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STRUCTURE-GUIDED IDENTIFICATION OF A LAMININ BINDING SITE ON THE LAMININ RECEPTOR PRECURSOR

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Abstract

The 37/67 kDa human laminin receptor (LamR) is a cell surface receptor for laminin, prion protein, and a variety of viruses. Because of its wide range of ligands, LamR plays a role in numerous pathologies. LamR overexpression correlates with a highly invasive cell phenotype and increased metastatic ability, mediated by interactions between LamR and laminin. In addition, the specific targeting of LamR with small-interfering RNAs (siRNAs), blocking antibodies, and Sindbis viral vectors confers anti-tumor effects. We adopted a structure-based approach to map a laminin binding site on human LamR, by comparing the sequences and crystal structures of LamR and *A. fulgidus* S2p, a non-laminin binding ortholog. Here, we identify a laminin binding site on LamR, comprising residues Phe32, Glu35, and Arg155, which are conserved among mammalian species. Mutation of these residues results in a significant loss of laminin binding. Further, recombinant wild-type LamR is able to act as a soluble decoy to inhibit cellular migration toward laminin. Mutation of this laminin binding site results in loss of migration inhibition, which demonstrates the physiological role of Phe32, Glu35, and Arg155 for laminin binding activity. Mapping of the LamR binding site should contribute to the development of therapeutics that inhibit LamR interactions with laminin, and may aid in the prevention of tumor growth and metastasis.

The 37/67 kDa human laminin receptor (LamR), was originally identified as a non-integrin cell surface receptor for the extracellular matrix molecule laminin^{1;2;3}. Laminins, other glycoproteins, collagen IV and proteoglycans constitute a tight network to form the basement membrane. Laminin-1, a 900 kDa glycoprotein, contains many bioactive domains involved in binding both integrin and nonintegrin receptors and is vital for basement membrane assembly⁴. Interactions between LamR and laminin play a major role in mediating changes in the cellular environment that affect cell adhesion⁵, neurite outgrowth⁴, and tumor growth and metastasis⁶.

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Competing Interests Statement

Some of the authors have competing interests. Specifically, the contents of this paper are being utilized for a patent. According to the rules and regulations of New York University School of Medicine, if this patent is licensed by a third party, some of the authors (D.M., S.R.H., K.V.J.) may receive benefits in the form of royalties or equity participation.

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Overexpression of LamR has been shown in many cancers, including lung⁷, breast⁸, gastric⁹, colon^{10;11}, ovarian^{12;13}, uterine¹⁴, thyroid¹⁵, prostate¹⁶, liver¹⁷, and melanoma^{18;19}. LamR expression correlates with a highly invasive cell phenotype and increased metastatic ability, mediated by the high-affinity interactions between LamR and laminin^{20;21;22;23;24}. Interactions between LamR and laminin contribute to tumor cell attachment to the basement membrane. These properties render LamR a prognostic factor in determining the degree of malignancy of human cancer patients²⁰. Understanding how LamR and laminin interact may provide insight into tumor invasion and metastasis.

LamR has been observed to act as the cell surface receptor for pathogenic prion protein²⁵ and a variety of viruses, including Sindbis virus^{26;27;28}. LamR has also been reported to bind EGCG, a polyphenol extract found in green tea²⁹. Interestingly, both human LamR and the p40 ribosomal protein are encoded by the same gene, 37LRP/p40³⁰. Thus, human LamR precursor protein has a dual function as a component of the translational machinery and as a cell surface receptor for laminin and other molecules. Intracellular LamR is found on the 40S ribosome and polysomes^{31;32} and in the nucleus³³, which suggests that LamR is a multifunctional protein. Silencing LamR expression in mammalian cells *in vitro* induces G1 cell cycle arrest, protein translation shutdown, and loss of LamR association with the polysomes³². Sequence conservation of 37LRP/p40 genes across species has been demonstrated, with evolution of the C-terminal tail convergent with vertebrates³⁰. At the cell surface, LamR, which is targeted to the membrane via fatty-acid acylation³⁴, exists as both a monomer (37 kDa) and a dimer (67 kDa)²⁵. Although the homo- or hetero-dimeric state of LamR has not been fully resolved, both 37 kDa and 67 kDa LamR bind laminin^{35;36}.

Characterization of LamR interactions with laminin has not been fully conclusive. Several LamR segments, including residues 161–180 (peptide G)³⁷, residues 205–229³⁸, and TEDWS-containing C-terminal repeats³⁹ have been implicated as binding epitopes for laminin. We previously determined a high-resolution crystal structure of the majority of LamR, residues 1 through 220 (abbreviated LamR220)³⁶ (PDB code 3BCH). LamR220 binds laminin with similar affinity as full-length LamR and inhibits Sindbis virus infection *in vitro*³⁶. It has also been shown that some of the phenotypic effects of silencing endogenous LamR can be rescued by exogenous full-length LamR and LamR220⁴⁰. Based on analyses of sequence conservation and of the crystal structures of human LamR and *A. fulgidus* S2 ribosomal protein, we introduced a number of point mutations into LamR220 to probe LamR binding to laminin. In the current study, we have mapped a laminin binding site on LamR, comprising residues Phe32, Glu35, and Arg155.

Expression and characterization of LamR mutants

We previously solved the crystal structure of a biologically active domain of human LamR, LamR220³⁶. LamR220 binds laminin with similar affinity to full-length LamR (LamR295)³⁶, demonstrating that LamR220 is sufficient for laminin binding. For these studies, LamR220 was used for mutagenesis due to its higher level of expression and purity compared to LamR295. Despite the implication in an earlier study³⁷ of residues 161–180 in laminin binding, the laminin binding site has remained elusive since the crystal structure of human LamR revealed that this domain is not solvent accessible³⁶. To better understand LamR interactions with laminin, we designed LamR mutants based upon analysis of sequence conservation and the crystal structures of human LamR220³⁶ (PDB code 3BCH) and its non-laminin binding ortholog, *Archaeoglobus fulgidus* S2 ribosomal protein⁴¹ (PDB code 1VI6).

In total, 14 mutants were analyzed individually (Table 1, Fig. 1a). Protein expression and purification of each mutant, including by gel filtration, was similar to that of wild-type

LamR220, which indicates that the mutations did not interfere with protein folding or stability. The purity of the proteins was monitored by SDS-PAGE (Fig. 2b). All mutants were examined for their ability to bind immobilized laminin. Purified recombinant LamR220 (greater than 95% purity) was incubated in serial dilution on pre-coated laminin plates, and binding was detected by anti-His HRP. As a control for binding specificity, we tested laminin binding for *A. fulgidus* S2p ribosomal protein, which should not interact with laminin. *A. fulgidus* S2p does not exhibit laminin-binding activity, as expected (Fig. 2a). These data support our hypothesis that differences between human LamR and *A. fulgidus* S2p are important for laminin binding.

Mapping of the laminin-binding region of LamR by site-directed mutagenesis

Structural and sequence analyses afforded selection of solvent-exposed residues that could contribute to laminin binding. Our studies examined the role of three categories of mutations: structurally divergent regions between human LamR and *A. fulgidus* S2p, residues previously implicated in laminin binding, and non-conserved residues between human LamR and *A. fulgidus* (Fig. 1a). Human LamR and its ortholog *A. fulgidus* S2p share 32% sequence identity. Superimposition of the structures of LamR220 and *A. fulgidus* S2p revealed two areas in which the structures are divergent (Fig. 1b): a segment between $\beta 4$ and $\beta 5$ (residues 111–118 in LamR) and a segment after the last α helix (αE) (residues 190–194 in LamR), in which LamR contains a five-residue insertion relative to *A. fulgidus* S2p. In *A. fulgidus* S2p, the segment between $\beta 4$ and $\beta 5$ (residues 111–118 in LamR) is stabilized in a folded-back conformation via a salt bridge between Arg113 and Asp93. These residues, which correspond to Arg117 and Thr97 in LamR (Fig. 1a and 1b), project away from the domain in LamR220 and instead pack against a symmetry (two-fold)-related molecule in the crystal structure. Within this crystallographic dimer, Ala114 packs into a tight pocket in the symmetry-related molecule and Phe116 is in van der Waals contact with Tyr139. We mutated LamR Ala114 to Lys and Phe116 to Ala (abbreviated A114F/K116A), the corresponding residues in the *S. cerevisiae* S2 ribosomal protein ortholog. Wild-type LamR220 bound laminin with an K_d of 2.3 μ M, and *A. fulgidus* S2p showed no binding to laminin (Table 1, Fig. 2a). A114F/K116A had no effect on laminin binding (Table 1, Fig. 2a).

The second region of structural divergence between human LamR and *A. fulgidus* S2p is a 5-residue insertion in human LamR, comprising residues Ser190 through Pro194 (Fig. 1b). Mutation of all five residues (Ser190-Pro194) to Ala resulted in no change in laminin binding compared to wild-type LamR220 (Table 1, Fig. 2a). In addition, we examined mutation of a previously identified putative laminin binding site, peptide G. Peptide G, residues 161–180, was implicated as a binding epitope in assays that utilized peptide segments of LamR to bind laminin³⁷. Of this segment, only residues Lys166 and His169 are solvent accessible in the LamR220 crystal structure³⁶. Mutation of Lys166 to Ala does not affect laminin binding (Table 1, Fig. 1a), which suggests that Lys166 is not essential for the laminin-binding function of LamR.

Lastly, we selected LamR residues Leu16, Phe32, Glu35, Arg53, Glu56, Lys57, Arg102, His131, Tyr139, Asn141, and Arg155 (Fig. 1a and 1c), which are solvent-exposed in the LamR220 structure and show sequence conservation among laminin-binding species³⁰, but are not conserved in the sequence of the non-binding *A. fulgidus* S2p. These mutations include changes in charge (for Glu35, Arg53, Glu56, Lys57, Arg102, His131, and Arg155) and in hydrophobicity (for Leu16, Phe32, Tyr139, and N141). Only mutations Phe32, Glu35, and Arg155 resulted in an appreciable loss of binding affinity (Table 1, Fig. 2a). Single point mutation of LamR Phe32 to Val (the corresponding residue in *S. cerevisiae* S2

ribosomal protein), or Glu35 to Lys, or Arg155 to Ala (the corresponding residues in *A. fulgidus* S2 ribosomal protein) resulted in a decrease in laminin binding (8- to 42-fold increase in K_d) (Table 1, Fig. 2a). Double point mutation of Phe32 and Glu35 (abbreviated F32V/E35K) or Phe32 and Arg155 (abbreviated F32V/R155A) resulted in a greater loss of laminin binding activity than the single mutants (Table 1, Fig. 2a). Mutation of His131, Tyr139, and Asn141, which are in close proximity to Arg155, Phe32, and Glu35, did not affect laminin binding (Table 1, Fig. 1c). These data demonstrate that Phe32, Glu35, and Arg155 (Fig. 1c) comprise a primary laminin binding site.

Physiological significance of LamR mutants

To assess the physiological significance of residues Phe32, Glu35, and Arg155 in laminin binding, we developed a cellular assay to test wild-type and mutant LamR function. Previously, it has been demonstrated that LamR localizes to both the cell membrane and cytoplasm^{32;42}. Silencing LamR expression in HT-1080 cells results in protein translation and cell cycle arrest⁴⁰. Although transfection of FLAG-tagged LamR cDNA into cells is able to rescue the effects of silencing endogenous LamR⁴⁰, transfected LamR does not localize to the cell membrane (D.M., unpublished data). These results render studying the role of mutations in LamR at the cell surface difficult. As a result, we utilized purified recombinant LamR as a soluble decoy to interfere with endogenous LamR cell surface interactions with laminin in a cell migration assay. Cell migration to laminin has been previously observed^{43;44}. We examined the ability of recombinant wild-type or mutant LamR220 to inhibit HT-1080 cell migration towards laminin in a Boyden chamber in which cells were added to the top chamber and laminin or laminin with recombinant protein were added to the bottom chamber. When recombinant wild-type LamR220 is added with laminin, HT-1080 cell migration towards laminin is 8% of the migration observed relative to migration to laminin alone (Fig. 3a, WT). These data suggest that recombinant LamR220 interacts with laminin in solution, inhibiting cellular migration towards laminin. Cellular migration to recombinant protein alone is not observed. Protein purity and equal loading was examined by SDS-PAGE. *A. fulgidus* S2p, which does not exhibit laminin binding activity *in vitro*, was utilized as a negative control for migration inhibition (Fig. 3a, Rps2). LamR220 mutants F32V/E35K and F32V/R155A, which exhibited loss of laminin binding *in vitro*, were examined for loss of inhibition and restoration of cell migration to laminin. Both LamR220 mutants F32V/E35K and F32V/R155A demonstrate a significant loss of inhibition of cell migration to laminin (Fig. 3a, F32V/E35K and F32V/R155A). LamR220 F32V/R155A behaves similarly to *A. fulgidus* S2p (negative control), with 70% migration observed compared to migration to laminin alone. LamR220 F32V/E35K results in 46% of migration compared to migration to laminin alone. Cell migration towards 10% FBS is not inhibited by wild-type LamR220, mutant LamR220, or *A. fulgidus* S2p, which suggests that inhibition of cell migration to laminin by wild-type LamR220 is specific to interactions between LamR and laminin (Fig. 3b). Together, these data demonstrate the physiological role of Phe32, Glu35, and Arg155 in laminin binding.

Summary

The interaction between LamR and laminin mediates changes in the cell environment that affect cell adhesion and tumor growth and metastasis^{5;6;22;42}. We utilized sequence and structure analyses to guide LamR mutagenesis to map a laminin binding site on LamR. Both the mutagenesis data in the context of the LamR crystal structure and cell migration data indicate a primary laminin binding site comprising residues Phe32, Glu35, and Arg155 (Fig. 2). These residues are conserved among mammalian laminin-binding species³⁰, but not in *A. fulgidus* S2 ribosomal protein ortholog, which does not bind laminin or inhibit cell migration to laminin. Interestingly, mutation of two regions of structural divergence between human

LamR and *A. fulgidus* S2p, the $\beta 4$ - $\beta 5$ linker and a five-residue insertion after αE , did not affect laminin binding, suggesting that they contribute to other LamR functions. In addition, mutation of Lys166 to Ala, which was previously implicated in laminin binding³⁷, does not affect binding *in vitro*.

In the setting of cancer, interactions between LamR and laminin contribute to modifications of the extracellular matrix structure that affect cancer cell growth and proliferation as well as tumor invasion and metastasis, and activate proteolytic enzymes and their regulators^{6;45;46}. The specific inhibition of LamR interactions with laminin has been shown to elicit anti-tumor effects^{32;42;47;48}. Targeting LamR in the tumor environment may be possible due to a larger percentage of unoccupied LamR sites compared to normal cells, on which laminin binding is saturated^{22;23;24}. Mapping the LamR binding site for laminin may contribute to the development of therapeutics that inhibit LamR interactions with laminin and aid in the prevention of tumor growth and metastasis.

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Figure 1. Analysis of LamR structural and sequence features. (a) Sequence alignment between 37LRP/p40 orthologs *H. sapiens* LamR (residues 1–220) and *A. fulgidus* S2p ribosomal protein (residues 1–198). Residue numbering is for human LamR. Mutated residues that cause a significant change in laminin binding are in red. Mutated residues that cause a slight or no reduction in affinity are in cyan. Secondary structure (α helices and β strands, magenta) for LamR220 is shown according to PROCHECK⁴⁹. Gray lines indicate the N- and C-terminal portions of the structure that are disordered. (b) Superimposition of human LamR220 (magenta) and *A. fulgidus* S2p (cyan) in ribbon format. α helices and β strands are labeled according to (a). The regions of structural divergence, between the β 4– β 5 loop (residues 111–118) and after α E (residues 190–194), are labeled. (c) Surface representation of LamR220 mutations. LamR220 (grey) with Phe32, Glu35, and Arg155 labeled (red) and shown in stick, mutations that do not affect laminin binding (cyan) are also labeled and shown in stick.

Figure 2.

Analysis of LamR binding affinity for laminin. **(a)** Binding of LamR and mutants to laminin. LamR220 (black), *A. fulgidus* S2p (light grey), LamR220 A114K/F116A (dark grey), LamR220 S190-P194 polyAla (purple), LamR220 Phe32Val (blue), LamR220 R155A (green), LamR220 E35K (orange), LamR220 F32V/R155A (pink), and LamR220 F32V/E35K (red). $n = 3 \pm$ SEM. **(b)** Coomassie blue staining of *A. fulgidus* S2p, LamR220 and mutants that affect binding for experimental loading control. Residues 1–220 of human 37 kDa LamR precursor protein (LamR220) and LamR220 mutants were expressed and purified as previously described³⁶. Residues 1–208 of *A. fulgidus* S2p were cloned, expressed and purified utilizing the same conditions as human LamR220. The LamR220 construct described above was used as the backbone for generating LamR220 point mutants in the LamR coding region with the QuikChange site-directed mutagenesis kit XL II (Stratagene). Complementary forward and reverse primers were designed to mutate LamR220 residues. PCR-based point mutations were made using thermal cycling according to the Stratagene protocol, the PCR product was digested with DpnI restriction enzyme (NE Biolabs) and transformed into *E. coli* strain XLII Blue (Stratagene). LamR220 vectors positive for mutations were verified by automated DNA sequencing. Wild-type and mutant LamR220 binding affinity for laminin were tested *in vitro*. Protein concentration was determined by Protein Dc Assay (Biorad). Clear polystyrene ELISA 96-well microplates, precoated with murine laminin (NEBiolabs), were blocked for 1 hr at 37° C with blocking buffer (2% FCS, 1mg/ml BSA, 0.1% sodium azide in PBS). Wells were incubated with increasing concentrations of purified wild-type or mutant LamR220, which all contain a 6XHis-tag, for 1 h at 37° C. Each well was washed three times with wash buffer (0.5% Tween in PBS). Penta-His HRP conjugate (1:500) (Qiagen) was incubated for 2 h at RT. After washing, substrate solution was added and fluorescent absorbance was detected at 490 nm on an ELISA plate reader (ELX800, Biotek Instruments, Inc.). A binding curve, K_d , and standard error for K_d were generated for wild-type and mutant LamR220 using GraphPad Prism software. The K_d was calculated using a one-site binding hyperbola and the equation $Y = B_{max} \times X / (K_d + X)$. Each group was tested in triplicate and binding affinity was determined by normalizing to background fluorescence.

Figure 3.

Recombinant wild-type LamR inhibits HT-1080 cell migration towards laminin. **(a)** Inhibition of cell migration to laminin by wild-type LamR, but not LamR mutants. Laminin alone (grey), laminin + *A. fulgidus* S2p (horizontal stripes), laminin + wild-type LamR220 (checked), laminin + LamR220 F32V/E35K (vertical stripes), laminin + LamR220 F32V/R155A (diagonal stripes). $n = 3 \pm$ SEM. The differences between wild-type LamR220 inhibition and either LamR220 F32V/R155A or F32V/E35K are both statistically significant ($P < 0.0001$). **(b)** HT-1080 cell migration towards FBS is not inhibited by recombinant wild-type or mutant LamR. FBS alone (grey), FBS + *A. fulgidus* S2p (horizontal stripes), FBS + wild-type LamR220 (checked), FBS + LamR220 F32V/E35K (vertical stripes), FBS + LamR220 F32V/R155A (diagonal stripes). $n = 3 \pm$ SEM. HT-1080 cells were tested for their ability to migrate to 10 μ g/mL purified laminin (Invitrogen) or 10% FBS using the CytoSelect™ 24-Well Cell Migration Assay (8 μ m, Colorimetric Format) (Cell Biolabs, Inc.). HT-1080 cells were obtained from the American Type Culture Collection (Mannassas, VA). HT-1080 cells were maintained in DMEM 4.5 g/ml glucose with 10% FCS. All basal media was supplemented with 100 μ g/ml of penicillin-streptomycin and 0.5 μ g/ml

amphotericin B (all from Mediatech). Briefly, 1.5×10^5 cells in unsupplemented media were added to the upper insert chamber of each well. For (a), the reservoir below contained 500 μ l of either unsupplemented media, purified laminin, or purified laminin and recombinant wild-type LamR, mutant LamR, or *A. fulgidus* S2p. For (b), the reservoir below contained 500 μ l of either unsupplemented media, media supplemented with 10% FBS, or 10% FBS and recombinant wild-type LamR, mutant LamR, or *A. fulgidus* S2p. All protein was at 1 μ M final concentration. Protein concentration was determined by Protein Dc Assay (Biorad). After the incubation at 37°C for 5 hours the upper membrane of each insert was thoroughly washed with dH₂O 3X to remove non-migratory cells and then incubated in cell stain solution for 20 minutes at room temperature. Inserts were washed again in dH₂O and then air-dried. After agitation for 10 minutes at RT in extraction solution, 100 μ l of each sample was transferred to an ELISA plate and read at 630nm. To calculate migration, background migration to unsupplemented media was subtracted. Migration to laminin or FBS in the presence of either wild-type LamR, mutant LamR, or *A. fulgidus* S2p was normalized to migration to laminin or FBS alone. Assays were performed in triplicate. Results were plotted using GraphPad Prism software and statistical significance was calculated using a standard Student's *t*-test. ($P < .0001$).

Table 1

Effect of mutations of LamR on laminin binding activity. Half-maximal binding (K_d) was generated using nonlinear regression of binding curves in GraphPad Prism. Relative binding refers to the percentage ratio of LamR220 wild-type K_d /mutant K_d . NB indicates mutations that result in loss of laminin binding activity.

Mutant	Half-maximal binding [μ M]	Relative binding (%age)
WT LamR220	2.3 $\pm$ 0.16	100
L16Q	2.8 $\pm$ 0.15	82
R53A E56A K57A	1.9 $\pm$ 0.21	121
R102A	2.7 $\pm$ 0.16	85
A114K F116A	3.5 $\pm$ 0.31	66
H131A	5.4 $\pm$ 0.28	43
Y139A	6.2 $\pm$ 0.9	37
N141A	2.2 $\pm$ 0.13	105
K166A	4.3 $\pm$ 0.92	53
S190-P194 polyAla	2.0 $\pm$ 0.17	115
F32V	97.0 $\pm$ 41.8	2
E35K	18.5 $\pm$ 2.9	12
R155A	52.8 $\pm$ 20.8	4
F32V E35K	NB	N/A
F32V R155A	NB	N/A