thereby preventing the β2 domain I region of the hybrid from coming into proximity of the full-length β2 chain.

These hybrid Ln chains provide a good model for studying the function of domain I as part of a full-length molecule. Again, with the addition of an α chain cDNA and antibodies to Ln α protein, these hybrids could be studied for their ability to form trimers. Their usefulness need not stop at the study of assembly. Any number of functions ascribed to the long arm of the Ln chain, such as cell attachment and neurite outgrowth stimulation (reviewed in Beck et al. 1990), could be restudied using a recombinant trimer with one or the other hybrid chains incorporated. Because studies of motor neuron adhesion to Ln β2 chain (D. Hunter et al. 1991) have been criticized for not utilizing a full-length or native glycoprotein (Brandenberger et al. 1996), the **$\gamma 1(\beta 2)_I$-HA** hybrid, which has the sequences proposed to bind motor neurons, could be utilized to re-evaluate

this binding to the β2 chain as part of a dimeric or trimeric molecule.

The β2-up/ HA/cDNA I vector (HA-vector) itself should be a useful tool for later studies. Since this vector contains the native β2 upper region in frame with the HA epitope sequence it can be utilized to tag full-length or truncated versions of the β2 chain, which could then be purified using the anti-HA antibody in affinity chromatography. And, since other laboratories have utilized signal sequences from non-Ln cDNAs to drive production of Ln chains (Niimi et al. 1997; Yurchenco et al. 1997) it seems that for *in vitro* studies this HA-vector could be used to epitope-tag any recombinant protein at its N-terminus.

The two-hybrid assay results support earlier work indicating Ln chain assembly is initiated by C-terminal sequences of the β and γ chains, which are part of the long arm of the Ln trimer. A 37 amino acid sequence near the C-terminus of the β2 chain (a chain not previously studied in this manner) is implicated as essential for specific interactions with the γ1 chain. Finally, by utilizing hybrid Ln chains with wild-type Ln chains in immunoprecipitations, it is concluded that β2 domain I and γ1 domain I, as part of full-length chains, are responsible for the specific β-γ dimers seen *in vivo*.

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ABSTRACT

Laminin, a major component of basement membrane, is a trimeric glycoprotein comprised of three chains - α, β and γ (Burgeson et al., 1994). An order for trimer assembly has been deduced: first, the β and γ chains bind to form a dimer and subsequently α is added to complete the trimer (I. Hunter et al., 1990 & 1992; Utani et al., 1994 & 1995). The C-terminal portions, found within the protein structural domain I of the β and γ chains, are implicated in dimer and trimer formation by biochemical studies performed extracellularly (Utani, et al., 1994 & 1995; Nomizu et al., 1995).

Using the yeast two-hybrid system, long arm fragments of the laminin chains β2, γ1, and α1 were assayed for their ability to form dimers. This assay confirmed the strong specific interactions between the β and γ chains seen in other studies of recombinant laminin fragments (Nomizu et al., 1994 & 1996; Utani et al., 1994 & 1995). Interactions of the α1 fragment with β2 or γ1 were weak or non existent in this assay. A region necessary for dimerization within the β2 chain was found between the C-terminal 75 and 38 amino acids, as the C-terminal 75 amino acids interacted strongly with γ1 domain I but the C-terminal 38 amino acids did not. Additionally, a domain I fragment of β2 containing a cysteine to serine substitution at amino acid 1765 (created to prevent disulfide bonding) was able to form dimers with γ1 domain I, indicating that non-covalent forces can mediate this interaction.

To determine the ability of domain I alone to mediate specific dimerization of the

β2 with the γ1 chain, the domain I regions of β2 and γ1 were switched to create two chimeric laminin chains. Epitope-tagged chimeras were tested for their ability to interact with the full-length wild-type β2 or γ1. In immunoprecipitation experiments wild-type β2 associated only with the chimera containing domain I of γ1 and wild-type γ1 co-precipitated only with the chimera containing domain I of β2. These results indicate that the domain I of laminin chains as part of a full-length chain can impart specificity to chain assembly within a cell.