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LIM domain-containing adaptor, leupaxin, localizes in focal adhesion and suppresses the integrin-induced tyrosine phosphorylation of paxillin

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Focal adhesion (FA) consists of multiple cellular proteins including paxillin and serves as a center for adhesion-mediated signaling. The assembly and disassembly of FAs is regulated by locally produced intracellular signals, and tyrosine phosphorylation of paxillin has been implicated in this process. A Lin-11 Isl-1 Mec-3 (LIM) domain-containing adaptor protein, leupaxin, a member of the paxillin family, is expressed in leukocytes as well as in certain cancer cells, and shares overall structural characteristics with paxillin. However, it remains unknown whether leupaxin and paxillin cooperate with or antagonize each other in integrin signaling. Here we show that leupaxin potently represses the tyrosine phosphorylation of paxillin. When expressed in mouse thymoma BW5147 cells bound to ICAM-1, leupaxin accumulated in FA-like patches in the cell periphery. When expressed in NIH3T3 and HEK293T cells, leupaxin localized to FAs upon cell adhesion to fibronectin and strongly suppressed the integrin-induced tyrosine phosphorylation of paxillin. In integrin-stimulated HEK293T cells, leupaxin's LIM3 domain appeared essential for selective FA localization and the suppression of paxillin tyrosine phosphorylation. Leupaxin's LD3 motif, which is critical for stable association with FAK, was dispensable for leupaxin's suppressive ability. In addition, leupaxin reduced the spreading of NIH3T3 cells on fibronectin, which required both the LD3 motif and LIM3 domain. When expressed in human leukocytic K562 cells, leupaxin significantly suppressed integrin α5β1-mediated cell adhesion to fibronectin and the tyrosine phosphorylation of paxillin. These findings indicate that leupaxin functions as a paxillin counterpart that potently suppresses the tyrosine phosphorylation of paxillin during integrin signaling. (*Cancer Sci* 2010; 101: 363–368)

Cell adhesion and migration are fundamental to cell and tissue dynamics in morphogenesis and immune defense, as well as cancer invasion and metastasis.[1] The cell-surface integrins play critical roles in cell adhesion and the subsequent transduction of signals into the cells.[2,3] Focal adhesion (FA) is a common type of adhesion contact that cells generate using integrins, and it serves as a center for integrin signaling.[4,5]

FAs are composed of multiple cytoplasmic plaque proteins and tyrosine kinases.[5] The FA protein paxillin contains multiple protein-binding domains and binds various signaling molecules, including focal adhesion kinase (FAK), prolin-rich tyrosine kinase 2 (Pyk2), and sarcoma kinase (Src).[6–8] The formation of FAs is closely associated with the temporally and spatially regulated tyrosine phosphorylation of paxillin, and phosphorylated paxillin provides additional binding sites for other adaptor proteins such as Crk and Cas, which recruit yet other signaling molecules to form large protein complexes.[5–8] Thus, paxillin not only provides a structural framework but also participates in the propagation of integrin-mediated adhesion signals through the sequential activation of signal-transducing components.

Leupaxin was originally identified as a leukocyte-specific isoform of paxillin,[9] and is a member of the paxillin family, which includes paxillin, Hic-5, and leupaxin.[6,7] Subsequent studies have shown that leupaxin is also expressed in non-hematopoietic lineage cells, including vascular smooth muscle cells[10] and certain cancer cells, such as prostate cancer cells.[11,12] In a subset of prostate cancers, leupaxin expression intensity is directly linked to cancer progression.[12] Like paxillin, leupaxin is composed of multiple functional modules, including leucine (L) and aspartate (D) motifs and LIM domains, suggesting that leupaxin also serves as a molecular adaptor that is involved in integrin-mediated signaling. In fact, studies by Gupta *et al.*[13] showed that leupaxin localizes to the podosomal signaling complex in murine osteoclasts and that it is likely to be involved in regulating the rearrangement of cytoskeletal components. In addition, leupaxin has been shown to interact with multiple components of FAs, including Pyk2, FAK, Src, lck/yes-related novel tyrosine kinase (Lyn), and protein tyrosine phosphatase proline-, glutamate-, serine-, and threonine-rich sequence (PTP-PEST), which play important roles in integrin signaling.[9,11,13,14] However, it is not clear whether leupaxin and paxillin functionally cooperate with or antagonize each other in integrin-mediated signal transduction pathways. In this study, we evaluated the functional significance of leupaxin and demonstrated that leupaxin counteracts the integrin-mediated tyrosine phosphorylation of paxillin. This counterregulatory activity is closely associated with leupaxin's ability to localize to FAs and requires its LIM3 domain, and appears to regulate cell adhesion and spreading.

Materials and Methods

Isolation of mouse leupaxin cDNA. A cDNA fragment of mouse leupaxin (GS9937: 261 bp) was isolated[15–17] (http://bodymap.ims.u-tokyo.ac.jp/), labeled with HRP (GE Healthcare, Waukesha, WI, USA), and used to screen a mouse spleen cDNA library (5′-Stretch plus; Clontech, Palo Alto, CA, USA). A 1.7-kb cDNA clone was isolated and fully sequenced (Genbank/DDBJ/EMBL AB071194).

cDNA constructs. Mutant forms of leupaxin lacking the LD3 motif (amino acids 92–104), the LIM3 domain (amino acids 270–303), or both, were generated by PCR. The nucleotide sequences of the cDNA fragments generated by PCR were confirmed by dideoxy sequencing. To prepare enhanced green fluorescent protein (EGFP)-tagged leupaxin proteins, the WT or mutated leupaxin cDNA was then ligated into pEGFP-N1 (Clontech) that had been cleaved with EcoRI and BamHI. The

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resultant plasmids were digested with EcoRI and XbaI to obtain chimeric cDNA fragments that were then inserted into the pEF-BOS vector.[18]

Expression of leupaxin in cultured cell lines. Mouse thymoma BW5147 cells and human erythroleukemia K562 cells were co-transfected with an expression plasmid for WT leupaxin-EGFP and pSV2-Neo, and selected in 0.5 mg/mL G418. Stable transformants were cloned by a limiting dilution method. The transient transfection of human embryonic kidney-derived HEK293T cells and murine fibroblastic NIH3T3 cells was performed using the FuGENE6 (Roche, Mannheim, Germany) or Lipofectamine (Gibco BRL, Grand Island, NY, USA) reagent. The expression of leupaxin-EGFP protein was confirmed by Western blotting with an anti-GFP antibody (Clontech).

Subcellular localization of leupaxin in transfectants. BW5147 transfectants were plated onto ICAM-1/IgG[19] (5 μg/mL)-coated glass coverslips in RPMI medium containing 0.1% BSA for 1 h at 37°C. To enhance the spreading and extension of BW5147 cells, PMA (10 ng/mL) was added to the medium. For HEK293T and NIH3T3 transfectants, cells in DMEM with 0.1% BSA were plated onto human plasma fibronectin (FN) (10 μg/mL) (Gibco BRL)-coated coverslips for 1 h at 37°C. The cells were then fixed with 3.7% formaldehyde in PBS, washed in PBS, and permeabilized with 0.1% Triton X-100. In some experiments, the cells were stained with anti-vinculin mAb (h-VIN-1) (Sigma, St. Louis, MO, USA) and Cy5-conjugated donkey antimouse IgG (Chemicon, Temecula, CA, USA). The cells were then further incubated with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR, USA), and analyzed by confocal microscopy (LSM-410 or LSM-510; Carl Zeiss, Oberkochen, Germany).

Tyrosine phosphorylation of paxillin. Cells were serum-starved for 2 h at 37°C, and then allowed to bind to plastic wells pre-coated with poly-L-lysine (PLL, 50 μg/mL) (Sigma), BSA (10 μg/mL), or FN (10 μg/mL) for 1 h at 37°C. The cells were then lysed in ice-cold lysis buffer (50 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 μg/mL aprotinin, 1 mM $NaVO_4$), and immunoprecipitated with an anti-paxillin mAb (clone 349) (BD Transduction Laboratories, Franklin Lakes, NJ, USA) and protein G Sepharose (GE Healthcare). The anti-paxillin mAb was found to cross-react with leupaxin and immunoprecipitated both paxillin and leupaxin (see Fig. 2 for details). The immunoprecipitates were subjected to SDS-PAGE under reducing conditions, and protein tyrosine phosphorylation was detected by an HRP-conjugated anti-phosphotyrosine mAb (RH20) (BD Transduction Laboratories) and ECL Western blotting detection reagents (GE Healthcare).

Co-immunoprecipitation of leupaxin and FAK. HEK293T cells transiently expressing EGFP or EGFP-tagged leupaxin proteins were lysed in ice-cold lysis buffer and subjected to immunoprecipitation with an anti-GFP antibody (Clontech). The immunoprecipitates were separated by electrophoresis and transferred to PDVF membranes. Western blotting with an anti-FAK mAb (clone 77; BD Transduction Laboratories) or anti-GFP antibody was performed to detect the immunoprecipitated proteins.

Cell spreading. Spreading of NIH3T3 cells on FN was assessed as described previously.[20] In brief, NIH3T3 cells that transiently expressed either EGFP alone or the EGFP-tagged leupaxin protein were incubated on the FN-coated coverslips for 1 h at 37°C. After gentle washing with pre-warmed PBS, the cells were stained with rhodamine-conjugated phalloidin and observed by confocal microscopy (LSM-510; Carl Zeiss). Cells that had spread were defined as those that had extended process and lacked a round morphology. The experiments were performed three times. In each experiment, >100 fluorescent transfected cells were analyzed.

Cell adhesion to FN. Parental and transfected K562 cells were labeled with 2 μM 3′-*O*-acetyl-2′, 7′-bis (carboxyethyl)-4 or 5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) (Dojindo, Osaka, Japan). The cells (1×10^5 cells/well) were added to 96-well plastic plates (Sumilon H type; Sumitomo, Tokyo, Japan) that had been coated with FN (10 μg/mL) and were then incubated for 1 h at 37°C. The wells were then filled with RPMI-1640 containing 10% FCS, sealed with Parafilm, and incubated at 37°C for 30 min in an inverted position. The supernatants were carefully aspirated to remove unattached cells, and adherent cells were lysed by adding 50 μL of 1% NP40 in PBS per well. The plates were measured using a Fluoroskan II (Labosystems, Helsinki, Finland).

Flow cytometry. Cells were incubated with anti-CD29 (clone K20) or anti-CD49e (clone SAM1), followed by biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) and avidin-PE. The cells were then analyzed by an EPICS-XL flow cytometer (Beckman Coulter, Miami, FL, USA). Primary mAbs were obtained through the VIth Human Leukocyte Differentiation Antigen Workshop (Kobe, Japan, 1996).

Statistical analysis. The Student's *t*-test was applied to compare the statistical difference within two groups.

Results

Leupaxin accumulates to FAs. In a search for genes regulating lymphocyte adhesion and migration, we identified the mouse ortholog of leupaxin. Mouse leupaxin is 87% identical to its human counterpart at the protein level and contains four potential LD motifs in its N-terminal half and four LIM domains in its C-terminal half, suggesting functional conservation of leupaxin between mice and humans. In close parallel with leupaxin's human counterpart, one of the LD motifs (LD2) of mouse leupaxin exhibits lower homology than is found among the other three LD motifs.[9]

When expressed in BW5147 cells that were bound to ICAM-1, EGFP-tagged leupaxin was found mainly in the FA-like patches in the F-actin-rich peripheral regions in the large cellular projections (Fig. 1a, arrowheads). When expressed in NIH3T3 cells that adhered to FN, leupaxin co-localized with the FA protein vinculin, which was distributed along with the actin fibers in the cell periphery (Fig. 1b, arrowheads). Leupaxin was also distributed to the perinuclear area where a relatively large cytoplasmic pool of paxillin exists. Leupaxin showed a similar subcellular distribution in HEK293T cells (Fig. 1c). No distinctive localization of leupaxin was observed in cells bound to PLL (data not shown). These results showed that leupaxin can be recruited to the FAs or FA-like structures upon cell adhesion to integrin ligands, and thus argue for the possible involvement of leupaxin in integrin signaling at specific cell sites.

Leupaxin negatively regulates the tyrosine phosphorylation of paxillin. Integrin engagement triggers rapid tyrosine phosphorylation of paxillin and generates SH2-binding sites (Y31 and Y118) for other SH2 domain-containing FA proteins. Although leupaxin shares overall structural characteristics with paxillin, it lacks tyrosine residues homologous to Y31 and Y118 in paxillin. Another paxillin family member Hic-5 also lacks tyrosine residues in homologous positions, and down-regulates the tyrosine phosphorylation of paxillin after integrin stimulation.[21,22] These observations led us to explore whether leupaxin also suppresses the cell adhesion-induced tyrosine phosphorylation of paxillin. As shown in Figure 2, in untransfected HEK293T cells that adhered to FN but not to PLL, strong tyrosine phosphorylation of paxillin was observed. When leupaxin was exogenously expressed by transfection, the FN-stimulated tyrosine phosphorylation of paxillin was significantly decreased in HEK293T cells (Fig. 2, top panel), although cell adhesion was not compromised (data not shown). In sharp contrast, no significant effect on the tyrosine phosphorylation of paxillin was observed when an irrelevant control EGFP fusion protein was expressed. Unlike

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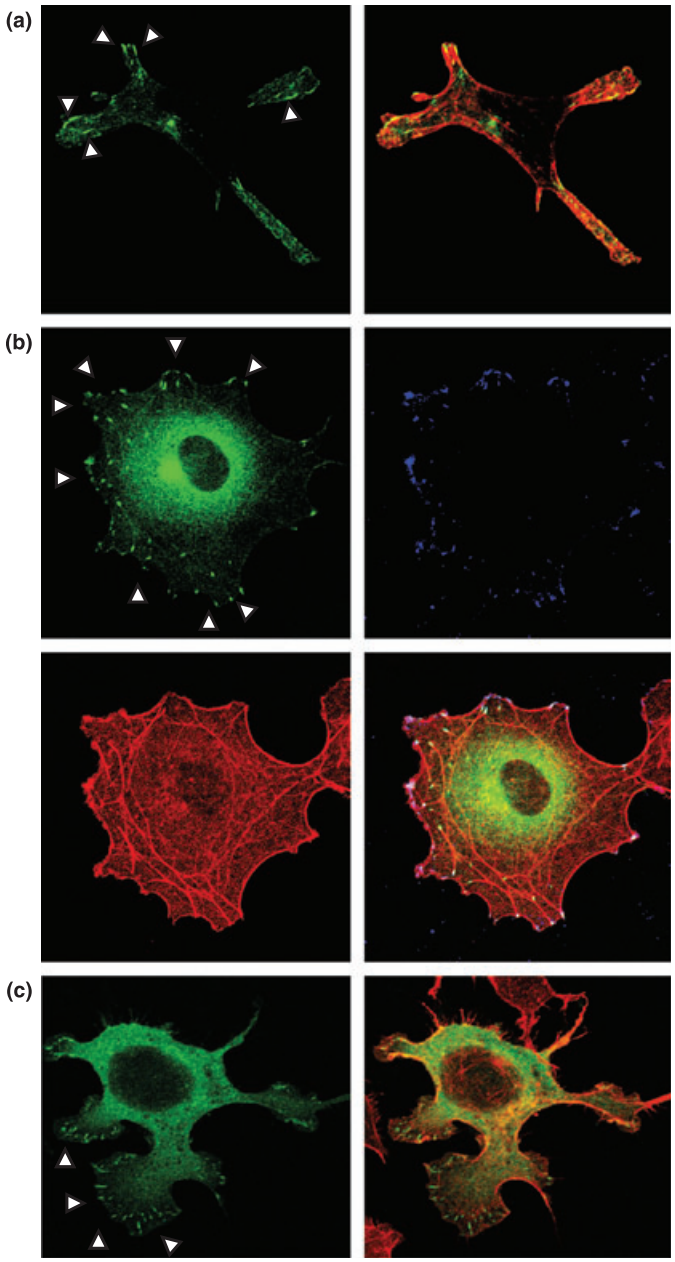

Fig. 1. Subcellular localization of leupaxin in cells adhering to integrin ligands. (a) Distribution of leupaxin in BW5147 cells. BW5147 cells expressing leupaxin-EGFP (enhanced green fluorescent protein) were stimulated with PMA and plated onto ICAM-1/IgG. Cells were stained with rhodamine-phalloidin (red), and analyzed by confocal microscopy. A merged image is shown in the right panel. (b,c) Accumulation of leupaxin in focal adhesion (FA) in NIH3T3 and HEK293T cells. NIH3T3 (b) and HEK293T (c) cells transiently transfected with leupaxin-EGFP were allowed to bind to fibronectin (FN). Cells were then stained with anti-vinculin (for NIH3T3, blue) and rhodamine-phalloidin (for NIH3T3 and HEK293T, red) and examined. Note that leupaxin-EGFP is condensed in FA or FA-like structures (arrowheads).

paxillin, leupaxin was barely tyrosine phosphorylated in HEK293T cells that were bound to FN (Fig. 2, top panel), suggesting that leupaxin cannot substitute for the tyrosine phosphorylation-dependent functions of paxillin. Similar results were obtained with NIH3T3 cells (data not shown). Taken together, these results indicate that leupaxin down-regulates the tyrosine phosphorylation of paxillin during integrin signaling.

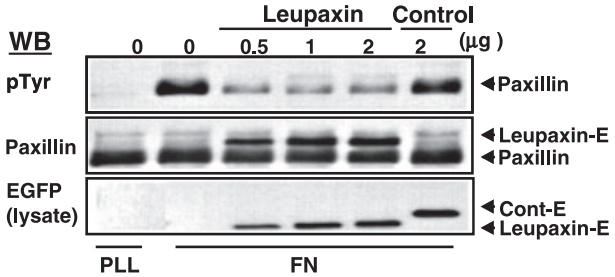

Fig. 2. Leupaxin reduced the tyrosine phosphorylation of paxillin. HEK293T cells were transfected with various amounts of an expression plasmid for leupaxin-EGFP (enhanced green fluorescent protein) or an irrelevant-EGFP fusion protein (control) or were not transfected, and were then plated onto immobilized poly-L-lysine (PLL) or fibronectin (FN). After immunoprecipitation with an anti-paxillin mAb, the tyrosine phosphorylation of paxillin was examined by Western blotting with an anti-phosphotyrosine mAb. Exogenously expressed leupaxin cross-reacted with the anti-paxillin mAb and was immunoprecipitated along with paxillin (middle panel). Note that expression of leupaxin strongly suppressed the tyrosine phosphorylation of paxillin in HEK293T cells that were bound to FN (top panel).

The LIM3 domain of leupaxin is critical for FA targeting and the suppression of paxillin tyrosine phosphorylation. Previous studies by others indicated that the LD4 motif and LIM3 domain are, respectively, critically important for paxillin's binding to FAK and its selective localization to the FAs.[23–26] Since the LD4 motif of paxillin and the LD3 motif of leupaxin are homologous to each other, as judged by amino acid alignment,[9] we generated EGFP-tagged leupaxin mutants lacking the LD3 motif and/or the LIM3 domain (Fig. 3a) and examined their subcellular localization and functions.

As shown in Figure 3(b,c), a leupaxin mutant lacking the LD3 motif (leupaxin *Δ*LD3) successfully accumulated in FAs and the perinuclear area, as seen with WT leupaxin (Fig. 1c), and potently suppressed the tyrosine phosphorylation of paxillin. In contrast, leupaxin mutants lacking the LIM3 domain alone (leupaxin ΔLIM3) or both the LD3 motif and LIM3 domain (leupaxin ΔLD3/LIM3) failed to localize to FAs (Fig. 3b). In addition, they showed much weaker suppression of paxillin tyrosine phosphorylation compared with WT leupaxin (Fig. 3c). These results indicate that the LIM3 domain of leupaxin is required for FA targeting and also for the effective suppression of the integrin-induced tyrosine phosphorylation of paxillin.

Leupaxin has been shown to interact with FAK.[13] To test directly the roles of leupaxin's LD3 motif and LIM3 domain in its association with FAK, we carried out immunoprecipitation analyses. As shown in Figure 4, WT leupaxin co-precipitated with FAK from the HEK293T cell lysates. In contrast, leupaxin *Δ*LD3 and leupaxin *Δ*LD3/LIM3 failed to co-precipitate with FAK, but leupaxin *Δ*LIM3 did co-precipitate with it (Fig. 4). These results indicate that leupaxin's LD3 motif plays a critical role in leupaxin's association with FAK, although it may be dispensable for the suppressive activity of leupaxin on paxillin tyrosine phosphorylation.

Leupaxin functions as a negative regulator in integrin-mediated cell adhesion events. Previous studies showed that Hic-5 down-regulates NIH3T3 cell spreading on FN, which is regulated by paxillin and FAK.[20] To test whether leupaxin also exerts suppressive functions, leupaxin and the leupaxin mutants were expressed in NIH3T3 cells, and their effects on cell spreading were assessed. As shown in Figure 5, a moderate but statistically significant ($P < 0.0001$) suppression of NIH3T3 cell spreading was observed upon expression of WT leupaxin. In contrast, leupaxin mutants lacking either the LD3 motif or the LIM3 domain, or both, failed to suppress the spreading of

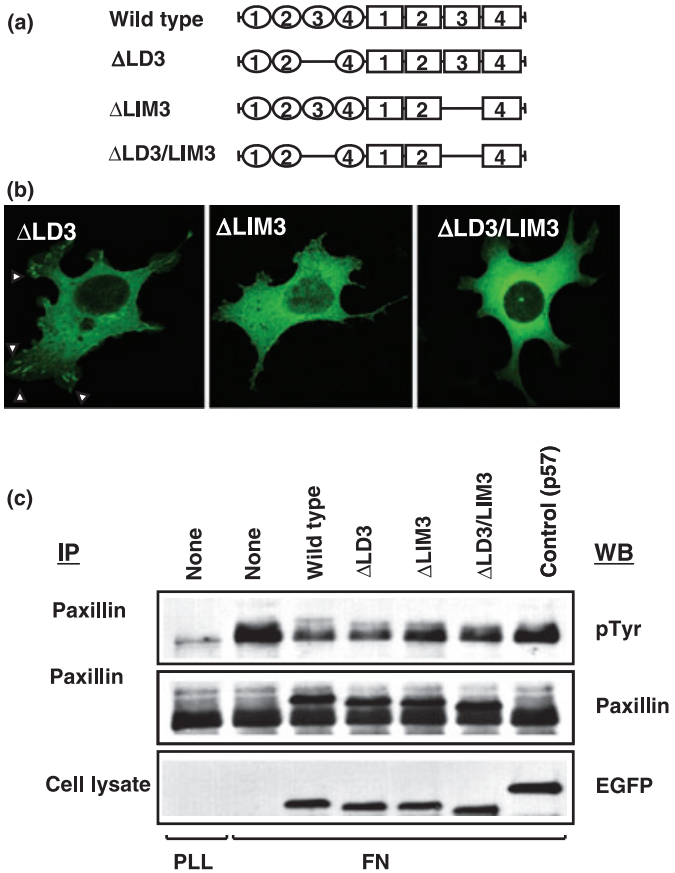

Fig. 3. Subcellular distribution of leupaxin mutants and their ability to suppress the tyrosine phosphorylation of paxillin. (a) Schematic structure of leupaxin mutants. (b) Confocal microscopic analysis of the subcellular distribution of the leupaxin mutants. HEK293T cells expressing the leupaxin-EGFP (enhanced green fluorescent protein) mutants ΔLD3 (right), ΔLIM3 (middle), and ΔLD3/LIM3 (left) were allowed to bind to FN and examined by confocal microscopy. (c) Western blotting analysis of the tyrosine phosphorylation of paxillin. Untransfected HEK293T cells or HEK293T cells expressing an EGFP-fusion protein of leupaxin, mutant leupaxin, or an irrelevant protein were serum-starved for 2 h. The cells were then allowed to bind to FN, and the tyrosine phosphorylation of paxillin was examined by Western blotting.

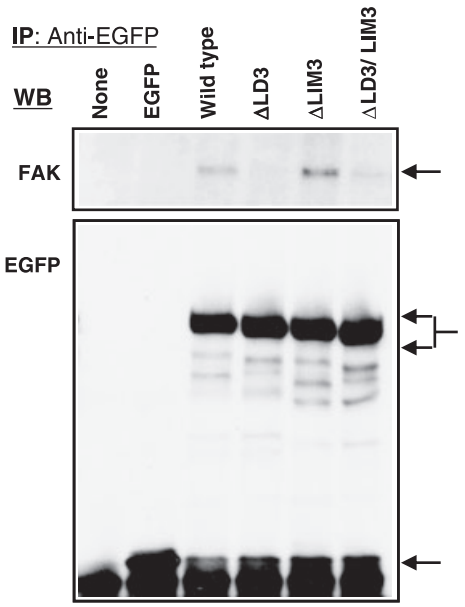

Fig. 4. Interaction of leupaxin and its mutants with FAK. Untransfected HEK293T cells or HEK293T cells transfected with enhanced green fluorescent protein (EGFP), EGFP-tagged leupaxin, or the leupaxin mutants were immunoprecipitated with an anti-EGFP antibody and the immunoprecipitates were subjected to Western blotting with anti-FAK (upper panel) or anti-EGFP (lower panel). Arrows indicate the position of FAK (upper panel), or the EGFP-tagged leupaxin proteins or EGFP alone (lower panel).

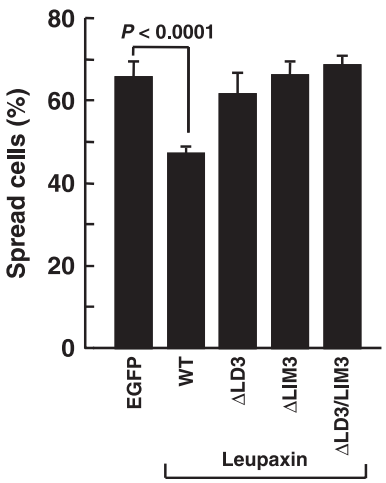

Fig. 5. NIH3T3 cells inhibited by leupaxin from spreading on fibronectin. NIH3T3 cells transiently expressing enhanced green fluorescent protein (EGFP) or EGFP-tagged leupaxin proteins were incubated for 1 h at 37°C. The cells were stained with rhodamine-phalloidin, and at least 100 transfected cells were examined by confocal microscopy. Cells that had spread were defined as those that had extended processes and lacked a round morphology. Data represent the mean ± SD of three independent experiments.

NIH3T3 cells. These results suggest that, like Hic-5, leupaxin can suppress NIH3T3 cell spreading and that both the LD3 motif and LIM3 domain of leupaxin are required for this suppression.

We next examined leupaxin's functions in hematopoietic tumor cells. Leupaxin was stably expressed in K562 cells expressing endogenous paxillin but lacking leupaxin[9] and Hic-5[27] (Fig. 6a), and leupaxin's influence on the tyrosine phosphorylation of paxillin was examined. As seen in Figure 6(b), cell adhesion to FN induced prominent tyrosine phosphorylation of paxillin in the parental K562 cells and also in K562 cells that expressed a control EGFP protein (E-B2 and E-D3). In contrast, the FN-stimulated tyrosine phosphorylation of paxillin was significantly reduced in two independent K562 transfectants that expressed EGFP-tagged leupaxin (L-7 and L-18) (Fig. 6b).

We next asked if leupaxin affects integrin-mediated K562 cell adhesion to FN. An approximately 40% reduction of cell adhesion was observed in K562 cells expressing leupaxin-EGFP, compared with the parental K562 cells or K562 cells expressing EGFP alone (Fig. 6c). The expression of integrin $\alpha 5\beta 1$, which is responsible for the binding of K562 cells to FN,[28] remained unaltered by the expression of leupaxin (Fig. 6d). These results collectively suggest that leupaxin functions as a negative regulator of integrin-mediated cell adhesion events such as cell spreading and binding.

Discussion

In this study, we have shown that leupaxin selectively accumulates in FAs and functions as a potent repressor of the tyrosine

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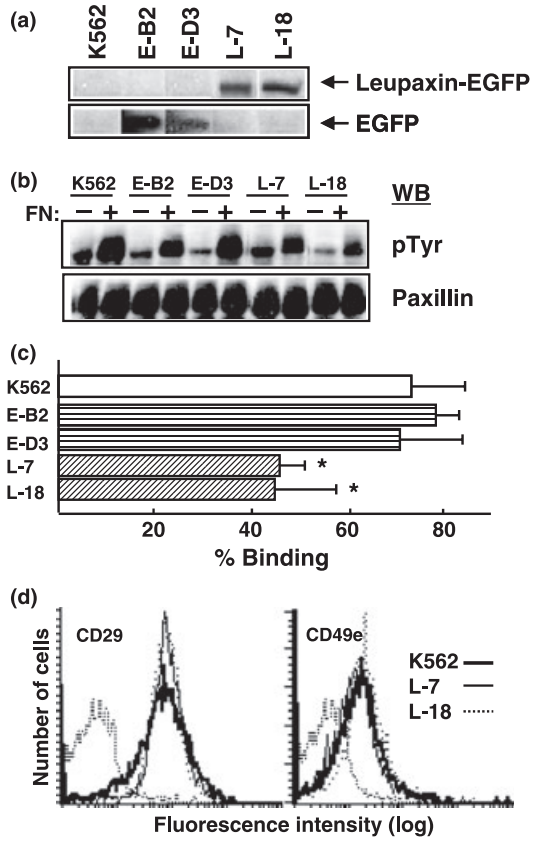

Fig. 6. Suppression of integrin α5β1 function by leupaxin in K562 cells. (a) Stable K562 transfectants expressing enhanced green fluorescent protein (EGFP) or leupaxin-EGFP. The parental K562 cells and their stable transfectants expressing EGFP (E-B2 and E-D3) or leupaxin-EGFP (L-7 and L-18) were analyzed with an anti-GFP antibody. (b) Leupaxin suppressed the tyrosine phosphorylation of paxillin in K562 cells. The cells were allowed to bind to plastic wells coated with BSA or fibronectin (FN), and their lysates were immunoprecipitated with an anti-paxillin mAb. Tyrosine phosphorylation of paxillin was analyzed by Western blotting. (c) Effects of leupaxin on the adhesion of K562 cells to FN. Fluorescently labeled cells were allowed to bind to FN. Results represent the mean ± sd from triplicate experiments. *$P < 0.05$ *versus* parental K562 cells. (d) Expression of integrin α5β1 in K562 transfectants. Cells were incubated with an anti-CD29 or anti-CD49e mAb, and stained with biotinylated antimouse IgG and avidin-PE. Histograms shown on the left side of each panel represent K562 cells that were not treated with the primary antibody.

phosphorylation of paxillin in integrin signaling in HEK293T and NIH3T3 cells. We also showed that leupaxin suppressed integrin-dependent cell adhesion in a hemopoietic tumor cell line, K562. These results strongly suggest that leupaxin is a negative regulator of paxillin in integrin-mediated cell adhesion events.

Among the paxillin family members, leupaxin and Hic-5 are highly homologous to each other in their structure and functions.[6,8] For instance, they both lack SH2 and SH3 binding motifs that are present in paxillin and suppress paxillin's tyrosine phosphorylation. However, the mRNA expression patterns of leupaxin and Hic-5 are almost mutually exclusive. Leupaxin mRNA is largely restricted to cells of the leukocytic lineage and certain cancer cells, whereas Hic-5 mRNA is found in other cell types.[9,27] These observations suggest that leupaxin and Hic-5 both serve as counter-regulators for paxillin, but function in different cell subsets.

Paxillin tyrosine phosphorylation appears to be mediated by multiple tyrosine kinases, including FAK, Src, C-terminal Src kinase (Csk), and abelson kinase (Abl).[29–32] Previous studies have suggested that Hic-5 strongly binds FAK and competes with paxillin for FAK binding, thereby suppressing paxillin tyrosine phosphorylation.[21,22] However, our results with leupaxin mutants indicate that leupaxin's accumulation in FAs, but not its stable association with FAK, correlates with leupaxin's suppressive activity of the tyrosine phosphorylation of paxillin. This raises the possibility that leupaxin affects signaling components other than FAK. In this regard, it is of note that leupaxin has been shown to interact with a tyrosine phosphatase, PTP-PEST, which is implicated in the dephosphorylation of paxillin.[13] Future investigation should be directed to understanding the molecular mechanisms by which leupaxin suppresses the tyrosine phosphorylation of paxillin.

Leupaxin suppressed NIH3T3 cell spreading on FN, and the inhibitory effects required both leupaxin's LIM3 domain and LD3 motif. Because the leupaxin LD3 motif is important for its stable association with FAK, sequestration of FAK from its substrates, in concert with the LIM3-domain-dependent reduction of paxillin's tyrosine phosphorylation, appeared to be critical to the leupaxin-mediated suppression of NIH3T3 cell spreading. This is consistent with the important positive regulatory role of the tyrosine kinase activity of FAK and paxillin's tyrosine phosphorylation in the spreading of NIH3T3 cells.[20]

Although exogenously expressed leupaxin significantly suppressed the binding of K562 cells to FN, it did not compromise the binding of HEK293T cells. The apparently different outcomes of exogenously expressing leupaxin in the adhesion of these cells may be attributable to a quantitative difference in the endogenous expression of paxillin in K562 and HEK293T cells. In addition, it is also possible that HEK293T cells express multiple adhesion receptors for FN that are absent in K562 cells. These possibilities should be tested experimentally in future studies.

In contrast to the report by Lipsky *et al.*[9] that leupaxin is tyrosine phosphorylated in a lymphoblastoid cell line, JY8, we did not observe tyrosine phosphorylation of leupaxin in HEK293T, NIH3T3, or K562 cells, even after integrin stimulation. This apparent discrepancy might be explained partly by the different cells used by Lipsky *et al.* and we used. Gupta *et al.*[13] reported that leupaxin associates with FAK and Pyk2 but is only marginally tyrosine phosphorylated in murine osteoclasts. These observations suggest that, unlike paxillin, leupaxin is a poor substrate for tyrosine kinases that are activated by integrin stimulation. A lack of tyrosine phosphorylation upon integrin stimulation was also noted with Hic-5.[21,22]

The turnover of FAs is a complex process that is regulated by the fine-tuning of the tyrosine-specific phosphorylation and dephosphorylation of various FA components, including paxillin.[5] Thus, it is tempting to speculate that leupaxin contributes to a rapid turnover of FAs by counterbalancing the tyrosine phosphorylation of paxillin, and thereby regulating integrin-mediated cell adhesion and migration. In this regard it is of note that in certain prostate carcinoma cells, knockdown of leupaxin significantly decreased cell migration whereas overexpression of leupaxin increased cell migration.[11,12] Further investigation into the molecular basis of leupaxin's functions in cell adhesion and migration is needed.

In conclusion, we have identified leupaxin as an adaptor protein with a potent suppressive activity on the tyrosine phosphorylation of paxillin. Because the expression of leupaxin is largely restricted to leukocytes and certain malignant cells including prostate cancer cells, our findings indicate that leupaxin serves as a cell type-specific negative regulator of paxillin. Recently, leupaxin as well as other paxillin family members have been shown to undergo regulated nucleocytoplasmic shuttling and contribute to gene transcription.[10,12] Future investigation will

be necessary to gain insights into the functional interplay between leupaxin and paxillin in the integrin-dependent adhesion and migration.

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Disclosure Statement

All authors have no conflict of interest.

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