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Ectopic Expression of LIM-nebulette (LASP2) reveals roles in cell migration and spreading

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Abstract

LIM-nebulette (LASP2) is a small focal adhesion protein and a member of the nebulin family of actin binding proteins. This recently identified splice variant of the nebulette locus is widely expressed and highly enriched in neuronal tissue. Other than the facts that LIM-nebulette is a focal adhesion protein and interacts with zyxin, nothing is known about its function. Given that LIM-nebulette has an identical modular organization and overlapping tissue distributions to that of LASP1, we have analyzed the role of LIM-nebulette in comparison with that of LASP1. We find that LIM-nebulette is a dynamic focal adhesion protein that increases the rate of attachment and spreading of fibroblasts on fibronectin coated surfaces. Additionally, LIM-nebulette is recruited from the cortical cytoskeleton in non-motile cells to focal adhesions at the leading edge of stimulated cells. In confluent cultures of HeLa and NIH3T3 cells, LIM-nebulette co-localizes with α -catenin in putative adherens junctions, whereas LASP1 is devoid of these areas. Interestingly, overexpression of LIM-nebulette in PC6 cells inhibits neurite outgrowth in response to growth factors. Collectively, our data indicate that LIM-nebulette and LASP1 have distinct roles in the actin cytoskeleton.

Keywords

Nebulin; actin binding proteins; focal adhesions; zyxin; neurite outgrowth

Introduction

LIM-nebulette (LASP2) and LASP1 are members of the nebulin family of actin binding proteins (Chew et al., 2002; Li et al., 2004). All of the family members are involved in anchoring actin filaments to dynamic cytoskeletal structures, such as the Z-lines of striated muscle or the focal adhesions of migratory cells. Although LIM-nebulette is a splice variant of the nebulette gene (Katoh, 2003; Li et al., 2004; Terasaki et al., 2004), LIM-nebulette exhibits homology at the domain level with LASP1 (Panaviene and Moncman, 2007), in that both proteins contain an N-terminal LIM domain, a central repeat domain comprised of nebulin modules, a serine rich linker region and a C-terminal Src homology 3 (SH3) domain (Figure 1A). The presence of the nebulin modules in the repeat domain suggests the ability of both LIM-nebulette and LASP1 to interact with actin. This hypothesis has been substantiated by in vitro experiments that demonstrate that both LIM-nebulette (Terasaki et al., 2004; Zieseniss et al., 2008) and LASP1 (Chew et al., 2002; Schreiber et al., 1998; Terasaki et al., 2004) sediment with actin filaments in co-sedimentation assays. Additionally, both proteins appear to be components of focal adhesion complexes when

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expressed in fibroblasts (Chew et al., 2002; Lin et al., 2004; Panaviene and Moncman, 2007; Schreiber et al., 1998; Terasaki et al., 2004).

LIM-nebulette was identified as a zyxin interacting protein from a yeast two hybrid screen (Li et al., 2004) and by *in silico* analysis of the human genome (Katoh, 2003). The tissue distribution of LIM-nebulette overlaps with that of LASP1, in that it is ubiquitously expressed (Li et al., 2004; Terasaki et al., 2004) and in fact, both proteins are highly expressed in the brain (Li et al., 2004; Phillips et al., 2004; Terasaki et al., 2004). The function of LIM-nebulette is currently unknown. The cellular distribution of LIM-nebulette has been examined in fibroblasts and cardiomyocytes (Li et al., 2004; Panaviene and Moncman, 2007; Zieseniss et al., 2008). In fibroblasts, GFP tagged LIM-nebulette incorporates into focal adhesions as demonstrated by its co-localization with vinculin and FAK (Li et al., 2004; Panaviene and Moncman, 2007; Terasaki et al., 2004). We have previously shown that LIM-nebulette localizes to long fibrous structures at the cell-substrate contact and with the pre-myofibrils at the peripheral edge of spreading cardiomyocytes (Panaviene and Moncman, 2007). Due to its identity with the nebulette C-terminus, it was not surprising that LIM-nebulette incorporates into the myofibrils at the level of the Z-line (Panaviene and Moncman, 2007; Zieseniss et al., 2008).

LASP1 was identified as an mRNA that is highly expressed in breast carcinoma (Tomasetto et al., 1995) and the protein is overexpressed in 8–12% of breast carcinomas in humans. Interestingly, LASP1 has been shown to have a role in cellular migration. Decreased levels of LASP1 by siRNA in both breast cancer cell lines and fibroblasts results in decreased migration of the cells (Chew et al., 2002; Grunewald et al., 2006; Lin et al., 2004). Overexpression of LASP1 also appears to decrease rates of cellular migration in some fibroblasts (Lin et al., 2004). However, overexpression of LASP1 in cells containing low endogenous levels also can enhance migration (Grunewald et al., 2006). LASP1 is found in high levels in parietal cells of the gastric system and in platelets and is believed to play a role in vesicular secretions (Chew et al., 2000; Chew et al., 1998). It is also expressed in the brain and has been shown to interact with actin filopodia of differentiating NG108-15 cells (Phillips et al., 2004; Terasaki et al., 2004). In all cases, LASP1 has been demonstrated to be a dynamic protein that is involved in ruffled membranes at the peripheral edge of spreading cells and in focal adhesions at the leading edge of migratory cells.

In this report, we have analyzed the cellular roles of LIM-nebulette by comparing its cellular distribution to that of LASP1 during cell attachment and migration in fibroblasts. We find that LIM-nebulette is localized to focal adhesions at the leading edge of migrating cells and increases the rate of attachment and spreading in fibroblasts. In confluent cultures of epithelial cells, LIM-nebulette is found associated with α -catenin enriched cell:cell contacts. We have also examined the role of LIM-nebulette during neuronal growth factor (NGF) induced neurite outgrowth in PC6 cells. Although LIM-nebulette is expressed in brain tissue, the overexpression of LIM-nebulette dramatically decreases both neurite outgrowth and branching of these structures. Both neurite outgrowth and branching in PC6 cells overexpressing LASP1 were equivalent to that of cells expressing GFP alone. These data suggest that although LIM-nebulette and LASP1 have similar cellular and tissue distributions, each protein has a specific cellular function. These data represent the first reports of the functions of LIM-nebulette in eukaryotic cytoskeletal dynamics and report the role of this protein in cellular communication.

Materials and Methods

Cell Culture

NIH3T3 and HeLa cells were gifts from Dr. Rick McCann (University of Kentucky) and PC6 cells were a gift from Dr. Doug Andres (University of Kentucky). NIH3T3 cells are an embryonic mouse fibroblast cell line (ATCC#CRL-1658). NIH3T3 cells were grown in 10% FBS in DME plus glutamine and gentamycin. For migration and spreading assays, the transfected cells were allowed to recover for 24 hours post-transfection and then replated on fibronectin coated coverslips at a density of 2×10^5 cells per 35 mm plate. For spreading assays, the cells were fixed 30 min-two h post plating and processed for immunofluorescence microscopy. Attempts to fix the zero timepoint resulted in the loss of the majority of the cells from the coverslip, thus for the initial diameter measurements post plating, the cells were plated in Delta T dishes (Bioprotech, Inc.) coated with fibronectin and immediately observed while maintaining the temperature at 37°C.

For migration assays, the transfected cells were serum starved overnight and then treated with 40 ng/ml platelet derived growth factor (PDGF-BB homodimer) (Sigma, Inc.) for 20 min prior to fixation. For live cell imaging, the cells were plated onto fibronectin coated Delta T culture dishes and the temperature was maintained using a Bioprotech Delta T temperature controlled stage. Fluorescent images were captured prior to addition of PDGF containing media. Upon stimulation with PDGF, 10–15 DIC images were captured over a 10–15 min period and then a final fluorescent image was taken.

PC6 cells are derived from the rat pheochromocytoma cell line PC12 (ATCC#CRL-1721). PC6 cells were grown in 10% FBS, 5% HS in DME plus glutamine and gentamycin as described in (Shi and Andres, 2005). Induction of differentiation was achieved using neuronal growth factor (β -NGF) (R&D Systems, Inc.) at 100 ng/ml (Shi and Andres, 2005). COS-7 cells were cultured as previously described (Panavien and Moncman, 2007).

HeLa cells are a human epithelial cell line that was derived from a cervical carcinoma (ATCC#CCL-2). HeLa cells were grown according to ATCC recommendations. The cells were plated onto fibronectin coated coverslips prior to transfection and allowed to grow to confluency prior to fixation.

Transfections

GFP-LIM-nebulette and GFP LASP1 were prepared as described in Panavien and Moncman (2007). All transfections were performed with CsCl purified DNAs. NIH3T3, HeLa and COS7 cells were transfected with Eugene6 (Boehringer-Mannheim, Inc.) as described in Moncman and Wang (Moncman and Wang, 1999). PC6 cells were transfected with Effectene (Qiagen, Inc.) as described in (Shi and Andres, 2005). In all cell types, the transfection efficiencies were 10–30% of the cells.

Immunofluorescence Microscopy

All cells were processed for immunofluorescent microscopy by fixation with paraformaldehyde and Triton X-100 permeabilization as described in Moncman and Wang (Moncman and Wang, 1995). NIH3T3 cells were probed with antibodies to vinculin (1:1000, hVIN, Sigma, Inc.), focal adhesion kinase (FAK) (1:300, BD Transduction Laboratories), and phalloidin (Sigma, Inc.). HeLa cells were probed with an anti- α -catenin Ab (1:100, Zymed, Inc.). The PC6 cells were counterstained with anti-neurofilament M protein (1:200, Santa Cruz, Inc.), anti-TRK-A (1:200, Upstate Cell Signaling Solutions, Inc.) and phalloidin. Detection of Ab binding was performed with goat-anti-mouse or rabbit IgG conjugated with TRITC (1:300, Jackson ImmunoResearch Inc.)

Analysis of cell migration and spreading

Random fields of cells were captured using an Orca ER camera attached to a Zeiss Axiovert 200M using a 10 or 20X objective to gather large fields of cells. For spreading assays, the largest axis through the cell body was measured and the results were compared to cells transfected with GFP alone. A cell was considered spread if it measured 2X greater than the average diameter for all of the data sets at time zero. All cells that had extensions greater than the length of the cell body were considered aberrant. For the migration assays, the length of the cells, as well as the length of the cellular extensions was monitored. All measurements were performed using OpenLab (Improvision, Inc.). Three individuals, blind to the construct being tested, performed all measurements. All statistical analysis was performed using KaleidaGraph (Synergy Software, Reading, PA).

Immunoblot assays

COS-7, HeLa, PC6 and NIH3T3 cells were transfected with the LASP-1 and LIM-nebulette constructs and then the cells were harvested 48–60 hours later. Whole cell lysates were prepared for electrophoretic separation by placing the lysates into SDS sample buffer as described in (Panaviene and Moncman, 2007). The cell extracts were separated on a 10% tris-glycine gel and then transferred to nitrocellulose using a semi-dry transfer technique (Kyhse-Anderson, 1984). The gel replicas were then probed with an anti-GFP Ab (1:1000, Clontech, Inc) or anti-LASP1 (1:1000, Chemicon, Inc). Secondary Abs were conjugated with horseradish peroxidase (1:5000, Jackson ImmunoResearch, Inc.) and the binding was detected using enhanced chemiluminescence (Kirkegaard and Perry, Inc.).

Results

Expression of GFP tagged LIM-nebulette

Although LIM-nebulette is derived from the nebulette locus, it shares homology at the level of domain organization with LASP1 (Figure 1A). We have generated vectors to express both LIM-nebulette and LASP1 as GFP fusion proteins in mammalian cells (Panaviene and Moncman, 2007). Expression of both constructs in COS7, PC6, HeLa and NIH3T3 cells results in the appropriate sized proteins as determined by western blot analysis using anti-GFP mAb as a detection reagent (Figure 1B). Using an anti-LASP1 Ab, we detect a band of 38 kDa in all of the cell lines tested; however, this Ab exhibited reactivity to both the LASP1 and LIM-nebulette proteins expressed by our constructs. As the immunogen used to generate this Ab is derived from a nebulin module, we also determined that the anti-LASP1 Ab detects nebulette and nebulin by western blot (Norris and Moncman, unpublished observation). The intensity of the band for the 38 kDa protein is greater than that of our expressed protein in all of the cell lines tested. We cannot conclusively state whether this is the endogenous expression of LASP1 or LIM-nebulette due to the crossreactivity; however, based on the molecular mass of 38 kDa, we would suggest that it is LASP1 and LIM-nebulette (34 kDa) is not present in any of the cell types used in this analysis. We can clearly detect this separation in mass for the GFP tagged constructs.

Cellular distribution in NIH3T3 cells

Both LIM-nebulette (Terasaki et al., 2004; Li et al., 2004; Panaviene and Moncman, 2007) and LASP1 (Chew et al., 1998; Schreiber et al., 1998) are known components of focal adhesions in fibroblasts. The PtK2 cells, used in our previous study (Panaviene and Moncman, 2007), are well spread flat cells that exhibit extensive cytoskeletal networks and do not express LASP1 according to Keicher *et al.* (2004). NIH3T3 cells do not develop the well defined stress fibers seen in PtK2 cells, but are considerably more motile and thus more suitable for assays involving migration. Lin et al. (2004) and our western blot analysis (Fig.

1) have demonstrated that NIH3T3 cells express LASP1. Thus to determine if the constructs would incorporate into focal adhesions in this background, we first expressed the GFP tagged LIM-nebulette, LASP1 and GFP alone in NIH3T3 cells to examine their distributions. Expression of GFP tagged LIM-nebulette (Fig. 2 A, C) also resulted in incorporation into the focal adhesions of the NIH3T3 cells as demonstrated by the co-localization with vinculin (Fig. 2D). Counterstaining for the stress fibers using phalloidin (Fig. 2B) indicates that the stress fiber incorporation of LIM-nebulette was not as pronounced in the NIH3T3 cells as that observed in PtK2 cells (Panavien and Moncman, 2007). A similar distribution was also observed for the expression of our LASP1 construct (Fig. 2E–H).

Spreading and attachment

LASP1 has been shown to redistribute from focal adhesions to the leading edge of migratory cells and to ruffled membranes of spreading fibroblasts (Lin et al., 2004). To assess if LIM-nebulette behaved in a manner similar to LASP1, we transfected NIH3T3 cells with the constructs and performed spreading assays. To evaluate the rate of attachment and spreading, NIH3T3 cells were transfected with GFP alone or GFP-tagged LIM-nebulette or LASP1. After 24 h recovery from transfection, the cells were passaged onto fibronectin coated coverslips and fixed at 30 min. intervals post plating over a 2 h time course. Consistent with the single time point, cells expressing LIM-nebulette exhibited a greater rate of attachment and spreading than the cells expressing LASP1 or GFP. Representative fields for LIM-nebulette (A–D) and LASP1 (E–G) are shown in Figure 3. Measurements of the cells at each time point using the criterion indicated above for quantization of spreading indicates that 50% of the cells expressing LIM-nebulette have a spread morphology by 1 h and 87 $\pm$ 3.1% of the cells are spread by 2 h (Figure 3I). In contrast, both GFP and LASP1 expressing cells exhibited ~30% spreading at the 1 h time point and ~60% by 2 h (Figure 3I). There was no statistical difference between the cell populations at the zero timepoint.

We next examined the distribution of the GFP tagged proteins in the spreading NIH3T3 cells relative to the distribution of the actin filaments and of focal adhesion proteins, such as vinculin and FAK (Figure 4). GFP alone exhibited a diffuse cytoplasmic distribution in the spreading cells and did not appear to co-localize with either the cytoskeleton or focal adhesions (data not shown). LIM-nebulette co-localized with vinculin (note arrows in Fig. 4 A, B) and FAK (not shown) in focal adhesions at the peripheral edge of the cells. Additional small internal adhesions and some cortical actin distributions were observed in the LIM-nebulette expressing cells. The recombinant LASP1 expressed in these cells gave a similar distribution (Fig. 4E, F). Comparison of the distributions of the recombinant proteins with actin demonstrates the focal adhesion distribution for both LIM-nebulette and LASP1 just beyond the cortical actin in the spreading cells (note arrows in Fig. 4 C, D, G, H).

Migration

Stimulation of non-motile cells using PDGF results in a translocation of LASP1 from the focal adhesions to the leading edge of the cell (Lin et al., 2004). As LIM-nebulette enhances cellular attachment and spreading, it was of interest to determine if expression of LIM-nebulette would affect cell migration and exhibit a dynamic re-distribution during migration. Cells transfected with GFP alone or GFP-tagged LIM-nebulette were serum starved overnight, forcing the cells into quiescence and then were fed with fresh media containing serum and PDGF to stimulate migration. In serum starved cells, both LIM-nebulette and LASP1 were associated with the cortical actin fibers (note arrowheads in Fig. 5 A, E). This continuous cortical staining also co-localizes with actin found at the cell periphery (data not shown). We also noted the presence of LIM-nebulette in focal adhesions within the body of the cells that co-localized with vinculin (note arrows in Fig. 5A, B). Upon stimulation with

PDGF, LIM-nebulette redistributed to focal adhesions at the leading edge of the cells (Fig. 5 C, D and Fig. 6). Live imaging of cells expressing LIM-nebulette (Fig. 6) demonstrate both loss of LIM-nebulette from the retracting edge of the cell and an accumulation of this protein to the leading edge of migrating cells into well-defined focal adhesions. Immunofluorescence analysis of cells fixed at 20 min. post stimulation demonstrates the distribution of GFP-LIM-nebulette in vinculin rich focal adhesions at the leading edge of the cells (Fig. 5 C, D). We also noted that there was a decrease in the number of LIM-nebulette positive adhesions within the mid-body of the cells and a decrease in the stress fiber incorporation. A similar redistribution was observed for LASP1 (Fig. 5E–H).

Communication

In our initial assays, we noted that the distribution of LIM-nebulette seemed greater in positions of the cell that represented cell to cell contacts suggesting that LIM-nebulette may be a component of adherens junctions. To test this hypothesis, we expressed LIM-nebulette in both NIH3T3 cells (not shown) and HeLa cells (Figure 7), allowed the cells to reach confluence prior to fixation and counterstained for the distribution of α -catenin, a known component of adherens junctions (Figure 7). The recombinant GFP-LIM-nebulette appeared to be concentrated in areas rich in α -catenin (note insets in Fig. 7 A, B). This distribution was not observed for LASP1 (Figure 7 C, D) or GFP alone (not shown).

Neurite outgrowth

As LIM-nebulette and LASP1 are highly expressed in the brain, we next tested the effects of overexpression of LIM-nebulette and LASP1 in PC6 cell growth and differentiation. PC6 cells are a pheochromocytoma cell line that has been used as a cell model for neurite outgrowth in response to β -NGF. The transfected cells were passaged to reduce the density prior to feeding the cells β -NGF. The cells were maintained in β -NGF for 5–7 days prior to fixation. In untreated PC6 cells, both LASP1 and LIM-nebulette displayed diffuse cytoplasmic distribution (Figure 8). In a small percentage of LIM-nebulette expressing cells, there was a distinctive focal adhesion distribution in large flattened cells (Figure 8A). This distribution was never observed in the LASP1 or GFP expressing cells. When the cells were grown in the presence of β -NGF, the cells expressing GFP alone (not shown) or LASP1 (Figure 8 G, H) produced long branched neurite outgrowths. Greater than 50% of the cells expressing either construct exhibited this differentiation process in response to β -NGF; whereas spontaneous neurite outgrowth was observed in 10–15% of the non-treated expressing cells (Figure 9). Interestingly, the cells expressing LIM-nebulette (Figure 8 C, D and Figure 9) did not develop neurite outgrowths in the presence of β -NGF and the percentage of cells displaying neurites was not statistically different from the rate of spontaneous growth ($21 \pm 5.4\%$ vs. $14 \pm 3.3\%$). These data would suggest that LIM-nebulette interferes with the differentiation response to β -NGF in these cells. In all cases, the cells treated with β -NGF exhibited expression of neurofilament M protein (Figure 10 A–D) and there were no observable differences in the distribution of the NGF receptor between the cells expressing any of the constructs tested (Figure 10 E–H).

Discussion

The newest members of the nebulin family, LIM-nebulette and LASP1, have an identical domain layout containing an N-terminal LIM domain, nebulin modules, a serine rich linker region and a C-terminal SH3 domain. Although LIM-nebulette is known to be a component of focal adhesion complexes, its functions are unknown. Both LASP1 and LIM-nebulette have been identified as zyxin (Li et al., 2004) and actin (Chew et al., 2002; Schreiber et al., 1998; Terasaki et al., 2004) binding proteins and both proteins appear to be ubiquitously expressed. The overall sequence conservation between these two proteins is further

highlighted by the crossreactivity of the anti-LASP1 Ab with LIM-nebulette. In an effort to sort out similar but distinct functions of these proteins, we have analyzed the effects of ectopic expression of LIM-nebulette on spreading and migrating fibroblasts and differentiating PC6 cells. Our results indicate that LIM-nebulette and LASP1 share some overlapping function, but also exhibit distinct behaviors in the cellular function that we have tested.

Expression pattern of LIM-nebulette

Li et al. (2004) demonstrated the presence of the LIM-nebulette message in brain, lung, kidney, heart, and pancreas by northern blot in both humans and mice. Western blot analysis of a variety of chicken organs also demonstrated the presence of LIM-nebulette in various tissues (Terasaki et al., 2004). Our western blot analysis using the anti-LASP1 Ab that crossreacts with LIM-nebulette failed to detect LIM-nebulette in any of the cell lines that we have tested. Both the studies by Li et al (2004) and Terasaki et al. (2004) indicate that the expression levels for LIM-nebulette are relatively low in all tissues except for brain. Thus if these cell lines do indeed express LIM-nebulette, the expression levels are clearly below our current detection limits.

Spreading and attachment

LIM-nebulette (LASP2) was first identified as a zyxin binding partner (Li et al., 2004) and as a putative protein expressed in brain by in silico analysis (Katoh, 2003). Both analyses indicate that LIM-nebulette is present in focal adhesion; however, there is little information on its role in cytoskeletal dynamics and function. Consistent with our previous studies (Panavien and Moncman, 2007) and those of others (Li et al., 2004; Terasaki et al., 2004), we find that LIM-nebulette localizes in focal adhesions of NIH3T3, despite the presence of endogenous LASP1. Our data indicates that LIM-nebulette affects the rate of attachment and spreading for NIH3T3 cells. Cells expressing LIM-nebulette appear to spread at about twice the rate of those expressing LASP1 or GFP. These data would suggest that LIM-nebulette has a role in the focal contacts that affect the rate of attachment and spreading on the fibronectin coated surfaces. LASP1 is known to be located in the ruffled membranes of spreading cells; however, it does not appear to stimulate of the rate of attachment and spreading in fibroblasts (this study; (Lin et al., 2004)). In cells that are starting to attach, LIM-nebulette is localized to vinculin rich focal adhesions just below the ring of cortical actin.

Migration

LIM-nebulette, like LASP1, displays dramatic rearrangements in response to serum starvation and stimulation with growth factors. Lin et al. (2004) and Chew et al. (2000) have indicated that LASP1 is a dynamic cytoskeletal protein that moves from the cortical actin to focal adhesions in response to growth factor stimulation of non-motile, quiescent cells. Stimulation of non-motile cells expressing LIM-nebulette resulted in a recruitment of this protein to focal adhesions at the leading edge of migrating cells. In serum starved cells, LIM-nebulette was associated with the cortical actin cytoskeleton and to some extent with focal adhesions found within the midbody of the cells. The recruitment of LIM-nebulette to the leading edge of stimulated cells suggests that this protein is also dynamic and involved in regulation of cytoskeletal dynamics.

In the present study, we observed a distribution of LASP1 consistent with cortical actin distribution and ruffled membranes. Our analysis is indeed consistent with the idea that LASP1 is a dynamic cytoskeletal protein in its redistribution from the cortical cytoskeleton in non-motile cells to focal adhesions at the leading edge of cells in response to hormone stimulation. Lin et al. (2004) have suggested that overexpression of LASP1 in cells that

normally express this protein may result in abnormal distributions of LASP1 that could ultimately disrupt normal cell polarity and migration. Knock-down of LASP1 by siRNA also decreases the rate of cellular migration (Chew et al., 2002; Grunewald et al., 2006; Lin et al., 2004). Thus the concentration of LASP1 is critical for the maintenance of its proper cytoskeletal functions.

In our immunoblot analysis using an anti-LASP1 Ab, we have demonstrated crossreactivity of this Ab with other members of the nebulin family of actin binding proteins. Additionally, we demonstrated the presence of a single band migrating at 38 kDa in all of the cell lines used in this study. These data suggest that if LIM-nebulette (34 kDa) was present in the cells, a doublet would have been expected in this region of the gel and this was not observed. This further suggests that LIM-nebulette is not present or is undetectable in these cells. While both the changes in the rate of attachment and distributions could possibly be due to disruptions in the LASP1 function by the presence of LIM-nebulette, all of the changes observed with LIM-nebulette were different than the disruptions observed for LASP1 overexpression and silencing and were not observed for the expression of GFP alone, which further suggests that LIM-nebulette plays a role in cell migration and attachment.

Communication

We noted in many of our micrographs that LIM nebulette was often distributed in an almost continuous manner in areas of the membrane skeleton that appeared to be involved in cell: cell contacts. Zieseniss et al. (2008) also noted the incorporation of GFP-LIM-nebulette in areas of cell: cell contact and staining with Abs to the endogenous LIM-nebulette also decorated the intercalated discs of cardiomyocytes. We have determined that LIM-nebulette colocalizes with α -catenin in presumptive adherens junctions involved in cell: cell communication in NIH3T3 and HeLa cells. This distribution was not observed with the expression of LASP1 (this study). The actin binding properties of LIM-nebulette associated with the adherens junctions may provide a link between the junctional complexes and actin filament bundles. Nakagawa et al. (2006) have suggested that both LIM-nebulette and LASP1 are involved in the stabilization of actin bundles within lamellipodia. We were unable to co-immunoprecipitate LIM-nebulette and α -catenin (Norris and Moncman, unpublished observation). However, this does not preclude the possibility that other components of the cadherin-catenin complex may directly interact with LIM-nebulette. Further work is needed to assess this role for LIM-nebulette.

Neurite outgrowth

Interestingly, we find that overexpression of LIM-nebulette in PC6 cells led to a dramatic decrease in neurite outgrowth in response to β -NGF. Instead these cells exhibited short protrusions and enlarged cell bodies as compared to cells expressing LASP1, GFP or non-transfected cells in the same field. The perturbation in the differentiation process of the PC6 cells does not appear to be a global shut down of the pathway in that the cells expressing LIM-nebulette still express neurofilament-M protein in response to β -NGF. This would suggest that the defect might be more directly linked to alterations in the actin cytoskeletal dynamics. Terasaki et al. (2004) examined the distribution of LIM-nebulette (LASP2) in NG108-15 cells and determined that LIM-nebulette distributed along actin filament bundles in filopodia. It is also interesting to note that the Phillips et al. (2004) study identified nebulette as a component of the post synaptic density. Although no further analysis was reported on this protein, the identification of nebulette as a component of the postsynaptic density may likely be further identification of LIM-nebulette. It is interesting to speculate that the increased rate of attachment and spreading observed with the ectopic expression of LIM-nebulette may play a role in the lack of neurite outgrowth in the PC6 cells.

Expression of LASP1 did not have an effect on neurite outgrowth in PC6 cells induced by growth factor stimulation. In both the differentiated and undifferentiated cells, LASP1 exhibited a diffuse distribution similar to GFP alone. We did not observe a distribution consistent with focal adhesions of this protein in undifferentiated or differentiated PC6 cells. Terasaki et al. (2004) examined the expression of LASP1 in the neuroblastoma cell line NG108-15 and found that LASP1 was associated with the actin filament bundles at the ends of filopodia, but did not distinguish between differentiated or non-differentiated cells. Phillips et al. (2004) identified LASP1 as a component of the central nervous system synapses and dendritic spines using MudPIT analysis of postsynaptic densities. Immunofluorescent labeling of primary hippocampal neurons demonstrated the presence of LASP1 in growth cones shortly after plating and that the distribution of LASP1 changed with time in culture to a punctate or clustered distribution along the dendrites (Phillips et al., 2004). The differences that we observed in the LASP1 expression in the PC6 cells may simply reflect differences between the neuroblastoma cell line used in the Terasaki et al. (2004) work, the primary neurons used in the Phillips et al. study (Phillips et al., 2004) and the pheochromocytoma cell line used in the present study. Additionally, both the Terasaki study (2004) and the present study used a strategy of overexpression of tagged LASP1 which may mask the endogenous distribution observed by the Phillips et al. study (2004).

Concluding Remarks

LIM-nebulette is a dynamic focal adhesion protein. We have demonstrated that LIM-nebulette plays a role in cellular attachment and spreading to fibronectin coated surfaces and that this protein localizes to presumptive adherens in junctions in confluent cultures. Although LIM-nebulette and LASP1 have similar domain organization and overlapping distributions, we have demonstrated that each protein performs unique functions in three different cell model systems. Like many of the focal adhesion proteins, increases and decreases in their expression levels alter both focal adhesion function and cytoskeletal dynamics. Identification of other binding partners and the role of phosphorylation in their function should further help to delineate the differences in function between these two highly homologous proteins.

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Figure 1.

A. Schematic of LASP1 and LIM-nebulette. Both LASP1 and LIM-nebulette are comprised of four distinct domains: an N-terminal LIM domain, a central repeat domain containing nebulin modules, a serine rich linker region and a C-terminal SH3 domain. **B.** Western blot of expressed proteins in the cell lines used. COS7, NIH3T3, HeLa and PC6 cells were transfected with constructs encoding GFP alone or GFP tagged LASP1 or LIM-nebulette. Forty eight hours post transfection the cells were harvested and taken up in SDS sample buffer. After separation on 10% polyacrylamide gels and transfer to nitrocellulose, the replicas were probed with an anti-LASP1 Ab or anti-GFP mAb. The anti-LASP1 Ab detected a 37 kDa band in the extracts of the COS7, NIH3T3 and PC6 extracts. This Ab also exhibited crossreactivity with both the GFP tagged LASP and LIM-nebulette. ECL detection indicates that the appropriate sized proteins were produced from the expressed constructs with GFP being 26 kDa, GFP-LASP1 at 67 kDa and GFP-LIM-Nebulette at 63 kDa. There is a low level of proteolysis in the extracts, but the majority of the protein was full length.

Figure 2.

Expression of LIM-nebulette and LASP1 in NIH3T3 cells. NIH3T3 cells were transfected with GFP-LIM-nebulette (A–D) or LASP1 (E–H). The cells were fixed and processed for immunofluorescence 48 h post transfection. The GFP expressing cells were counterstained for the distribution of actin using rhodamine phalloidin (B, F) or the distribution of vinculin using a vinculin Ab (D, H). Both LIM-nebulette and LASP1 displayed a pronounced presence in the focal adhesions of the NIH3T3 cells (arrows in all panels). Although the display of stress fibers was not as prominent in the NIH3T3 cells, association with the stress fibers was noted (asterisks in A, B, E, F). Bar = 5 μm.

Figure 3.

Time course of spreading for GFP tagged LIM-nebulette and LASP1 expressing cells. NIH3T3 cells transfected with GFP-LIM-nebulette (A–D), GFP-LASP1 (E–H) or GFP alone were trypsinized and plated onto fibronectin coated coverslips. The cells were fixed at 30 min. intervals and monitored for the extent of spreading at each time point. Representative fields for LIM-nebulette (A–D) and LASP1 (E–H) are shown. Statistical analysis of this data is given in panel I, which demonstrates the statistically different populations observed for LIM-nebulette as compared to LASP1 and GFP. Each time point represents the analysis of at least 3 different experiments and the blind scoring of 3 individuals for each time point. Asterisks indicate statistically different populations at each time point. The error bars represent the standard deviation for all data sets. For each time point N = over 300 cells scored. Bar = 50 μ m.

Figure 4.

Morphology of spreading cells expressing LIM-nebulette or LASP1. Cells transfected with LIM-nebulette (A–D) and LASP1 (E–H) were passaged 24 hours post transfection and allowed to settle onto fibronectin coated coverslips for 60 min. The cells were then fixed and processed for immunofluorescence. The GFP expressing cells were counterstained for either the focal adhesion protein, vinculin (B, F) or actin using rhodamine phalloidin (D, H). Although significant cortical actin staining was not noted in either treatment group, both proteins exhibited focal adhesion distribution (arrows in all panels). Consistent with our time course analysis, cells expressing LIM-nebulette were significantly flatter and better spread than the cells expressing LASP1. Bar = 10 μ m.

Figure 5.

Distribution of LIM-nebulette and LASP1 in serum starved and stimulated cells. Cells transfected with GFP-LIM-nebulette (A–D) or GFP-LASP1 (E–H) were passaged 24 h post transfection. The cells were then allowed a 12 h recovery period prior to serum starvation. Both recombinant constructs displayed distributions that were consistent with the membrane cytoskeleton and with focal adhesions as defined by vinculin rich focal contacts after serum starvation (A, B, E, F). LIM-nebulette (C, D) and LASP1 (G, H) expressing cells were induced to migrate and then counterstained for the distribution of vinculin (D, H). Both LIM-nebulette (C) and LASP1 (G) were recruited to focal adhesions at the leading edge of

the cells that co-localized with vinculin. Arrowheads point to cortical actin distributions and arrows point to focal adhesions. Bar = 5 μ m in A–H

Figure 6.

Live cell imaging of migrating cells expressing GFP-LIM-nebulette. Transfected cells were passaged onto a fibronectin coated DeltaT dish, serum starved and then stimulated to migrate. The initial distribution of LIM-nebulette rich structures was shot prior to stimulation (0 min). Upon stimulation, a DIC timelapse movie was shot and a final fluorescent image to examine the GFP-LIM-nebulette distribution (12 min). The black outline is the outline of the cell prior to stimulation and the white outline is the periphery of the cell following 12 min of stimulation. The cell is clearly moving upward and demonstrates the recruitment of LIM-nebulette to the leading edge of the cell. Bar=10 μ m

Figure 7.

Cell: cell junctions. HeLa cells transfected with LIM-nebulette (A, B) or LASP1 (C, D) were grown to confluency and then the distribution of the recombinant proteins were compared with that of the endogenous α -catenin. LIM-nebulette (A) co-localized with α -catenin (B) at cell: cell junctions. LASP1, in contrast did not exhibit this distribution (C, D). Insets in all images are the edge of the cell involved in the contact. Bar =10 μ m and 5 μ m in the insets.

Figure 8.

Expression of LIM-nebulette and LASP1 in PC6 cells. PC6 cells were transfected with LASP1 or LIM-nebulette cDNAs and cultured 7 days post transfection +/- β -NGF. Most of the cells expressing LIM-nebulette (A, B) displayed a diffuse cytoplasmic distribution; however in about 20% of the cells a distinct focal adhesion distribution was observed (arrowheads in A). After stimulation of differentiation, the majority of the cells expressing LIM-nebulette (C, D) did not develop neurites and often displayed short protrusions with distinctive focal adhesion distribution of LIM-nebulette (arrowheads in C, D). The non-differentiated cells expressing LASP1 (E, F) also displayed a diffuse cytoplasmic distribution. However, after exposure to β -NGF, the cells expressing LASP1 (G, H)

developed numerous branched neurites (arrows in G, H). The distribution of LASP1 remained diffuse throughout the cells. Bar = 10 μ m.

Figure 9.

Statistical analysis of the neurite outgrowth study. Random fields of cells were scored for the number of cells expressing the GFP-tagged proteins that exhibited neurite outgrowth for each construct and treatment. Both cells expressing GFP alone or GFP-LASP1 showed a significant increase in the number of cells with neurites after treatment with β -NGF. In contrast, the cells expressing LIM-nebulette did not respond to this developmental stimulus. All data represents, 3 independent experiments with 3 individuals blind to the treatment scoring the samples. The open columns represent the percentage of GFP expressing cells that exhibited spontaneous neurite outgrowth and the stippled columns represent the

percentage of cells with neurite outgrowths after treatment with β -NGF. Bars indicate the standard deviation of the sample. For each treatment N=over 300 cells.

Figure 10.

Expression of Neurofilament protein M and the NGF receptor TrkA in cell expressing LIM-nebulette and LASP1. PC6 cells were transfected with LIM-nebulette (A, B, E, F) or LASP1 (C, D, G, H) cDNAs and cultured 7 days post transfection $\pm$ β -NGF. The cells were fixed and counterstained for the distribution of neurofilament protein M (B, D) or TrkA (D, H). Both the cells expressing LIM-nebulette and LASP1 expressed neurofilament protein M in response to NGF. TrkA was observed to have a punctate distribution on the cell surface independent of the constructs expressed. Bar = 10 μ m.