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Comprehensive Proteomic Analysis of Non-Integrin Laminin Receptor Interacting Proteins

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Abstract

Human non-integrin laminin receptor is a multifunctional protein acting as an integral component of the ribosome and a cell surface receptor for laminin-1. Laminin receptor is overexpressed in several human cancers and is also the cell surface receptor for several viruses and pathogenic prion protein, making it a pathologically significant protein. This study focused on the proteomic characterization of laminin receptor interacting proteins from *mus musculus*. The use of affinity chromatography with immobilized recombinant laminin receptor coupled with mass spectrometry analysis identified 45 proteins with high confidence. Following validation through co-immunoprecipitation, the proteins were classified based on predicted function into ribosomal, RNA processing, signal transduction/ metabolism, protein processing, cytoskeleton/ cell anchorage, DNA/ chromatin and unknown functions. A significant portion of the identified proteins is related to functions or localizations previously described for laminin receptor. This work represents a comprehensive proteomic approach to studying laminin receptor, and provides an essential stepping-stone to a better mechanistic understanding of this protein's diverse functions.

Keywords

laminin receptor; RPSA; ribosome; cancer; extracellular matrix; mass spectrometry; chromatography

INTRODUCTION

Non-integrin laminin receptor (LamR), also referred to as p40 and ribosomal protein SA (RPSA), was originally identified as a 67 kDa cell surface protein with the ability to interact with laminin-1 in the extracellular matrix [1–3]. Since its discovery, multiple functions and subcellular localizations have been described. LamR is important for cellular translation as an integral component of the 40S ribosomal subunit and it is conserved across species from

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The data associated with this manuscript may be downloaded from ProteomeCommons.org Tranche, https://proteomecommons.org/tranche/, using the following hash: Password: LamR2012
1. For specific binding proteins
eJ6IH/CZjr3pZ+l+HbJ3t21AJrv2xy2Z4o240vPPpxtCR7bk7aRhM51HNk0RcUubJM1xMlZAmTiHWnuvq9vl90KDpTcAAAAAAhcETA==
2. For non-specific binding proteins
Ci5JXnTjDHxWe8HByWK2fRwQUpco+aSEJLD30B2HRlDLL/i4mHAIL+Wdf+pFA/I/imB3S7BVntTmwFVtvjSxira1bVcAAAAAAhc0TA==

plants to mammals [4–8]. LamR also plays a role in ribosomal RNA processing and is critical for maturation of the 40S subunit [4, 9, 10]. Additionally, LamR has been observed in the nucleus bound to histone proteins and it is hypothesized to play a role in chromatin regulation [11].

LamR has several functions at the cell membrane. It acts as the cell surface receptor for several viruses including Sindbis virus [12], Venezuelan equine encephalitis virus [13], dengue virus [14, 15], adeno-associated virus serotypes 2, 3, 8 and 9 [16], as well as for prion proteins [17, 18], and cytotoxic necrotizing factor type 1 [19]. Several studies also show that LamR plays a role in cell motility [20–26]. More specifically, a recent study from our laboratory indicated that LamR co-localizes with actin at lamellipodia structures and could be a component of focal contacts [27].

Overexpression of LamR is prevalent in a number of human cancers [28–35] and correlates with poor patient prognosis [31, 36, 37]. LamR plays a role in several functions related to cellular transformation. There is a direct relationship between tissue vascularity and LamR expression [31, 38]. Ribosomal association enables LamR to play a role in maintaining the increased metabolic needs of tumor cells [6, 7]. Additionally, LamR plays a central role in tumor invasion and metastasis [39–42].

Even with its multi-functional nature and many observed localizations relatively little is known about LamR-specific interacting proteins. This study used recombinant LamR protein coupled with affinity chromatography and mass spectrometry analysis to isolate novel binding partners. The new knowledge of LamR interacting partners will hopefully shed some light onto previous observations and foster a better understanding of this complex protein.

MATERIALS AND METHODS

Protein Purification

Recombinant LamR (rLamR) was purified as described previously [43]. Briefly, a construct expressing human full length LamR was transformed into *E.coli* strain BL21 (DE3*) and grown in Luria broth to OD_{600} of 0.6 at 37°C with constant agitation. Protein expression was induced by the addition of 0.1 mM, isopropyl-thiogalactopyranoside, at 20°C for 18 hours. Cells were harvested and lysed by French press. The lysate was cleared by centrifugation at 16,000 RPM for 30 minutes and filtered through a 0.45 μm filter. The protein was purified using the AKTA purification system (General Electric) in two steps: by Ni-NTA chromatography (General Electric) followed by gel filtration chromatography (Superdex 75) (Amersham). Purified protein sample was run on a polyacrylamide gel and stained with Imperial protein stain (Pierce) to assess quality and purity of the purified protein sample.

Cell Line

NIH 3T3 cells were obtained from the American Type Culture Collection. Cells were maintained in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal calf serum, 100 μg/ml of penicillin-streptomycin and 0.5 μg/ml amphotericin B (all from Mediatech).

Cell Lysate Preparation

Cell lysates were collected using Mammalian Protein Extraction Reagent (Pierce) with the addition of 150 mM NaCl and supplemented with EDTA-free complete protease inhibitor (Roche) according to the manufacturer's instructions. For chromatography samples, 20 mM

imidazole was also added. Lysates were cleared by centrifugation at 16,000 RPM for 30 minutes and filtered through a 0.45 μm filter. Protein content of the resulting supernatant was measured using BioRad D_c Protein Reagent according to manufacturer instructions (BioRad). For chromatography, lysates containing 15 mg total protein were precleared with Ni-NTA beads (Qiagen) by incubation for 2 hours at 4°C with end-over-end shaking. Precleared supernatant was collected by gravity flow separation from the Ni-NTA beads.

Chromatography

Purified full length LamR was concentrated in spin concentrators (Millipore) and 1 mg protein was rebound to the Ni-NTA column. Five column volumes of buffer were flowed over the column to remove unbound protein. Ultraviolet readout was monitored throughout. Fifteen mg of whole cell lysate was collected from NIH 3T3 cells (as described above) and injected onto the column at a flow rate of 0.5 ml/min. Column was washed with 10 column volumes of buffer to remove unbound protein. Bound proteins were eluted from the column with the addition of imidazole. For LamR only control sample, purified LamR was bound and eluted under the same conditions without the addition of cell lysate. For the lysate only sample, whole cell lysate was flowed over an empty column and then eluted under the same conditions as above.

Mass Spectrometry

Samples corresponding to peak elution fractions from the chromatography experiment (described above) were run on a 10–20% polyacrylamide gel and stained with Imperial protein stain (Pierce). Each lane was excised and cut into 16 slices. Each slice was analyzed using LC/MS/MS at the Rockefeller University Proteomics Facility. Gel samples were reduced, alkylated and then subjected to in-gel proteolytic digestion with trypsin. Peptides were extracted with 50% acetonitrile + 0.1% trifluoroacetic acid. Peptides were resuspended in water and subjected to liquid chromatography/ mass spectrometry (LC/MS/MS) analysis. LC: Ultimate 3000 system (Dionex), with in-house made C18 analytical column (75 μm diameter beads), C18 5 μm trap column from LC Packings, 60 min gradient mixture of water + 0.1% formic acid and acetonitrile + 0.1% formic acid. Flow rate through trap column was 30 μl/min, flow rate through analytical column was 0.2 μl/min. MS/MS: LTQ Orbitrap XL (Thermo Scientific), mass range 400–1600 m/z, ion trap used for MS/MS, 5 μl injections. The precursor scan was carried out at a mass resolution of 30,000. Data was recorded in profile mode. Seven precursors from each scan were selected for fragmentation. Dynamic exclusion was used to resolve the less intense components of the sample with the following parameters: exclusion list size 500, duration, 60 seconds, exclusion by mass with both high and low exlusion mass widths of 1.5. The normalized collision energy of the ion trap was 35. Raw data was used to create .dta files. These files were used by Mascot (Matrix Science) to search nr.fasta (selected for Mus musculus, 2.2.25, 139163 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.80 Da and a parent ion tolerance of 25 PPM. Iodoacetamide derivative of cysteine was specified in Mascot as a fixed modification. Oxidation of methionine was specified in Mascot as a variable modification. Mascot files were loaded into Scaffold for further analysis. X! Tandem (version 2007.01.01.1) (The GPM) was run in subset mode during the Scaffold analysis using the same parameters as those used for the Mascot search (listed above). Scaffold (version Scaffold_3.2.0, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the Peptide Prophet algorithm [44]. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. Protein probabilities were assigned by the Protein Prophet algorithm [45]. Proteins that

contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

Co-Immunoprecipitation

Whole cell lysate was collected from NIH 3T3 cells (as described above). For co-immunoprecipitation 250 μg cell lysate was incubated with 10 μg of anti-LamR antibody (Santa Cruz) for 2 hours at 4°C with agitation. Dyna beads (Invitrogen) were washed with phosphate-citrate buffer and added to the lysate-antibody complex and incubated overnight at 4°C with agitation. Beads were washed three times with phosphate buffered saline containing 0.05% Tween20. Proteins were eluted with protein sample buffer and heated at 95°C for 10 minutes. Immunoprecipitation samples were run on polyacrylamide gels (BioRad) under reducing conditions. Protein was transferred to polyvinylidine fluoride membrane (Millipore). Membranes were blocked with non-fat dry milk and probed with anti-myosin light polypeptide 6 (MyL-6), anti-erbb2 interacting protein (ERBIN), anti-p21 activated kinase interacting exchange factor (β-PIX), anti-ribosomal protein, small subunit (RPS) 17, anti-cortactin, anti-SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 1 (SMARCC1)/BRG1 associated factor 155 (BAF155) or anti-zona occludens protein 2 (ZO-2) (all from Santa Cruz). Proteins were detected using horseradish peroxidase conjugated secondary antibodies (Santa Cruz) and then exposed by chemiluminescence (Pierce).

RESULTS

Affinity Purification of LamR Binding Proteins from NIH 3T3 Cell Extracts

Full length LamR was expressed and purified from E.coli. In previous studies this methodology has proven effective in producing a highly pure sample of functional protein [43]. The purified protein contains an N-terminal Histidine tag, which was used to rebind purified recombinant LamR to the Ni-NTA column. Whole cell lysate, extracted from NIH 3T3 cells, was used as a source of cellular proteins. Lysate was injected onto the column containing immobilized rLamR at a flow rate of 0.5 ml/min to facilitate binding. Proteins and rLamR were eluted with the addition of imidazole. To control for background LamR protein and non-specific binding to the column matrix, protein was eluted from a column with only immobilized rLamR and another with lysate only respectively. Elutions were analyzed by SDS-PAGE and stained with Imperial protein stain (Figure 1). Numerous proteins bound to the column containing both immobilized LamR and lysate. The control sample, lysate only, had relatively few proteins bound and the LamR only control column had predominantly three bands, which correspond to the full length LamR protein, and two C-terminal degradation products.

This methodology generated a reproducible staining pattern with Imperial stain and, therefore, these samples were used for mass spectrometry analysis. Each gel lane was divided into 16 slices and following digestion, tryptic peptides were extracted from the gel and analyzed by LC/MS/MS. Data was then analyzed using the Scaffold program for protein identification and to enable inter-sample comparison. Proteins present in the control samples were treated as non-specific and excluded from further analysis. An average of 8 peptides were identified for each protein, with a range from 2–34 peptides. Protein identifications were accepted if they could be established at greater than 99.0% probability and contained at least 2 identified peptides. These parameters yielded a false discovery rate for peptides of 0.4% and for proteins of less than 0.01% as calculated by Scaffold. This study identified 45 unique proteins that met these stringent criteria. The predicted functions and localization of these proteins are summarized in Figure 2 and tables I–VII. Additionally, lists of the

individual peptides identified and the non-specific proteins are available in the supplemental material.

Classification of LamR Binding Protein Functions and Cellular Localizations

LamR is a multifunctional protein that plays a role in translation, as a component of the ribosome, cell motility, as a cell surface receptor, and nuclear functions through its ability to bind to histone proteins. Results of this study support and extend the current literature. Functional classification of the identified proteins indicated interacting partners involved in ribosomal functions, RNA processing, nuclear functions and cytoskeletal functions as well as some new classifications such as protein processing and signal transduction (Figure 2A). Additionally, the ability to perform these diverse functions is supported by LamR's multiple cellular localizations, which were mirrored in the identified interacting partners (Figure 2B).

Validation of Mass Spectrometry Results

This study identified proteins from diverse cellular processes many of which were not previously known to be interacting partners of LamR. To determine the validity of these newly identified proteins co-immunoprecipitation from whole cell extracts was used. The use of co-immunoprecipitation also evaluates the ability of these proteins to interact in the context of the cellular environment rather than the screening method, which used recombinant LamR. In total 7 proteins, approximately 15% of those identified were chosen for validation. The selected proteins are from different functional classifications and localizations as not to bias the validation process. Co-immunoprecipitations were performed from whole cell extracts using LamR-specific antibodies and matched normal IgG as a negative control. Each of the 7 selected proteins interacted with LamR over three separate experiments (Figure 3). These data serve to validate the proteins identified from the mass spectrometry screen.

Specific LamR Binding Proteins Identified by Mass Spectrometry

Tables I–VII summarize the LamR binding proteins identified by mass spectrometry. Tables are functionally grouped, each listing the protein, accession number and related references. The number of peptides identified with ≥ 95% confidence by Scaffold is also listed. Additionally, two molecular weight assignments have been listed for each protein, the observed molecular weight, which is based on the gel migration and the calculated molecular weight, which is the predicted molecular weight value for each protein.

Table I lists the identified proteins involved in translation and ribosomal functions. LamR is an integral component of the small ribosomal subunit [6]. As expected, several other components of the 40S ribosomal subunit were also identified including RPS2, RPS17, RPS15a, RPS27, RPS29, RPS16, RPS23, and RPS25. Additionally, proteins from the large ribosomal subunit were also identified ribosomal protein, large subunit (RPL) 15, RPL18, RPL36 and RPL23a. Presumably these proteins were detected in the LamR binding fractions because they were in complex with the ribosome.

Table II describes proteins related to RNA processing and transcription that were identified from the LamR binding fraction. The Bms1-putative endonuclease protein, a GTPase that is part of the U3 RNA processing complex [46] and small subunit processome component 20, another component of the U3 complex [47], were identified. The U3 complex is critical for rRNA processing and ribosome biogenesis [48]. Aquarius, and splicing factor 3B (subunit 2), two intron-binding proteins, required for small nucleolar ribonucleoprotein (snoRNP) assembly were also identified [49, 50]. In addition to being a component of the ribosome, LamR plays a role in rRNA processing [9, 10]. It is possible that interactions with spliceosome components are related to the processing of ribosomal RNA. Large subunit GTPase 1

homolog, which is required for nuclear export of the formed 60S subunit was also identified. Again, this interaction is most likely related to LamR's role in ribosome biogenesis. U2-associated protein (SR140) and pre-mRNA processing factor 40 both associate with pre-mRNAs in the nucleus [51, 52]. B cell lymphoma 2 (bcl-2) associated transcription factor 1, a ribonucleoprotein involved in mRNA splicing was also isolated [53].

Table III lists proteins functioning in signal transduction and metabolism that were identified from the LamR binding fraction. β-PIX is an exchange factor for Rac/ cell division control protein 42 (Cdc42). It promotes lamellipodia formation and the turnover of focal adhesions both of which stimulate cell migration [54, 55]. Studies show that LamR plays a role in cell migration [40, 56, 57] and localizes to lamellipodia structures in migrating cells [27]. The LamR-β-PIX interaction may be important for regulating the dynamics of focal contacts and facilitating cell movement. ERBIN, a cytoskeleton-bound protein [58], regulates the erbb2 protein and disrupts the ras/raf interaction [59]. Literature also indicates that ERBIN plays a role in the maintenance of cell junctions [60], which coincides well with tight junction protein 2 (Table V), also identified in this study. These interactions suggest that LamR plays a role in signal transduction for cell motility.

Table IV describes proteins involved in protein processing. Several components of the T complex protein 1 (TCP-1) chaperone complex were isolated including TCP-1 alpha, TCP-1 theta and TCP-1 zeta. Heat shock protein 65 (hsp65) chaperone protein was also identified. Chaperones bind to nascent polypeptides and facilitate folding [61]. These proteins may have bound to LamR due to C-terminal degradation of the recombinant protein, as indicated by lower molecular weight species in Figure 1. This degradation could result in a loss of protein structure, which would likely bind chaperone proteins. Ribophorin II, a component of the N-oligosaccharyl transferase complex, which links mannose to proteins [62] and transmembrane emp24-like trafficking protein 10, a yeast protein with homology to TMP21 [63], which is involved in vesicle trafficking, were also identified. Higher molecular weight forms of LamR have been observed, including the originally isolated 67 kDa LamR [64, 65]. It is possible that ribophorin II and emp24-like trafficking protein 10 play a role in the maturation of LamR.

Table V represents the LamR binding proteins classified as cytoskeleton and cell anchorage. Alpha tubulin, a major component of microtubules and vimentin, an intermediate filament protein, were both identified in this study. Previous studies have shown that ribosomes [20–26] and LamR specifically [27, 66] bind to components of the cytoskeleton. Centrosomal protein KARP-1 binding protein, a component of the centrosome and part of the microtubule organizing center was also identified. Immunofluorescence staining from a previous study indicated that LamR may be present at the microtubule organizing center [27], therefore, it may bind centrosomal protein KARP-1 binding protein in that context. Dynein cytoplasmic 1 intermediate chain 2, a molecular motor protein that converts ATP and transports proteins along microtubules was also isolated [67]. Additionally, proteins associated with the actin cytoskeleton, cortactin, which is involved in rearrangement of the actin cytoskeleton and plays a role in cell migration [68], and MyL-6, a structural component of muscle that can signal actin remodeling through integrin signaling [69] were isolated. Tight junction protein 2, also referred to as ZO-2, an integral component of intercellular junctions, which links the extracellular space with the actin cytoskeleton [70] was identified. LamR was shown to associate directly with actin filaments through *in vitro* binding assays and was also shown to co-localize through immunofluorescence studies [27]. Previous work indicated that LamR stabilized lamellipodia structures by bridging the extracellular matrix and actin, however, these interactions could also be important for signaling cytoskeletal rearrangement.

Table VI lists the proteins involved in DNA/ chromatin maintenance. Histone proteins H2A, H3 and H4 were all identified in this study. LamR localizes to the nucleus [71] and has been shown to bind directly to Histones H2 and H4 [11]. SWI/SNF complex subunit SMARCC1/ BAF155, which is thought to regulate transcription through alteration of chromatin structure [72] was isolated. It has also been shown that the BAF155 homolog in *D. melanogaster* is associated with nacent preRNPs, which could affect preRNA processing [73]. As described above, LamR plays a role in RNA processing and this interaction may relate to this function. Alternatively, its association with both histones and a chromatin modifying protein could indicate a direct role in chromatin maintenance or modification.

Table VII describes proteins with unknown functions that were identified in this study. The ubiquitin-associated protein 2-like protein, [74] atlastin GTPase 3, thought to play a role in golgi-ER morphogenesis [75] and sugen kinase protein 269.

DISCUSSION

This study identified 45 LamR-binding proteins from NIH 3T3 whole cell extracts. Several factors indicate that our methodology was effective in isolating authentic LamR-binding proteins. For this study the purity and activity of the recombinant protein was confirmed through SDS-PAGE and *in vitro* binding assays as described previously [43], (data not shown). The chromatography/ SDS-PAGE analysis showed a reproducible band pattern. Additionally, known LamR binding proteins were isolated from the experiment including alpha tubulin, Histone H2 protein and components of the ribosome.

The identified proteins were grouped based upon predicted function as ribosomal functions, RNA processing, signal transduction/ metabolism, protein processing, cytoskeleton/ cell anchorage, DNA/ chromatin or unknown (Tables I–VII). Several of these categories were expected based on the literature. Previous studies have described the ribosomal function of LamR [6, 7] as well as the related cytoskeletal [27] and RNA processing functions [4, 9, 10]. The DNA/ chromatin function has been reported as well [11]. There is also some evidence for a role for LamR in signaling after binding and internalization of cytotoxic necrotizing factor-1 [76]. However within these functional classifications some novel interacting partners have been identified that could shed some light on the specific mechanisms behind LamR functions.

LamR plays a role in intracellular functions, such as translation, through its role as a component of the 40S ribosomal subunit [6, 7]. As expected, several components of both the small and large ribosomal subunits were identified. Recently the crystal structure of the eukaryotic 40S ribosomal subunit was published indicating that LamR interacts directly with RPS2, RPS17 and RPS21 [77]. RPS2 and RPS17 were both identified in this study, however RPS21 was not. RPS21 is a peripheral protein on the ribosome and its binding affinity for LamR may be low. In this study, to reduce non-specific binding, more stringent lysis and wash conditions were used, which could have inhibited the LamR- RPS21 interaction. Presumably, the other ribosomal proteins were identified because they were part of a bound ribosomal complex.

In addition to its integral ribosomal functions, LamR plays a role in rRNA processing. The LamR homologs in yeast are essential for maturation of the 40S ribosomal subunit [4, 9, 78]. In this study several processome components, responsible for RNA processing were identified. Two components of the small subunit processome, which is involved in the production of 18S rRNA [79], bms1 putative endonuclease and small subunit processome component 20 homolog were identified. Bms1 putative endonuclease is the only GTPase required for maturation of the 40S subunit. It forms a complex bound to pre-rRNA and remains bound

till the 35S rRNA is cleaved to form 20S rRNA [80]. As LamR is involved in the 20S to 18S cleavage step [9, 10], it is possible that bms-1 recruits LamR to the processing complex. Additionally, identification of two components of the U2 spliceosome that bind and remove introns from cellular mRNA, splicing factor 3B and U2 associated protein SR140, could indicate a role for LamR in mRNA processing. The large subunit GTPase 1 homolog was also detected indicating that LamR could play a role in mediating export of the large ribosomal subunit as well.

In addition to its ribosomal functions, LamR has several extra-ribosomal functions, it is a cell surface receptor for several viruses and laminin-1. Although LamR was originally discovered as a 67 kDa protein, its gene encodes a 295 amino acid, 37 kDa precursor protein [81, 82]. One study suggests that both isoforms exist within the mouse brain and are capable of binding prion proteins [83]. Conflicting data exists about the composition of the higher molecular weight species. Mass spectroscopy analysis indicates that LamR exists as a homo-dimer at the cell surface [64]. Another study asserts that LamR hetero-dimerizes with galectin3 [84]. While a crystallographic dimer interface exists within the 37 kDa LamR [43], LamR does not associate with itself in a yeast two hybrid screen [65]. There is evidence that LamR is post-translationally modified by fatty acid acylation [64,84], this modification may be required for formation of a dimeric species and/or membrane association. This study used purified recombinant LamR, which is not post-translationally modified. The unmodified state of the protein could have biased against some interactions. LamR was first identified as a laminin binding protein and characterized based on its cell surface functions. This study did not identify laminin protein and also found relatively few membrane interactions. It is possible that use of LamR in its unmodified state enabled the isolation of proteins involved in the modification process. This study did identify proteins associated with processing functions such as transmembrane emp24 like trafficking protein 10, which is related to vesicle trafficking and membrane delivery. This interaction could be important for transporting LamR to the cell membrane. Additionally, ribophorin II, which is an oligosaccharide linkage protein, was also identified. 120–140 kDa glycoprotein (Gp120/140), another laminin binding protein requires the addition of oligosaccharides to interact with laminin [85]. It is possible that these proteins play a role in maturation of LamR and production of the 67 kDa isoform.

As mentioned previously, LamR is a receptor for laminin-1 in the extracellular matrix and therefore plays a role in adhesion [1–3]. This study also indicates that LamR binds to ZO-2, an integral component of tight junctions. ZO-2 serves to link the extracellular environment with the actin cytoskeleton; perhaps LamR aids in this process because it also binds extracellular laminin-1 [1–3, 43, 86] and the actin cytoskeleton [27]. Additionally, ZO-2 has been shown to inhibit cell proliferation through cyclin D [87]. LamR also plays a role in the maintenance of cell viability [9, 88–90]. Ablation of LamR expression with targeted siRNA results in arrest of the cell cycle [90] and in some cases apoptosis [89]. It is possible that interactions with ZO-2 are important for intercellular adhesion as well as cell proliferation.

The cell surface functions of LamR extend beyond just cell adhesion through binding of laminin-1 to include a role in cell motility [20–26]. It has been shown that LamR co-localizes with actin at lamellipodia and may be a component of focal contacts [27]. This study indicates that LamR binds to βPIX and ERBIN, two proteins that play a role in cell motility signal transduction. Interaction with these proteins indicates a more active role for LamR in cell motility, possibly in signal transduction.

LamR has also been observed in the nucleus bound to histone proteins [11]. In addition to histone proteins this study also identified the BAF 155 protein, a component of the SWI/SNF-A chromatin modification complex [72]. These interactions indicate a role for LamR in

chromosomal maintenance or modification and have interesting implications for LamR in regulating gene expression.

CONCLUSION

LamR is a complex protein with multiple functions and cellular localizations. It is well documented that LamR is upregulated in a number of cancers. Literature also indicates that LamR expression is intimately linked to a variety of processes altered in the tumor environment. Many of these observations lack a mechanistic understanding required to effectively develop anti-cancer therapies. Proteins identified in this study will serve to gain a better mechanistic understanding of LamR. The methodology presented here can also serve as a guide for further study of LamR interactions in different cell types or physiological conditions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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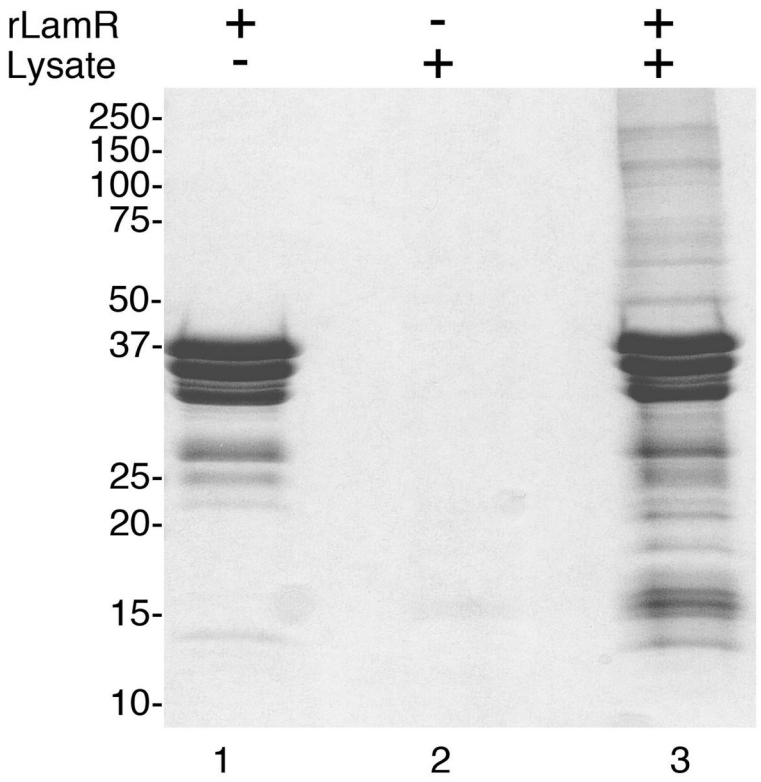

Figure 1. Affinity purification of LamR binding proteins

Stained elution fractions from affinity chromatography using immobilized recombinant LamR and NIH 3T3 whole cell extract. LamR only and Lysate only (lanes 1 and 2 respectively) served as controls for proteins related to rLamR protein and non-specific binding respectively. Lane 3 represents NIH 3T3 proteins that specially bound to immobilized rLamR protein. The molecular weight in kDa is indicated at the left.

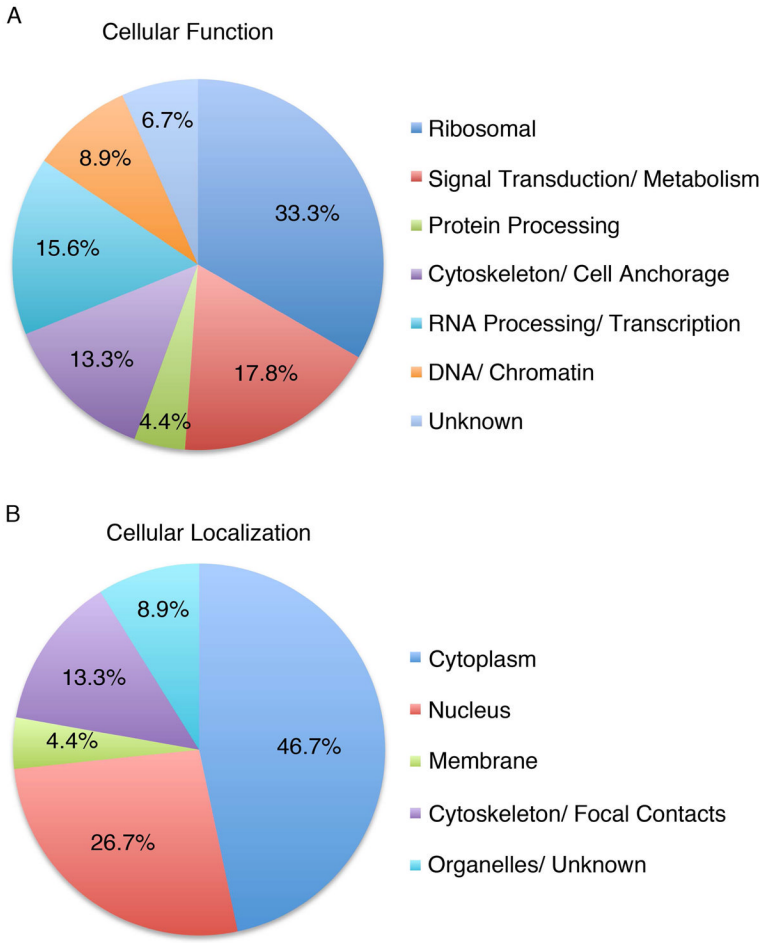

Figure 2. Distribution of LamR binding proteins

(A) Chart depicting the distribution of LamR binding proteins based on predicted function. (B) Distribution of LamR binding proteins grouped by theoretical cellular localization.

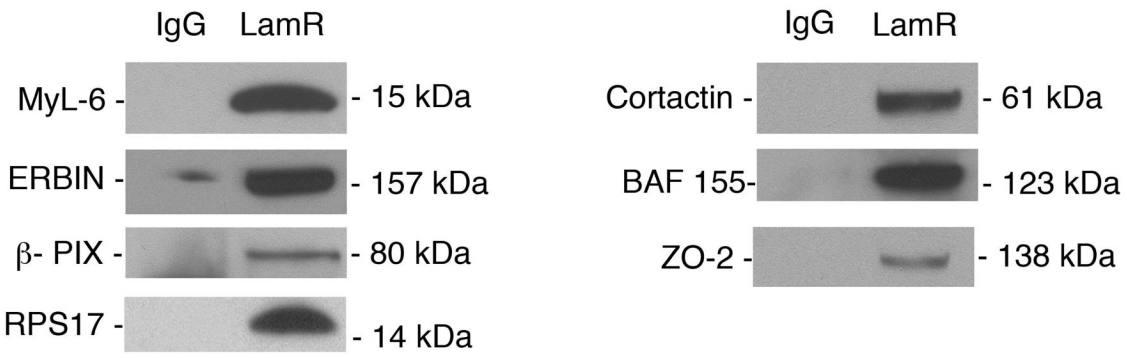

Figure 3. Validation of a subset of LamR binding proteins

Co-immunoprecipitation was employed to validate the protein-protein interactions predicted by chromatography/ mass spectrometry analysis. LamR-specific antibodies were used to capture LamR-protein complexes. Co-immunoprecipitation with normal rabbit IgG served as a negative control. Interactions between LamR and MyL-6, ERBIN, β-PIX, RPS17, Cortactin, BAF 155 and ZO-2 were successfully validated.

Table I

Ribosomal proteins that were identified in the LamR-binding fraction. Listed peptides represent those identified with ≥ 95% confidence by Scaffold

Protein	Accession Number	Number of Peptides	Observed Molecular Weight	Calculated Molecular Weight	References
Laminin Receptor (RPSA)	gi|171948782	30	33 kDa	33 kDa	4–8
Ribosomal Protein S2	gi|148705022	2	25 kDa	25 kDa	4–8
Ribosomal Protein S17	gi|148675005	12	14 kDa	14 kDa	4–8
Ribosomal Protein S15a	gi|30109302	7	17 kDa	15 kDa	4–8
Ribosomal Protein S27	gi|4506711	5	10 kDa	9 kDa	4–8
Ribosomal Protein L15	gi|13385036	4	24 kDa	24 kDa	4–8
Ribosomal Protein S29	gi|4506717	5	7 kDa	7 kDa	4–8
Ribosomal Protein S16	gi|200796	10	17 kDa	16 kDa	4–8
Ribosomal Protein L18	gi|51980472	8	22 kDa	22 kDa	4–8
Ribosomal Protein S23	gi|12846275	11	16 kDa	16 kDa	4–8
Ribosomal Protein L36	gi|149249185	3	12 kDa	12 kDa	4–8
Ribosomal Protein L23a	gi|20071865	16	18 kDa	18 kDa	4–8
Ribosomal Protein L24	gi|94386657	6	18 kDa	18 kDa	4–8
Ribosomal Protein S25	gi|4506707	3	14 kDa	14 kDa	4–8
Ribosomal Protein L27	gi|148702098	2	17 kDa	17 kDa	4–8

Table II

Proteins involved in RNA processing that were identified in the LamR-binding fraction. Listed peptides represent those identified with ≥ 95% confidence by Scaffold.

Protein	Accession Number	Number of Peptides	Observed Molecular Weight	Calculated Molecular Weight	References
Bms1- putative endonuclease	gi|39930555	5	146 kDa	146 kDa	9, 10, 46
small subunit processome component 20 homolog	gi|62510597	3	320 kDa	318 kDa	9, 10, 47
aquarius	gi|123123505	3	171 kDa	171 kDa	9, 10, 49
splicing factor 3B, subunit 2	gi|30794206	13	98 kDa	98 kDa	50
U2-associated protein SR140	gi|171460908	9	119 kDa	118 kDa	51
pre-mRNA processing factor 40	gi|148694968	5	94 kDa	94 kDa	52
bcl-2- associated transcription factor 1	gi|24496776	7	106 kDa	106 kDa	53
large subunit GTPase 1 homolog	gi|30017373	2	74 kDa	73 kDa	9

Table III

Proteins involved in signal transduction or metabolism that were identified in the LamR-binding fraction. Listed peptides represent those identified with ≥ 95% confidence by Scaffold.

Protein	Accession Number	Number of Peptides	Observed Molecular Weight	Calculated Molecular Weight	References
betaPix-b (Guanine nucleotide exchange factor)	gi|10504263	2	74 kDa	80 kDa	27, 54–56, 91
Erbb2 interacting protein (ERBIN)	gi|148686562	3	155 kDa	157 kDa	58, 60

Table IV

Proteins involved in protein processing that were identified in the LamR-binding fraction. Listed peptides represent those identified with ≥ 95% confidence by Scaffold.

Protein	Accession Number	Number of Peptides	Observed Molecular Weight	Calculated Molecular Weight	References
TCP-1-alpha	gi|110625624	9	61 kDa	60 kDa	
TCP-1-theta	gi|126723461	11	60 kDa	60 kDa	
TCP-1-zeta	gi|62948125	6	58 kDa	58 kDa	
Hsp65	gi|183396771	2	61 kDa	61 kDa	
ribophorin II	gi|123297068	5	68 kDa	68 kDa	62, 64
transmembrane emp24-like trafficking protein 10	gi|148670919	5	25 kDa	26 kDa	63, 64

Table V

Proteins associated with the cytoskeleton or cell anchorage that were identified in the LamR-binding fraction. Listed peptides represent those identified with ≥ 95% confidence by Scaffold.

Protein	Accession Number	Number of Peptides	Observed Molecular Weight	Calculated Molecular Weight	References
alpha tubulin	gi|34740335	21	51 kDa	50 kDa	[27]
Vimentin	gi|2078001	20	54 kDa	52 kDa	[22]
centrosomal protein (KARP-1 binding protein)	gi|143955299	2	176 kDa	175 kDa	[27]
dynein cytoplasmic 1 intermediate chain 2	gi|123207569	6	69 kDa	71 kDa	[27, 67]
cortactin	gi|75677414	16	61 kDa	61 kDa	[27, 68]
myosin light polypeptide 6 (MyL-6)	gi|148664460	2	18 kDa	15 kDa	[69]
tight junction protein 2	gi|148709667	5	134 kDa	138 kDa	[27, 70]

Table VI

Proteins associated with DNA or chromatin maintenance that were identified in the LamR-binding fraction. Listed peptides represent those identified with ≥ 95% confidence by Scaffold.

Protein	Accession Number	Number of Peptides	Observed Molecular Weight	Calculated Molecular Weight	References
Histone H2A	gi|56238118	11	14 kDa	14 kDa	11, 71
Histone H3	gi|78070549	5	16 kDa	15 kDa	71
Histone H4	gi|117167984	15	11 kDa	11 kDa	11, 71
SMARCC1 (BAF 155)	gi|112421097	7	121 kDa	123 kDa	71–73

Table VII

Proteins of unknown function that were identified in the LamR-binding fraction. Listed peptides represent those identified with ≥ 95% confidence by Scaffold.

Protein	Accession Number	Number of Peptides	Observed Molecular Weight	Calculated Molecular Weight	References
Ubiquitin- associated protein 2-like	gi|148683226	13	117 kDa	117 kDa	[74]
atlastin GTPase 3	gi|81902439	3	64 kDa	60 kDa	[75]
Sugen kinase protein 269 (SgK269)	gi|118572720	4	193 kDa	191 kDa	