Leupaxin Is a Novel LIM Domain Protein That Forms a Complex with PYK2*

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We have identified a novel cytoplasmic protein, leupaxin, that is preferentially expressed in hematopoietic cells and is most homologous to the focal adhesion protein, paxillin. Leupaxin possesses two types of protein interaction domains. There are four carboxyl-terminal LIM domains in leupaxin that share 70% amino acid identity and 80% similarity with those in paxillin. Paxillin LIM domains mediate localization to focal contacts. In the amino-terminal region of leupaxin there are three short stretches of approximately 13 amino acids that share 70–90% similarity with paxillin LD motifs. Paxillin LD motifs have been implicated in focal adhesion kinase (FAK) and vinculin binding resulting in the localization of FAK to focal adhesions. Leupaxin is expressed in cell types, such as macrophage, that lack FAK. We demonstrate here that leupaxin associates with a second FAK family member, PYK2. As leupaxin and PYK2 are both preferentially expressed in leukocytes they may therefore form a cell type-specific signaling complex. We also demonstrate that leupaxin is a substrate for a tyrosine kinase in lymphoid cells and thus may function in and be regulated by tyrosine kinase activity. Leupaxin is thus a phosphotyrosine protein with LD and LIM binding motifs most homologous to paxillin that may assemble and regulate PYK2 signaling complexes in leukocytes.

Cell adhesion, spreading, and migration are mediated by integrin interactions with extracellular and cell surface ligands (1, 2). In adherent cell types such as epithelial cells and fibroblasts a complex of cytoplasmic proteins localizes to the termini of actin bundles at sites of integrin-dependent close cell contact with substratum or extracellular matrix proteins (3). These complexes, designated focal adhesions, have been implicated in the regulation of cell locomotion, survival, and proliferation (4, 5).

Focal adhesions are rich in tyrosine kinases and tyrosine-phosphorylated proteins, which may reflect a role for tyrosine kinases in integrin signaling (6, 7). Protein-tyrosine kinases that are focal adhesion proteins include focal adhesion kinase (FAK),³ Src, and Src family kinases. A second FAK family member, PYK2, has been identified by several groups and designated CAKβ (8), RAFTK (9), FAK2 (10), and CADTK (11). PYK2 and FAK are closely related in their overall structure, and both are phosphorylated on tyrosine in response to integrin engagement, T cell receptor engagement or chemokine stimulation (12–15). These stimuli also modulate integrin-dependent adhesion. PYK2 and FAK both associate with paxillin, p130cas, and Src (16, 17). Although PYK2 possesses a focal adhesion targeting domain that is highly homologous with FAK, the majority of PYK2 displays a more diffuse cytoplasmic distribution with a small percent present in focal adhesions (18). This indicates that FAK and PYK2 may possess both overlapping and unique functions.

Tyrosine phosphoproteins present in focal adhesions include FAK, vinculin, zyxin, and paxillin. A second paxillin family member, Hic-5, has recently been shown to localize to focal adhesions (18). As phosphorylation of tyrosine on focal adhesion proteins can regulate their interaction with proteins that possess SH2 and phosphotyrosine-binding domains, tyrosine kinase activity can regulate some protein-protein interactions in these dynamic focal adhesion complexes (7, 19). Another domain utilized by several focal adhesion proteins to mediate interactions with other proteins is the LIM domain (20). LIM domains are approximately 50 residues in length and contain conserved cysteine, histidine, and aspartate residues that form zinc binding modules (21–23). Paxillin, Hic-5, zyxin, and cysteine-rich protein contain a tandem array of 3–4 LIM domains in their carboxyl-terminal region. Individual LIM domains demonstrate specificity in binding different proteins or motifs. The first amino-terminal LIM domain of zyxin, LIM1, supports an interaction with the cysteine-rich protein CRP (20), and paxillin LIM3 has been shown to be critical to the localization of paxillin to focal adhesions (24). LIM domains in Enigma, a protein that interacts with the insulin receptor and the receptor tyrosine kinase Ret, binds to specific tyrosine containing tight-turn motifs (25). Recently, sequences of approximately 13 amino acids in the amino-terminal region of paxillin have been implicated in binding to FAK and vinculin (24). These sequences have been designated LD motifs because they contain an invariable leucine-aspartate pair near their amino terminus. Paxillin LD motifs appear to be responsible for the localization of FAK to focal adhesions. Thus, focal adhesion proteins such as paxillin contain multiple binding domains and likely serve as scaffolds to focus and regulate specific effector molecules such as FAK to a subcellular site.

We have identified a novel cytoplasmic protein, leupaxin, that is a substrate for a tyrosine kinase and thus may be functionally regulated by a protein-tyrosine kinase. Leupaxin contains LIM and LD motifs which are most homologous to paxillin, suggesting that it may serve as an adapter to regulate signaling at sites of adhesion. We demonstrate that leupaxin associates with PYK2 in lymphoid cells. Both PYK2 and leupaxin are preferentially expressed in leukocytes and have a...
diffuse cytoplasmic distribution. Thus leupaxin and PYK2 may form a cell type-specific signaling complex.

**MATERIALS AND METHODS**

Isolation of Leupaxin cDNA—A random screening sequencing of a human macrophage library resulted in the isolation of a partial 1.2-kb leupaxin cDNA clone. The insert from this clone was 32P-labeled using a Random Primed Labeling Kit (Boehringer Mannheim) and was used as a hybridization probe to obtain a cDNA clone containing a complete coding region from a spleen cDNA library (pCDNA1/Amp (Invitrogen)) (26). The 32P-labeled leupaxin probe was added to colony replicas in hybridization buffer (5 × SSC, 5 × Denhardt’s, 1% SDS, and 45% formamide) and hybridized overnight at 42 °C. The final wash (0.5 × SSC, 0.1% SDS) was at 50 °C. Two independent 1.9-kb clones recovered after a secondary screening were fully sequenced and determined to contain identical and complete coding sequences.

Expression and Subcellular Localization of Leupaxin in JYS—The entire leupaxin coding region was fused in frame to the 3' end of the enhanced green fluorescence protein (GFP) coding sequence isolated from pEGFP-C1 (CLONTECH). The resulting GFP-leupaxin chimeric sequence was subcloned into pCEP4 (pEGFP-PX2-CEP4). A lymphoblastoid line expressing an IL-8 receptor, JY8 (42), was electroporated with a probe to isolate additional cDNAs from a spleen library. Two independent 1.9-kb clones recovered after a secondary screening were fully sequenced and determined to contain identical and complete coding sequences.

**RESULTS AND DISCUSSION**

Isolation of cDNAs Encoding Leupaxin—To identify novel macrophage proteins a random sequencing screen of a human macrophage cDNA library was conducted. From this screen a cDNA was isolated that contains an in-complete coding region homologous to the 3' region of paxillin. This cDNA was used as a probe to isolate additional cDNAs from a spleen library. Two
Leupaxin, a Novel Focal Adhesion Protein

Leupaxin, a novel focal adhesion protein, is discussed in this section. The protein appears to be a relative of paxillin that is expressed preferentially in leukocytes (discussed below), we designate it leupaxin. The overall amino acid identity between leupaxin and paxillin is 37%; however the carboxyl-terminal half, residues 151–385, possesses 70% identity and 80% similarity (Fig. 1A). This region of leupaxin and paxillin contains four LIM domains. The homology between individual LIM domains range from 67 to 76% identity. Other proteins possessing less homologous LIM domains include Hic-5 (54% identity), PINCH protein (42% identity), and Enigma (36% identity). LIM domains are approximately 50–60 amino acids in length and contain three conserved residues, cysteine, histidine, and aspartate, spaced such that they form a structure that chelates zinc. Leupaxin LIM domains each contain two such zinc fingers with the consensus motif CX_2CX_16HX_2(C/H)X_6CX_2CX_17CX_2(D/H/C) (Fig. 1A). LIM domains have been implicated in interactions with other LIM domains as well as with tyrosine-containing motifs in tight turns of membrane proteins (25). Recently, paxillin LIM3 has been shown to mediate localization to focal adhesions and LIM2 appears to cooperate in this localization (24), but the focal adhesion ligand for paxillin LIM3 has not yet been identified.

The amino-terminal region of leupaxin is shorter than paxillin and demonstrates three short stretches of high homology (Fig. 1A and B). Residues 1–15, 85–102, and 127–149 share 53%, 56%, and 63% identity with paxillin. Considering conserved substitutions the similarity between these sequences is 90%, 72%, and 75% (Fig. 1B). Within these homologous sequences are LD motifs, first identified as a 13-amino acid motif in paxillin (24). These motifs are designated LD due to the invariable leucine and aspartate pair near their amino terminus. The homology around leupaxin and paxillin LD motifs extends into flanking residues. Leupaxin possesses three LD motifs which align, with gaps between motifs, to paxillin LD1, LD3, and LD4 (Fig. 1A). There is a potential fourth LD motif in leupaxin at residues 39–51. However, unlike other LD motifs, this sequence only contains the three invariable residues and no additional homology (Fig. 1, A and B). Another paxillin-related protein Hic-5 also possesses four LD motifs (18) that can be aligned with leupaxin and paxillin (Fig. 1B). Thus the positioning and degree of homology between LD motifs can differ, and this may impart unique binding properties to leupaxin, paxillin, and Hic-5.

LD motifs were first characterized in paxillin and are implicated in binding to FAK and vinculin (24, 27). Sequences encompassing paxillin LD2 and LD3 appear to bind to FAK whereas LD2 alone appears to mediate association with vinculin. Binding to paxillin LD2 and LD3 appears to mediate localization of FAK to focal adhesions (24). Thus paxillin functions as an adapter via LD and LIM motif interactions. Leupaxin does not possess a LD motif with significant homology to paxillin LD2 (Fig. 1B). In the absence of a LD motif corresponding to LD2 a potential interaction between leupaxin and FAK or vinculin would therefore be predicted to be relatively weak. Leupaxin would not be predicted to associate with FAK in monocytes or macrophages that express leupaxin but not FAK (28, 29). Additional candidate leupaxin-binding proteins might include other paxillin ligands such as Src (30), Csk (31), Lyn (32), Crk (33), and talin (34). The structural similarities between members of the paxillin family suggest that leupaxin may also serve an adapter function and localize signaling molecules to certain subcellular sites.

Cell Type and Expression of Leupaxin—To determine the range of cell types and tissues that express leupaxin the leupaxin cDNA was used to probe blots of mRNA isolated from various sources. Consistent with the size of the leupaxin cDNA...
the probe hybridized to a 2.4-kb mRNA (Fig. 2). This mRNA was present in lymphoid tissues including spleen, lymph node, thymus, and appendix. There was markedly less leupaxin mRNA in bone marrow and fetal liver which required 4 times longer exposure of film to hybridized blots for detection (data not presented).

Leupaxin mRNA was also detected in peripheral blood lymphocytes and the hematopoietic cell lines HL60, Molt4, and Raji (Fig. 2). Leupaxin mRNA in four different epithelial cell lines was only detected after 4 times longer exposure of hybridized blots to film (data not presented). Leupaxin mRNA levels therefore appear to be markedly greater in lymphoid tissues and certain hematopoietic cell lines relative to non-hematopoietic cell types. It is interesting that bone marrow cells appear to contain low levels of leupaxin mRNA relative to lymphocytes and lymphoid tissues suggesting that leupaxin expression in hematopoietic cells may increase with differentiation.

**Localization of Leupaxin in Lymphoid Cells**—To begin to determine a subcellular localization where leupaxin may function we examined the localization of a GFP-leupaxin chimeric protein in a lymphoblastoid cell line, JY8. To facilitate subcellular localization JY8 cells were incubated on an ICAM-1-coated substrate to promote cell spreading. ICAM-1 is a ligand for the only CD18 integrin, CD11a/CD18, expressed in JY8 (42). Leupaxin was found to be diffusely distributed in the F-actin cortical cytoskeleton that stains with rhodamine-phalloidin (Fig. 3). Leupaxin was present in the proximal regions of filopodia-like projections but was excluded or less represented in their distal tips. Leupaxin also localizes to a region adjacent to the F-actin-rich cortical cytoskeleton in JY8. Thus GFP-leupaxin is rather diffusely distributed in JY8 corresponding with the distribution of an associated protein, PYK2 (discussed below). GFP is uniformly distributed throughout the cytoplasm and nucleus (data not presented). It remains to be determined if leupaxin is present in complexes that approximate those in adherent cell-type focal adhesions. Focal adhesions in highly motile cells such as leukocytes have been suggested to be relatively diffuse, much smaller, greater in number, and more uniformly distributed (1, 35).

**Leupaxin Is Phosphorylated on Tyrosine**—Tyrosine phosphorylation of paxillin may support signaling following integrin engagement. Leupaxin contains 10 potential tyrosine phosphorylation sites (Fig. 1). To determine if leupaxin is a tyrosine kinase substrate the GFP-leupaxin chimera expressed in JY8 was tested for reactivity to a phosphotyrosine-specific mAb.

The GFP-leupaxin chimera migrates as a 78-kDa protein on SDS-PAGE whereas GFP migrates as a 33-kDa protein (Fig. 4, right blot). The difference between GFP-leupaxin and GFP, 45 kDa, is consistent with the calculated size of 43 kDa for leupaxin. The GFP-leupaxin fusion protein immunoprecipitated from lysates of JY8 transfectants bound an anti-phosphotyrosine mAb (Fig. 4). The binding of the anti-phosphotyrosine mAb was inhibited by phenyl phosphate (500 μM) confirming the specificity of the anti-phosphotyrosine mAb interaction. There was no detectable binding of the phosphotyrosine mAb to GFP which was expressed at equivalent or greater levels than leupaxin. These results indicate that leupaxin is a substrate for a tyrosine kinase in lymphoid cells.

Leupaxin function may be regulated by a tyrosine kinase activity. Paxillin is also phosphorylated on tyrosine, and this...
phosphorylation can be induced with cell adhesion or by engagement of integrins with antibodies (36–38), by cell transformation (30), and in response to mitogens that signal through G protein-coupled seven transmembrane receptors (38, 39). Thus, tyrosine phosphorylation of paxillin occurs in the process of cell adhesion, motility, and growth. The regulation of leupaxin phosphorylation by various stimuli and its functional consequences are currently being addressed.

**Leupaxin Associates with PYK2 in Lymphoid Cells**—Leupaxin contains LD motifs homologous to those in paxillin and Hic-5 suggesting that it may interact with FAK or PYK2. The leupaxin cDNA, however, was isolated from macrophages where FAK expression has not been detected (28, 29). We therefore determined whether leupaxin associated with PYK2, a protein-tyrosine kinase closely related to FAK and expressed preferentially in leukocytes and brain. Immunoblots clearly demonstrate that PYK2 co-immunoprecipitated with GFP-leupaxin (Fig. 5A). This association was specific for leupaxin because PYK2 did not co-immunoprecipitate with GFP although equivalent levels of GFP and GFP-leupaxin were immunoprecipitated from JY8 lysates. We also demonstrate in the reciprocal experiment that GFP-leupaxin was co-immunoprecipitated with PYK2 from lysates of JY8 transfectants (Fig. 5B). Again, this association was specific for leupaxin as GFP was not found to associate with PYK2 even though PYK2 was immunoprecipitated at equivalent levels from lysates of JY8 cells expressing GFP-leupaxin or GFP (Fig. 5B). In addition, control antibodies did not co-immunoprecipitate either PYK2 or leupaxin (data not presented). Thus when overexpressed leupaxin complexes with PYK2 in JY8 cells.

The association of leupaxin with PYK2 may specifically support PYK2 functions in leukocytes. PYK2 is a FAK family member that is expressed preferentially in leukocytes and brain (9, 17). Leupaxin, however, is expressed preferentially in leukocytes and is expressed at relatively very low levels in brain (data not presented). Thus the potential role of leupaxin in PYK2 function may predominate in hematopoietic cells. PYK2 may be predicted to mediate functions overlapping with those of FAK including cell motility, cell spreading, and apoptosis. Leupaxin may serve to localize PYK2 to different subcellular sites to support or regulate these functions. It will be of interest to determine the role of leupaxin in PYK2 function relative to paxillin and Hic-5 (18).

Leupaxin possesses LD and LIM binding motifs which are highly homologous to those in paxillin that appear to function in the assembly of focal adhesions (24, 37, 40). Similar to paxillin and Hic-5, leupaxin associates with PYK2 and is phosphorylated on tyrosine. We suggest a role for leupaxin in localizing or modulating PYK2 containing signaling complexes in response to integrin engagement or G-protein-coupled receptor stimulation.

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