Zyxin Interacts with the SH3 Domains of the Cytoskeletal Proteins LIM-nebulette and Lasp-1*

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Zyxin is a versatile component of focal adhesions in eukaryotic cells. Here we describe a novel binding partner of zyxin, which we have named LIM-nebulette. LIM-nebulette is an alternative splice variant of the sarcomeric protein nebulette, which, in contrast to nebulette, is expressed in non-muscle cells. It displays a modular structure with an N-terminal LIM domain, three nebulin-like repeats, and a C-terminal SH3 domain and shows high similarity to another cytoskeletal protein, Lasp-1 (LIM and SH3 protein-1). Co-precipitation studies and results obtained with the two-hybrid system demonstrate that LIM-nebulette and Lasp-1 interact specifically with zyxin. Moreover, the SH3 domain from LIM-nebulette is both necessary and sufficient for zyxin binding. The SH3 domains from Lasp-1 and nebulin can also interact with zyxin, but the SH3 domains from more distantly related proteins such as vinexin and sorting nexin 9 do not. On the other hand, the binding site in zyxin is situated at the extreme N terminus as shown by site-directed mutagenesis. LIM-nebulette and Lasp-1 use the same linear binding motif. This motif shows some similarity to a class II binding site but does not contain the classical PXXP sequence. LIM-nebulette reveals a subcellular distribution at focal adhesions similar to Lasp-1. Thus, LIM-nebulette, Lasp-1, and zyxin may play an important role in the organization of focal adhesions.

Focal adhesions are essential cellular structures of metazoan organisms that link the extracellular matrix to the cytoskeleton. The controlled assembly and disassembly of focal adhesions is critical for a diversity of biological functions, including cell adhesion, motility, and spreading. Typically, focal adhesions are found at sites where a number of proteins from different signaling pathways converge to respond to extracellular signals (1). Thus far, more than 50 different focal adhesion proteins have been identified (2). Among them are several adapter proteins with protein-protein interaction motifs that play an important role in the dynamic assembly of focal adhesions.

One of these adapter proteins is zyxin. Zyxin was originally identified in chicken fibroblasts as a protein associated with stress fibers and focal adhesions (3). The mammalian homologue of avian zyxin was subsequently cloned from a subtracted cDNA library enriched with clones that are down-regulated in transformed fibroblasts (4). At the same time, human zyxin was also cloned from umbilical vein endothelial cells by cross-hybridization (5). Analysis of the protein sequence revealed the typical structure of a multidomain protein. At the C terminus, zyxin contains three LIM domains arranged in tandem. LIM domains are cysteine-rich motifs with two zinc finger-like structures that mediate specific protein-protein interactions. Three different binding partners for the LIM domains of zyxin have been identified, namely, the cysteine-rich protein CRP, the adapter protein p130cas, and the tumor suppressor protein H-warts/LATS1 (6–8). At the N terminus, zyxin features an extended, proline-rich domain, which contains a nuclear export signal and several proline clusters. By virtue of these proline clusters, zyxin binds to proteins of the Ena/VASP family, which control the assembly and disassembly of actin filaments (9). The proline clusters also serve as docking sites for the oncoprotein Vav, a nucleotide exchange factor for the Rho family of GTPases (10). Moreover, the extended N-terminal domain is responsible for the interaction of zyxin with the actin-bundling protein α-actinin (11). We have recently demonstrated that a linear sequence motif found at the extreme N terminus of zyxin is essential for this interaction (12, 13). Our observation is supported by the fact that LPP (lipoma preferred partner), another member of the zyxin family containing the same linear motif, binds to α-actinin, too (14).

Several pieces of evidence from different lines of investigation suggest that zyxin might play an important role in the organization of the actin cytoskeleton. Zyxin induces the polymerization of actin on the surface of mitochondria when artificially targeted to these ectopic sites (15, 16). Actin polymerization and the resulting tension appear to be crucial for the dynamic assembly of focal adhesions (1). On the other hand, zyxin is one of the first molecules that dissociate from dissolving focal adhesions (17). This observation raises the intriguing possibility that zyxin might represent one of the target molecules responsible for the controlled disassembly of focal adhesions.

Although tremendous efforts have been made to unravel the molecular mechanisms by which zyxin regulates the organization of the actin cytoskeleton, no details are yet known. The identification of binding partners for zyxin might provide some valuable clues. In this paper, we report on the interaction of zyxin with the sarcomeric protein nebulette. Furthermore, we identify a novel splice variant of nebulette, termed LIM-nebulette, which closely resembles Lasp-1. Similar to zyxin, nebulette and Lasp-1 are cytoskeletal proteins found in focal adhesions. Nebulette is specifically expressed in the cardiac muscle of vertebrates, where it interacts with actin filaments (18–20). It contains an acidic N terminus, an extended ring of nebulin-
like repeats, and a C-terminal SH3 domain. It has been postulated that the SH3 domain of nebulette, located 25–30 nm within the Z-disk, might play a crucial role in the assembly of Z-disks in cardiac muscles (18). Lasp-1 is composed of an N-terminal LIM domain, two nebulin-like repeats, and a C-terminal SH3 domain (21, 22). In contrast to nebulette, however, Lasp-1 is ubiquitously expressed in most cell types. It might therefore play a more general role in the regulation of the actin cytoskeleton (23, 24).

Here we provide evidence that all the related proteins nebulette, LIM-nebulette, and Lasp-1 interact with zyxin and that the C-terminal SH3 domain of the three proteins is responsible for this interaction. The fact that zyxin interacts with a multitude of actin-associated proteins suggests that these molecules cooperatively regulate the organization of the actin cytoskeleton.

**Materials and Methods**

cDNA Cloning and Plasmid Construction—A full-length cDNA for human zyxin (GenBank accession number X95755; Ref. 4) was cloned previously and characterized in our laboratory. Expressed sequence tags for mouse LIM-nebulette (IMAGE ID 5249766), human Lasp-1 (IMAGE ID 2301763), nebulin (IMAGE ID 4245063), vinexin (IMAGE ID 4328970), and SNX 9 (IMAGE ID 4869158) were obtained from the German Resource Center for Genome Research (Berlin, Germany).

For two-hybrid interactions in yeast, the matchmaker system from Clontech was utilized. Selected constructs were prepared by PCR with the help of synthetic oligonucleotide primers (Microsynth GmbH). The fragments for zyxin were subcloned into the bait vector pAS2-1 downstream of the sequence for the DNA-binding domain of GAL4. Likewise, selected fragments for LIM-nebulette, Lasp-1, nebulin, vinexin, and SNX 9 were subcloned into the prey vector pGADT7 downstream of the transactivation domain of GAL4.

For expression in bacteria, GST-tagged fusion constructs were prepared. A cDNA fragment corresponding to amino acid residues 1–42 of human zyxin was subcloned into the expression vector pGEX-5X-2 (Amersham Biosciences) downstream of the GST gene. Likewise, two nearly full-length cDNA clones for zyxin that lacked the codons for amino acids 19–42 (a1) or 261–283 (a2), respectively, were placed into pGEX-5X-2 (2).

Fusion constructs with GFP were prepared by ligating full-length or partial cDNA sequences for Lasp-1 and LIM-nebulette, respectively, into the expression vector pEGFP-C2 (Clontech) downstream of the GFP reporter gene. Authenticity and reading frame of all constructs were verified by DNA sequencing.

**Northern Blotting**—The multiple tissue Northern blots used in this study contained 2 μg of poly(A)+ RNA from various healthy adult human and mouse tissues (Clontech). The membranes were hybridized at 42 °C with different cDNA probes in a buffer containing 50% formamide (4). The probes corresponded to the N-terminal portion of human LIM-nebulette (residues 1–57, human 5′ probe), the C-terminal portion of human LIM-nebulette (residues 126–270, human 5′ probe), the LIM domain of mouse LIM-nebulette (residues 1–61, mouse 5′ probe), and the SH3 domain of mouse LIM-nebulette (residues 202–270, mouse 3′ probe). The fragments were labeled with [α-32P]dCTP by the random primed oligolabeling method. After overnight hybridization, the nylon membranes were washed in 1× sodium citrate buffer and exposed to X-Omat AR x-ray film. Electronic pictures were taken with filter settings optimized for green (515–565 nm) and red (>590 nm) light emission, respectively, and merged with the help of a computer software program.

**Results**

Identification of Human and Mouse LIM-nebulette—Extensive screening for potential binding partners of human zyxin utilizing the yeast two-hybrid system had previously led to the identification of 54 positive cDNA clones coding for a total of 14 independent proteins (13). One of these clones (novel 26) that appeared to encode a novel protein was analyzed in more detail in the present study. It contained an open reading frame of 810 nucleotides that could be translated into a polypeptide of 270 amino acid residues (Fig. 1A, GenBank accession number AJ580772). A detailed analysis with the simple modular architecture research tool SMART (26) predicted a protein of 31 kDa with an N-terminal LIM domain, three central nebulin-like repeats, and a C-terminal SH3 domain (Fig. 1B). The gene for the novel protein was identified on human chromosome 10 in...
band 10p12 by comparing the cDNA sequence with the complete human genome sequence. This gene spanned 390 kb and encompassed seven exons. Surprisingly, the last three exons of the novel gene were found to be identical to three exons from the 3′ part of the human gene for the cytoskeletal protein nebulette (27). We therefore concluded that the novel protein must represent a splice variant of human nebulette. Consequently, it was termed LIM-nebulette to distinguish it from regular nebulette, which does not possess an N-terminal LIM domain. The mRNA of LIM-nebulette comprised the sequences of four novel exons located at the 5′ end of the nebulette gene as well as the sequences of exons 24, 27, and 28 from the 3′ end of this gene. Exons 1–23 and 25–26 of the nebulette gene were missing in LIM-nebulette. Because the 5′-untranslated region of the LIM-nebulette mRNA differed substantially from that of the nebulette mRNA, it seemed likely that the two mRNAs were transcribed from the nebulette gene by usage of two alternative promoters.

To exclude the possibility that LIM-nebulette was simply the result of a cloning artifact, we also searched the mouse expressed sequence tag data bank for homologous sequences. In fact, we were able to identify several expressed sequence tag clones that coded for mouse LIM-nebulette (Fig. 1A). At the amino acid level, human and mouse LIM-nebulette differed only at seven positions and shared an overall identity of 97%. A comparison with the mouse genome sequence further demonstrated that the gene for mouse LIM-nebulette was located on mouse chromosome 2 in region 2A2, which is syntenic to human region 10p12. Similar to the human homologue, the mouse LIM-nebulette gene encompassed seven exons and spanned 390 kb. The first four exons were found to be located in the 5′ upstream region of the mouse nebulette gene, whereas the other three exons were identical to exons 24, 27, and 28 of the mouse nebulette gene.

A comparison of the amino acid sequences from human and mouse LIM-nebulette with all entries of the Swiss Protein Database revealed a striking similarity with the cytoskeletal protein Lasp-1 (Fig. 1A). Similar to LIM-nebulette, Lasp-1 has a modular structure with an N-terminal LIM domain, two central nebulin-like repeats, and a C-terminal SH3 domain (Fig. 1B). At the amino acid level, human LIM-nebulette and human Lasp-1 showed 66% sequence identity or 73% sequence similarity if conserved amino acid substitutions were included (Fig. 1A). The highest degree of similarity (78% identity) was observed between the SH3 domain of LIM-nebulette and that of Lasp-1. Thus, LIM-nebulette is a novel splice variant of nebulette that displays a modular structure similar to Lasp-1.

**Tissue Expression of LIM-nebulette**—To determine what tis-
sues might express LIM-nebulette, Northern blotting experiments were performed utilizing two different probes. One probe (human 5’ probe) was specific for LIM-nebulette and encoded the N-terminal sequence that is not found in regular nebulette (depicted in gray in Fig. 1B). The other probe (human 3’ probe) was designed to recognize the mRNAs for both LIM-nebulette and nebulette and corresponded to the sequence of the C-terminal half of the molecule (depicted in black in Fig. 1B). After hybridization to a human multiple tissue Northern blot, the 5’ probe detected a transcript of ~7 kb that was expressed in a wide range of tissues, including kidney, lung, brain, placenta, and pancreas (Fig. 2A). When the same blot was stripped and hybridized with the human 3’ probe, a different expression pattern was observed (Fig. 2B). Three prominent bands of 10, 8, and 4 kb were detected in addition to the 7-kb transcript. The 7-kb transcript occurred as a faint band in the samples from kidney, lung, brain, placenta, and pancreas as observed above, but the additional bands of 10, 8, and 4 kb were exclusively detected in the sample from heart. Thus, nebulette is specifically expressed in heart as described previously, whereas the novel splice variant LIM-nebulette is expressed at a relatively low level in a wide range of tissues.

To corroborate our results, a similar Northern blotting experiment was performed with probes specific for mouse LIM-nebulette. On a mouse multiple tissues Northern blot, the 5’ probe coding for the domain of mouse LIM-nebulette hybridized to a transcript of ~7 kb that occurred in the samples from lung, brain, and kidney (Fig. 2C). In contrast, the 3’ probe coding for the SH3 domain of mouse LIM-nebulette and nebulette detected a major transcript of 10 kb in the sample from heart as well as a minor transcript of 7 kb in the samples from lung, brain, and kidney (Fig. 2D). Thus, human and mouse LIM-nebulette show a very similar tissue expression.

**Zyxin Interacts with the SH3 Domain of LIM-nebulette**—The yeast two-hybrid system was used to map the binding site of zyxin in LIM-nebulette. To this end, several fragments of human LIM-nebulette cDNA were ligated into the prey vector pGADT7 downstream of the sequence for the GAL4 transactivation domain. The resulting constructs were introduced into yeast cells together with the bait vector pAS2-1 that contained the cDNA sequence for the N terminus of zyxin (residues 1–42) fused to the sequence for the GAL4 DNA-binding domain. A potential interaction of the resulting fusion proteins was analyzed by growth of yeast on histidine-deficient agar plates and by transcription of the reporter gene lacZ.

As expected, full-length LIM-nebulette showed a positive interaction with zyxin-(1–42) (Fig. 3A). In contrast, LIM-nebulette did not interact with lamin 5, which had been included as a negative control (data not shown). Furthermore, no binding to zyxin was detected with the N-terminal fragment of LIM-nebulette (residues 1–87) or with its central region (residues 126–219). However, a positive interaction was observed with the C-terminal half of LIM-nebulette (residues 126–270) as well as with the SH3 domain alone (residues 207–270). Thus, the SH3 domain, which occurs in nebulette and LIM-nebulette, appears to be sufficient for zyxin binding.

The results obtained with the two-hybrid system were confirmed by GST pull-down assays. To this end, the N-terminal fragment of zyxin (residues 1–42) was expressed as a GST fusion protein in bacteria and bound to glutathione beads. Various fragments of LIM-nebulette were produced by coupled in vitro transcription/translation in the presence of [35S]methionine and tested for their ability to bind to the GST-zyxin fusion protein. As shown in Fig. 3B, full-length LIM-nebulette was precipitated by GST-zyxin-(1–42). Likewise, the C-terminal half (residues 126–270) interacted with zyxin, whereas the N-terminal half (residues 1–87) did not. Further truncation of the C-terminal fragment indicated that the SH3 domain (residues 207–270) was sufficient for binding. In contrast, GST alone was not able to precipitate any of the fragments from LIM-nebulette used in this study (data not shown).

Taken together, the results obtained with the yeast two-hybrid system and the GST pull-down assay clearly demonstrate that zyxin interacts specifically with LIM-nebulette and that the SH3 domain of LIM-nebulette is sufficient for this interaction.
of the two proteins to precipitate full-length Lasp-1, indicating a direct interaction between Lasp-1 and zyxin. As outlined above, two different approaches were used, the yeast two-hybrid system and GST pull-down assays. For analysis with the yeast two-hybrid system, yeast reporter strain Y190 was co-transfected with a bait vector coding for the N terminus of zyxin (amino acids 1–42) and different prey vectors coding for various fragments of LIM-nebulette as indicated. Colonies that grew on histidine-, tryptophan-, and leucine-deficient plates were subjected to a qualitative β-galactosidase activity assay. B, the N terminus of zyxin (amino acids 1–42) was expressed as a GST fusion protein in bacteria and immobilized on glutathione-Sepharose. Full-length LIM-nebulette and various fragments derived thereof were prepared by coupled in vitro transcription/translation in the presence of [35S]methionine. The radiolabeled proteins were incubated with zyxin-GST beads. Bound proteins were dissolved in SDS sample buffer and analyzed by SDS-PAGE, followed by autoradiography. Aliquots of the in vitro translation products were analyzed in parallel on the same gel as input controls.

Zyxin Also Interacts with the SH3 Domain of Lasp-1—Because the structure of LIM-nebulette was highly related to that of Lasp-1, we investigated whether Lasp-1 would also interact with zyxin. As outlined above, two different approaches were used, the yeast two-hybrid system and GST pull-down assays. For the first set of experiments, cDNA sequences for full-length Lasp-1 and for two fragments thereof were inserted into the prey vector pGADT7. The resulting constructs were transfected into yeast together with the bait vector pAS2-1 that contained the sequence for zyxin-(1–42). Growth on histidine-, tryptophan-, and leucine-deficient plates demonstrated that full-length Lasp-1 was indeed capable of binding to zyxin-(1–42) (Fig. 4A). This interaction was specific because neither zyxin-(1–42) nor Lasp-1 showed any interaction with the unrelated proteins p53 or lamin A (data not shown). Truncation of full-length Lasp-1 further indicated that the SH3 domain (residues 187–261) was responsible for the interaction, whereas the other fragment containing the LIM-domain and the two nebulette repeats showed no binding.

In the second set of experiments, we performed GST pull-down assays with [35S]-labeled Lasp-1 and the GST-zyxin fusion protein. Fig. 4B demonstrates that GST-zyxin-(1–42) was able to precipitate full-length Lasp-1, indicating a direct interaction of the two proteins in vitro. Moreover, binding to zyxin was observed with the SH3 domain of Lasp-1, but not with the N-terminal fragment. On the other hand, GST alone was not able to pull down full-length Lasp-1 or any of its truncated fragments (data not shown). Lasp-1 can therefore bind, through its SH3 domain, to zyxin in a way similar to that of LIM-nebulette.

Binding Site in Zyxin—Next, we investigated the binding site of LIM-nebulette and Lasp-1 in zyxin. To this end, we prepared two deletion constructs. One construct (Δ1) lacked amino acid residues 19–42, and the other (Δ2) lacked residues 261–283. These sites were chosen because residues 19–42 are known to be involved in the binding of zyxin to α-actinin and because residues 261–283 show the best sequence similarity to the first region (12). GST pull-down experiments were performed with the two deletion constructs after transcription and translation in the presence of [35S]methionine. These experiments clearly indicated that construct Δ1 was not capable of binding and precipitating any LIM-nebulette or Lasp-1, whereas construct Δ2 had retained its binding activity (Fig. 5). These data demonstrate that amino acids 19–42 of zyxin are indispensable for binding to LIM-nebulette and Lasp-1.

In a further effort to map the crucial amino acids for LIM-nebulette and Lasp-1 binding, we performed site-directed mutagenesis. Individual amino acids at positions 17–52 of zyxin were replaced by serine, which carries an uncharged, hydrophilic side chain. Each mutated construct was tested for its
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activity to interact with LIM-nebulette or Lasp-1 in the yeast two-hybrid system. Quantitative analysis of the expression of the reporter gene lacZ demonstrated that substitutions at position 31, 33, and 39 yielded fusion proteins that had lost their binding activity for both LIM-nebulette and Lasp-1 (Fig. 6). In contrast, mutations at any other position barely compromised the interaction with LIM-nebulette and Lasp-1. Thus, amino acids 31–39 are crucial for the interaction of zyxin with LIM-nebulette and Lasp-1.

Subcellular Distribution of LIM-nebulette and Lasp-1—To compare the subcellular distribution of LIM-nebulette, Lasp-1, and zyxin, their cDNA sequences were cloned into a GFP expression vector and transfected into HeLa cells (Fig. 7). Staining of the transfected cells with antibodies against zyxin, followed by rhodamine-labeled secondary antibodies, specifically marked the focal adhesion plaques. When the fluorescence emitted from the GFP fusion proteins was analyzed, we found that LIM-nebulette as well as Lasp-1 localized to focal adhesions. Merging of the images demonstrated a strict co-localization of zyxin and LIM-nebulette on the one hand and of zyxin and Lasp-1 on the other hand (Fig. 7). Furthermore, the subcellular distribution of GFP-tagged Lasp-1 was very similar to that observed previously by indirect immunofluorescence with antibodies against Lasp-1 (23). Analogous results were obtained with HT1080 cells (data not shown). Thus, all three proteins (zyxin, LIM-nebulette, and Lasp-1) are localized at focal adhesions.

In an effort to identify the domains that are crucial for the correct subcellular distribution of LIM-nebulette, cDNA fragments corresponding to individual domains were cloned separately into the GFP expression vector and analyzed as described above (Fig. 8). We found that only the wild-type (Fig. 8, WT) protein with its full-length sequence showed specific distribution at focal adhesions. The LIM domain (residues 1–86) or the LIM domain plus the three nebulin-like repeats (residues 1–172) did not localize to focal adhesions but yielded a diffuse staining pattern throughout the cytoplasm of the cells. Likewise, the SH3 domain (residues 207–270) did not distribute at focal adhesions but accumulated within the nucleus (Fig. 8). This result is difficult to interpret, yet it is consistent with the observation that the GFP protein without any fusion peptide also showed a tendency to accumulate within the nucleus (data not shown). At any rate, none of the individual domains appears to co-localize with zyxin at focal adhesions. Thus, all the domains of LIM-nebulette are required to obtain the normal distribution of the full-length protein.

Interaction of Zyxin with other SH3 Domain-containing Proteins—Because both LIM-nebulette and Lasp-1 interacted with zyxin through their related C-terminal SH3 domain, it was of interest to test whether other SH3 domains would also be able to bind to zyxin. A search of the Swiss Protein Database with the amino acid sequence of the SH3 domain from human LIM-nebulette identified several related proteins with SH3 domains, including nebulin, SNX 9, and vinexin. The SH3 domain of human LIM-nebulette shared 75% sequence identity with that of nebulin and 31% with that of SNX 9 (Fig. 9A). Vinexin is known to contain three SH3 domains. The third SH3 domain displayed the highest degree of similarity (48% identity), whereas the first and the second showed only 34–35% identity with the SH3 domain of LIM-nebulette.

To test for a potential interaction with zyxin, the cDNA sequences of all these SH3 domains were inserted into the prey vector pGADT7. The resulting constructs were introduced into yeast together with the bait vector pAS2-1 that contained the N-terminal fragment of zyxin (residues 1–42). Based on the results obtained with the β-galactosidase assay, zyxin was able to bind to the SH3 domain of nebulin, whereas no binding was detected with the SH3 domain of SNX 9 or with any of the three SH3 domains of vinexin (Fig. 9B). Taken together, our data suggest that only SH3 domains that are closely related to LIM-nebulette can interact with zyxin. Other SH3 domains that do not share much sequence similarity with LIM-nebulette, such as those from vinexin and SNX 9, do not interact. It is therefore likely that the amino acid residues that are conserved in LIM-nebulette, Lasp-1, and nebulin but absent from SNX 9 and vinexin (as marked by asterisks in Fig. 9A) are directly or indirectly involved in zyxin binding.

DISCUSSION

Recently, we have started a systematic search for interaction partners of human zyxin. Utilizing the yeast two-hybrid system and cDNA libraries prepared from placenta and heart, we identified a number of potential candidates, including α-actinin, cyclophilin, and nebulin. In this report, we have carefully analyzed the interaction of zyxin with nebulin and its newly identified splice variant, LIM-nebulette.

Nebulette belongs to the nebulin family of actin-binding proteins. At present, this family includes the three large muscle-specific proteins nebulin, nebulin, and N-RAP (nebulin-related anchoring protein) as well as the relatively small non-muscle protein Lasp-1. Nebulin (800 kDa) is specifically found in skeletal muscle, where it is involved in maintaining the structural integrity of the sarcomeres. It has a modular structure composed of 185 nebulin repeats and a C-terminal SH3 domain (28). Nebulette (107 kDa) is exclusively expressed in cardiac muscle. This protein encompasses 23 nebulin repeats and a C-terminal SH3 domain (18, 19). N-RAP (185 kDa) occurs in both skeletal muscle and heart and appears to be involved in anchoring the actin filaments to the sarcolemma. It contains an N-terminal LIM domain and 40 nebulin repeats, but it lacks the C-terminal SH3 domain (29). The shorter protein Lasp-1 (30 kDa) is expressed in fibroblasts and epithelial cells. It
contains an N-terminal LIM domain, two nebulin repeats, and a C-terminal SH3 domain (21, 22).

In this report, we have identified a fifth member of the nebulin family, which we have termed LIM-nebulette. Similar to Lasp-1, it contains an N-terminal LIM domain, three nebulin repeats, and a C-terminal SH3 domain. A detailed comparison of its sequence with the completed sequence of the human genome clearly demonstrated that LIM-nebulette is not an independent gene product, but rather a novel splice variant of nebulette. With nebulette, it shares the C-terminal SH3 domain and one nebulin repeat. The N-terminal LIM domain and the first two nebulin repeats, however, are encoded by four separate exons located in the 5' upstream region of the nebulette gene on human chromosome 10p12. It is therefore likely that LIM-nebulette is transcribed from an upstream promoter that is active in non-muscle cells, whereas in cardiac cells, nebulette is transcribed from an internal promoter situated downstream of the four additional exons. The novel splice variant resembles Lasp-1 more closely than nebulette, although Lasp-1 is transcribed from a separate gene on chromosome 17q21. Thus, the LIM-nebulette gene represents a chimeric gene that might have been created during evolution by fusion of an ancestral LASP gene with an ancestral nebulette gene. Very recently, a similar conclusion has been drawn by Katoh and Katoh (30), who compared the sequences of expressed sequence tags with genomic sequences stored in public data banks.

LIM-nebulette is expressed in fibroblasts and HeLa cells in a fashion similar to Lasp-1. By indirect immunofluorescence, Lasp-1 has been localized to the leading edges of membrane ruffles and focal adhesions (23, 24, 31). It was therefore proposed that Lasp-1 might play a critical role in the dynamic organization of the actin cytoskeleton. Based on the striking similarity of its domain structure and its subcellular distribution, we speculate that LIM-nebulette might fulfill a similar function.

By a combination of three different approaches, we have been able to demonstrate that LIM-nebulette interacts with zyxin and that the C-terminal SH3 domain is responsible for this interaction. (a) When expressed in HeLa cells as a GFP fusion protein, LIM-nebulette shows a strict co-distribution with zyxin. (b) In the yeast two-hybrid system, LIM-nebulette interacts with zyxin and induces transcriptional activation of the reporter gene ß-galactosidase. When individual fragments derived from LIM-nebulette are tested, polypeptides containing the C-terminal SH3 domain are active, whereas those lacking this domain are inactive. (c) In a pull-down assay, zyxin is able to precipitate full-length LIM-nebulette as well as fragments that possess the SH3 domain. Fragments lacking this domain are not precipitated.

The ability to interact with zyxin is not confined to the SH3 domain of LIM-nebulette but extends to the SH3 domains of other nebulin family members. The SH3 domains from both nebulin and Lasp-1 show a positive reaction with zyxin in the two-hybrid system, whereas SH3 domains from more distantly

![Fig. 6. Identification of amino acids that are important for the interaction of zyxin with LIM-nebulette or Lasp-1. The bait vector pAS2-1 encoding the N terminus of zyxin (amino acids 1–81) was transfected into yeast reporter strain Y190 together with the prey vector pGADT7 encoding full-length LIM-nebulette or full-length Lasp-1. The codons for some amino acids of wild type zyxin were replaced with the codons for serine as indicated. Colonies that grew on tryptophan- and leucine-deficient plates were analyzed for transcription of the ß-galactosidase reporter gene using a quantitative, colorimetric assay. The results are expressed relative to the wild type zyxin sequence (100%) and represent the means ± S.D. from three independent determinations. Mutations at positions 17, 22, and 24 were tested in a separate experiment and yielded results similar to the V29S mutation.](image)

![Fig. 7. Subcellular distribution of LIM-nebulette, Lasp-1, and zyxin. HeLa cells were transiently transfected with the expression vector pEGFPC2 that contained the sequence of full-length LIM-nebulette or full-length Lasp-1, respectively, fused to the sequence of GFP. Two days after transfection, the cells were fixed, permeabilized, and stained with antibodies against zyxin. Green indicates direct fluorescence emitted from GFP, and red indicates indirect immunofluorescence emitted from anti-zyxin antibodies. The images were superimposed with the help of a computer to demonstrate the co-localization of the signals. Bar, 20 μm.](image)
related proteins are inactive. SH3 domains occur in a vast number of proteins and are involved in diverse cellular processes, including signal transduction, cytoskeletal assembly, and membrane localization (32). Typically, they bind to ligands with a proline-rich segment harboring the consensus sequence PXXP. The ligand adopts a helical polyproline type II conformation and interacts with the SH3 domain in either one of two opposite orientations (33). Accordingly, the ligands have been grouped in class I (consensus motif +XXPXP, where + denotes a positively charged amino acid) or class II (consensus motif PXXP+). Recent studies, however, have extended the repertoire of SH3 domain recognition beyond the classical, conserved binding motif PXXP (34). The three-dimensional structure of the SH3 domain from human nebulin has been determined in solution by nuclear magnetic resonance spectroscopy (35). In vitro, it binds selectively to peptides of class II as they occur in titin and myopalladin (36, 37). In the yeast two-hybrid system, myopalladin does in fact bind to the SH3 domain in either one of two opposite orientations (33). In vitro mutagenesis that amino acid residues 33–39 of the N terminus from zyxin are critical for interaction with the SH3 domain from LIM-nebulette. These amino acids occur within a motif that is highly conserved in human, mouse, and chicken zyxin: 28-PVVAPPKVNPPFR-40. This sequence shows some similarity to a class II motif but does not match it completely. It should be emphasized that the same motif is also found in the zyxin-related protein LPP (14). In fact, when tested in the two-hybrid system, LPP does interact with the SH3 domain of nebulin and nebulette.\(^2\)

Zyxin is typically found in focal adhesions of fibroblasts and other non-muscle cells. Thus far, there is no conclusive evidence that zyxin might also occur at the sarcomeric Z-disks of skeletal muscle (15). It is therefore likely that in fibroblasts, zyxin will interact with the SH3 domain of the non-muscle proteins LIM-nebulette and Lasp-1. In muscle cells, nebulin and nebulette might interact via their SH3 domains with another ligand such as the muscle-specific protein myopalladin. In this context, it is interesting to note that myopalladin has been found to interact with \(\alpha\)-actinin in a way analogous to

\(^2\) B. Li and B. Trueb, unpublished observations.
nebulin must therefore be located in very close proximity at binding due to steric hindrance.

LIM-nebulin caused by a mutation in the most 5’ermic Z-disks. It is therefore conceivable that the dysfunction of play a role in the assembly of focal adhesions similar to the role dilated cardiomyopathy, which is characterized by a thin-
gene are associated with the nonfamilial form of idiopathic
in skeletal muscle (39). Furthermore, defects in the nebulin
cause for the autosomal recessive form of nemaline myopathy
recently mapped the terminus of zyxin (12). By
in vitro the interaction (13). The binding sites for
prove that amino acids 26–29 are crucial for binding,
whereas substitutions at positions 31–39 did not compromise
the interaction (13). The binding sites for α-actinin and LIM-nebulin
must therefore be located in very close proximity at
the N terminus of zyxin. It remains to be determined whether
α-actinin and LIM-nebulin do in fact bind simultaneously to
zyxin, or whether the two proteins show mutually exclusive
binding due to steric hindrance.

Mutations in the nebulin gene have been identified as the
cause for the autosomal recessive form of nemaline myopathy
in skeletal muscle (39). Furthermore, defects in the nebulin
gene are associated with the nonfamilial form of idiopathic
dilated cardiomyopathy, which is characterized by a thinned-
walled heart (27). We have argued that LIM-nebulin might
play a role in the assembly of focal adhesions similar to the role
played by nebulin and nebulin in the assembly of the sarco-
meric Z-disks. It is therefore conceivable that the dysfunction of
LIM-nebulin caused by a mutation in the most 5’ part of the
gene might have severe consequences in non-muscle cells. It
will therefore be interesting to analyze the effects of a nonsense
mutation in one of the LIM-nebulin specific exons on cell
adhesion and the formation of focal contacts.

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Fig. 9. Interaction of zyxin with other SH3 domains. A, alignment of the amino acid sequences of the SH3 domains from LIM-nebulin, Lasp-1, nebulin, and nebulin (C-terminal SH3 domain), and SNX 9. Identical residues are boxed. Amino acids that are conserved in LIM-nebulin, nebulin, and Lasp-1 but not in nebulin and SNX 9 are indicated by asterisks. B, yeast reporter strain Y190 was co-transfected with a bait vector encoding zyxin (residues 1–42) and different prey vectors encoding various SH3 domains as indicated. Colonies that grew on histidine-, tryptophan-, and leucine-deficient plates were subjected to a qualitative β-galactosidase assay.
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