Regulation of cell migration and survival by focal adhesion targeting of Lasp-1

Yi Hsing Lin, Zee-Yong Park, Dayin Lin, Anar A. Brahmbhatt, Marie-Christine Rio, John R. Yates III, and Richard L. Klemke

1Department of Immunology and 2Department of Cell Biology, The Scripps Research Institute, La Jolla, CA 92037
3Institut de Génétique et de Biologie Moléculaire et Cellulaire, 67404 Illkirch, France

Large-scale proteomic and functional analysis of isolated pseudopodia revealed the Lim, actin, and SH3 domain protein (Lasp-1) as a novel protein necessary for cell migration, but not adhesion to, the extracellular matrix (ECM). Lasp-1 is a ubiquitously expressed actin-binding protein with a unique domain configuration containing SH3 and LIM domains, and is overexpressed in 8–12% of human breast cancers. We find that stimulation of nonmotile and quiescent cells with growth factors or ECM proteins facilitates Lasp-1 relocalization from the cell periphery to the leading edge of the pseudopodium, where it associates with nascent focal complexes and areas of actin polymerization. Interestingly, although Lasp-1 dynamics in migratory cells occur independently of c-Abl kinase activity and tyrosine phosphorylation, c-Abl activation by apoptotic agents specifically promotes phosphorylation of Lasp-1 at tyrosine 171, which is associated with the loss of Lasp-1 localization to focal adhesions and induction of cell death. Thus, Lasp-1 is a dynamic focal adhesion protein necessary for cell migration and survival in response to growth factors and ECM proteins.

Introduction

The direction of cell migration is controlled by chemokine and ECM gradients, and is exhibited during wound healing, angiogenesis, embryonic development, and immune function (Lauffenburger and Horwitz, 1996). Cells respond by local activation and amplification of signaling events on the side facing the attractant (Parent and Devreotes, 1999). This facilitates localized actin polymerization leading to morphological polarity and establishment of a dominant-leading pseudopodium and rear cell body compartment (Lauffenburger and Horwitz, 1996; Parent and Devreotes, 1999). Interestingly, the initial protrusion of a pseudopodium at the cell surface is independent of integrins and the ECM (Wyckoff et al., 2000). However, integrins tether the extending membrane to the substratum, which supports sustained and directional growth of a single dominant pseudopodium. Indeed, a pseudopodium that does not attach to the ECM rapidly retracts back to the cell body (Bailly et al., 1998). This suggests that formation of new integrin focal complexes at the leading front of the extending membrane provides necessary signals to fine-tune and maintain directional growth, while suppressing retraction mechanisms. Cell movement then commences as the cell undergoes repeated cycles of membrane extension and integrin ligation at the front and cell body retraction at the rear (Lauffenburger and Horwitz, 1996). The specific signaling proteins that regulate the spatial adhesive changes necessary for morphological polarity and directional cell translocation are poorly understood.

To help unravel the spatiotemporal organization of signaling cascades involved in cell polarization, including mechanisms of protein translocation, activation, post-translational modifications, and formation of complex multi-protein scaffolds, we developed a biochemical method to selectively isolate the pseudopodium and cell body of cells polarized toward a chemoattractant gradient using a microporous filter system (Cho and Klemke, 2002; Brahmbhatt and Klemke, 2003). Pseudopodia extension in this system, like traditional pseudopodia formation on two-dimensional surfaces, requires Cdc42 and Rac activity and shows normal actin cytoskeletal organization and focal adhesions. Cells extend pseudopodia projections through small openings in the vasculature and angiogenesis, embryonic development, and immune function.
ECM in vivo as a necessary process of immune cell intravasation as well as pathological processes associated with cancer cell metastasis (Wyckoff et al., 2000). Therefore, this model recapitulates physiological events associated with cell migration and is ideal for unraveling the spatial and temporal signaling mechanisms responsible for focal adhesion changes leading to cell polarity and directional movement.

In this report, we used this technique along with a new protein sequencing method called multidimensional protein identification technology (MudPIT; Washburn et al., 2001) for rapid and large-scale proteome analysis of purified pseudopodia. By using multidimensional liquid chromatography, tandem mass spectrometry, and database searching with SEQUEST algorithm, it is possible to identify large numbers of proteins (>1,000) directly from a complex protein lysate (Washburn et al., 2001). Here, we isolated pseudopodia and cell body proteins for comparison by MudPIT to reveal proteins uniquely present in these different cellular compartments. The novel cytoskeletal-associated Lim, actin, and SH3 protein (Lasp-1; GenBank/EMBL/DDBJ accession no. X82456) was identified as a component of the pseudopodium and further characterized for functional significance. Lasp-1 was initially identified from a breast cancer–derived metastatic lymph node cDNA library and is overexpressed in 8–12% of breast cancer (Tomasetto et al., 1995a,b). It is a ubiquitously expressed actin-binding protein with a unique domain configuration (Tomasetto et al., 1995a; Schreiber et al., 1998) that includes a LIM domain (Lin11, Isl-1, and Mec-3) in the NH$_2$-terminal region followed by two actin-binding repeats (R1, R2), and an Src homology 3 (SH3) domain in the COOH-terminal region. There are also two tyrosine phosphorylation sites (Y52 and Y152) corresponding to SH2-binding consensus motifs (YXXP; Tomasetto et al., 1995a), suggesting that Lasp-1 is tyrosine phosphorylated and may facilitate binding of SH2 effector proteins and their downstream signals. However, the biological function and regulation of this molecule has not yet been identified. Our findings demonstrate that Lasp-1 is a dynamic, spatially regulated protein necessary for cell migration. Furthermore, we show that the cytoskeletal regulatory

<table>
<thead>
<tr>
<th>Table I. Proteins identified in purified pseudopodia$^*$</th>
<th>Number of identified peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoskeletal-associated proteins</td>
<td></td>
</tr>
<tr>
<td>$\alpha$-Actinin</td>
<td>7</td>
</tr>
<tr>
<td>Tropomyosin 1</td>
<td>16</td>
</tr>
<tr>
<td>$\beta$-4-Tubulin</td>
<td>10</td>
</tr>
<tr>
<td>$\beta$-Actin</td>
<td>29</td>
</tr>
<tr>
<td>Transgelin 2</td>
<td>9</td>
</tr>
<tr>
<td>Lim and SH3 protein (Lasp-1)</td>
<td>1</td>
</tr>
<tr>
<td>Myosin 1B and C</td>
<td>1</td>
</tr>
<tr>
<td>Cofilin 1 and 2</td>
<td>1</td>
</tr>
<tr>
<td>Arp1 and 3</td>
<td>1</td>
</tr>
<tr>
<td>Caldesmon</td>
<td>1</td>
</tr>
<tr>
<td>Drebrin 1, isoform a and b</td>
<td>1</td>
</tr>
<tr>
<td>Filamin</td>
<td>1</td>
</tr>
<tr>
<td>Fascin</td>
<td>1</td>
</tr>
<tr>
<td>Adenomatous polyposis coli-binding protein EB1</td>
<td>1</td>
</tr>
<tr>
<td>Signaling proteins</td>
<td></td>
</tr>
<tr>
<td>Rho guanine nucleotide exchange factor 1</td>
<td>1</td>
</tr>
<tr>
<td>PI 3-kinase-related kinase SMG-1</td>
<td>1</td>
</tr>
<tr>
<td>Guanine nucleotide binding protein, $\beta$-type</td>
<td>1</td>
</tr>
<tr>
<td>RAB5A and C</td>
<td>1</td>
</tr>
<tr>
<td>A-kinase anchor protein 3</td>
<td>1</td>
</tr>
<tr>
<td>Oligophrenin 1, Rho-activating protein</td>
<td>1</td>
</tr>
<tr>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase 2</td>
<td>1</td>
</tr>
<tr>
<td>delta subunit</td>
<td>1</td>
</tr>
<tr>
<td>Insulin receptor substrate</td>
<td>1</td>
</tr>
<tr>
<td>Neuregulin 1: c-neu receptor</td>
<td>1</td>
</tr>
</tbody>
</table>

$^*$Complete list of all identified peptides and identified proteins in pseudopodia and cell body fractions is shown in Tables S1–S4 and Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200311045/DC1).
protein c-Abl tyrosine kinase (Woodring et al., 2003) directly phosphorylates Lasp-1, which regulates its localization to focal adhesions in apoptotic (but not migratory) cells.

Results
Characterization of the pseudopodial proteome and identification of Lasp-1
The ability to differentially isolate proteins from the leading pseudopodium and cell body and the recent development of MudPIT technology allowed us to undertake large-scale proteomics to characterize the pseudopodium and cell body proteomes. Five independent cell body and pseudopodial fractions were prepared and examined by MudPIT. From the tandem mass spectroscopy data, we generated 4,000 peptides leading to the identification of 980 total proteins. Although mass spectroscopy and MudPIT systems are limited in their ability to directly quantify proteins, they can be used to identify relative differences in protein profiles of two separate protein samples because an enriched protein will result in higher peptide identification. We considered a protein to be enriched if at least one peptide was identified in a fraction or at least 50% more peptides were sequenced in a given fraction. Comparison of the 980 proteins using DTA-select and Contrast software (Tabb et al., 2002) revealed 119 enriched proteins in the pseudopodial fraction and 203 enriched proteins in the cell body component. Notable proteins previously shown to be enriched in the pseudopodium of cells are shown in Table I, demonstrating the feasibility of this approach (a comparative list of all pseudopodia and cell body–associated proteins is shown in Tables S1–S4 and Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200311045/DC1). Of the proteins identified, Lasp-1 was further characterized for functional significance. Western blot analysis and densitometry confirmed that Lasp-1 protein was increased (approximately ninefold) in the pseudopodium compared with the cell body (Fig. 1 A). A Lasp-1 GFP fusion also revealed striking localization to the leading pseudopodial edge of spreading and migrating cells (Fig. 1, B and C). A similar pattern of Lasp-1 localization was observed using immunofluorescent staining and Lasp-1 antibodies (unpublished data; see also Chew et al., 2002). These findings confirm the purification and identification of this protein from the MudPIT system and demonstrate its localization to the leading pseudopodium of polarized cells.

Cell adhesion and exposure to growth factors induce the translocation of Lasp-1 from the cell periphery to focal complexes in migrating cells
It is notable that GFP Lasp colocalized to vinculin-positive focal adhesions primarily at the leading edge of the spreading membrane and was either absent or reduced in focal adhesions in the cell body proper (Fig. 1). In nonmotile serum-starved cells, Lasp-1 localized to the peripheral edge of...
cells, where it colocalized with cortical actin structures and weakly with F-actin cables (Fig. 2 A and Fig. 3), but not focal adhesions. However, exposure of these cells to growth factors to activate cell migration caused the rapid (1–2 min) relocalization of GFP Lasp from the cell periphery to focal adhesions (Fig. 2 A). At later times after stimulation (>15 min), GFP Lasp strongly localized to actin-rich membrane ruffles on the cell surface (Fig. 3).

Importantly, truncation of the COOH-terminal region of Lasp-1 (GFP LaspΔC) prevented translocation of Lasp-1 to focal adhesions (Fig. 2 B), but not to membrane ruffles, actin cables, or cortical actin structures in response to serum (Fig. 2 B) or cell spreading on the ECM (unpublished data). Thus, the COOH terminus of Lasp-1 is specifically responsible for translocation and targeting to focal adhesions, but not ruffles, in response to ECM proteins and growth factors. Expression of GFP fused to either the LIM or SH3 domains showed only diffuse cytoplasmic staining, suggesting these domains are not sufficient by themselves to target Lasp-1 to actin or focal adhesion structures under these conditions (unpublished data).

**Lasp-1 is necessary for cell migration (but not adhesion) to the ECM**

Although the function of Lasp-1 is not known, the above findings suggest that it is important for cell adhesion and migration on the ECM. To directly examine this possibility, we depleted cells of Lasp-1 using small interfering RNA (siRNA) technology (Elbashir et al., 2001). Cells exposed to anti-Lasp-1 siRNA showed an ~80–90% reduction in Lasp-1 protein compared with cells mock treated with control siRNA directed to GL2 luciferase. Total protein staining and Western blotting for either Lasp-1 or actin revealed that only Lasp-1 was reduced and not other cellular proteins (Fig. 4 A). Interestingly, although cells depleted of Lasp-1 showed little difference in adhesion and spreading, they did show a significantly impaired ability to migrate on the ECM (Fig. 4 A and B). These findings indicate that Lasp-1 protein is necessary for cell migration, but not cell adhesion and spreading. Similar findings were obtained with NIH 3T3 fibroblast cells depleted of Lasp-1 protein (unpublished data).

Interestingly, Lasp-1 expression has been reported to be increased in metastatic breast cancers, suggesting that protein amplification may contribute to the migratory properties of these cells (Tomasetto et al., 1995b). To investigate this, we ectopically expressed Lasp-1 in cells and monitored their ability to migrate in response to growth factors. Surprisingly, Lasp-1 amplification inhibited basal and growth factor-stimulated cell migration (Fig. 4 C) without affecting attachment to the ECM (unpublished data). Similar findings were obtained with HEK 293 (Fig. 4 D) and MCF-7 cells (unpublished data). Importantly, expression of LaspΔC failed to inhibit cell migration or adhesion, indicating that the COOH-terminal region of Lasp-1 is required for this process. Expression of the SH3 domain alone strongly inhibited cell migration, which supports this notion (Fig. 4 C). It is not yet clear why both depletion and amplification of Lasp-1 inhibits cell migration. However, it is possible that global amplification of Lasp-1 throughout the cell body disrupts signaling polarity and the normal restricted localization of this protein, as has been shown for Cdc42 (Allen et al., 1998). In this regard, disruption of signaling polarity by constitutive relocalization of Lasp-1 to the plasma membrane caused dramatic membrane blebbing and detachment from the ECM leading to cell death, whereas targeting of LaspΔC to the membrane failed to induce this response (Fig. 4 E). In any case, these findings demonstrate that Lasp-1 plays an important spatial role in cell migration, and that this process is regulated by the COOH-terminal region of Lasp-1 containing the SH3 domain.

**Abl kinase associates with the SH3 domain of Lasp-1 and phosphorylates tyrosine 171 in vivo**

Next, we performed a database and motif screen for potential regulatory proteins associated with Lasp-1 using the SH3 domain and putative tyrosine phosphorylation sites in the COOH terminus as targets. Lasp-1 was found to contain a conserved Abl consensus phosphorylation sequence at tyrosine 171 (VYxxP) and an SH3 domain that is predicted to bind to the proline-rich region of Abl family kinase (Liu et al., 1996; Wang et al., 1997; Barila et al., 2000). Furthermore, the fact that Abl and Arg regulate the actin cytoskelet-
ton, focal adhesions, and cell migration (Woodring et al., 2003) suggests these proteins may be part of a common signaling pathway. To begin to determine whether Abl and Lasp-1 associate in vivo, cells were transfected with HA-tagged Abl and full-length Lasp-1. HA-tagged Abl was then immunoprecipitated from these cells and immunoblotted for the presence of Lasp-1. In the reciprocal experiment, GST Lasp was precipitated from transfected cells and immunoblotted for HA-Abl. In both cases, Abl associated with and promoted increased tyrosine phosphorylation (>25-fold) of Lasp-1 in cells (Fig. 5 A). Tyrosine phosphorylation of Lasp-1 was accompanied by reduced mobility in SDS-PAGE, as indicated by the appearance of a higher molecular weight form of Lasp-1. This was also the case when purified activated Abl was allowed to phosphorylate bacterial purified Lasp-1 in vitro (Fig. 5 E). Importantly, LaspAC without the Abl binding and phosphorylation site Y171 failed to associate with Abl and showed no tyrosine phosphorylation as the result of Abl activation (Fig. 5, A and B). As expected, Abl with a truncated proline-rich domain (AblΔPro; Smith et al., 1999) failed to interact and phosphorylate Lasp-1 in cells (Fig. 5 C). Wild-type Abl and AblΔPro become auto-activated in cells due to protein amplification as previously reported (Smith et al., 1999; Kain and Klemke, 2001; Kain et al., 2003). In this regard, the constitutively activated oncogenic form of Abl (Bcr-Abl; Daley et al., 1990) tyrosine phosphorylates GST Lasp in cells (Fig. 5 D). K562 leukemic cells derived from a CML patient with endogenous Bcr-Abl (Grosved, et al., 1986) also show increased Lasp-1 tyrosine phosphorylation (unpublished data). As expected, exposure of cells to the Abl kinase inhibitor STI 571 (Buchdunger et al., 1996) inhibited Lasp-1 tyrosine phosphorylation, indicating that kinase activity is necessary for this event (Fig. 5 D). Most importantly, substitution of tyrosine 171 to pho-

Figure 4. Lasp-1 is necessary for cell migration, but not adhesion to the ECM. (A) Cos-7 cells were depleted of endogenous Lasp-1 protein using siRNA specific for Lasp-1 or control siRNA to GL2 luciferase. Cells were allowed to either migrate using Boyden chambers coated on the bottom with 10 μg/ml fibronectin or (B) to attach to culture dishes coated with the same ECM as described in Materials and methods. The number of migratory or adherent cells per microscopic field was counted. Each bar represents the mean ± SEM of cells in triplicate migration/adhesion chambers of three independent experiments. An aliquot of cell lysates prepared from cells treated as described above were Western blotted for expression of Lasp-1 or actin, which served as a loading control. (C) COS-7 cells were transfected with the empty vector (Mock), wild-type Lasp-1 (Wt), LaspΔC (ΔC), or the SH3 domain tagged with HA (SH3) along with a β-galactosidase reporter construct to mark transfected cells. Cells were allowed to chemotax with or without LPA in the lower chamber for 3 h. Migratory cells per microscopic field on the underside of the membrane were stained and counted. Each bar represents the mean ± SEM of blue cells in triplicate migration chambers of three independent experiments. An aliquot of cell lysates prepared from cells treated as described above were Western blotted for the expression of the appropriate protein or tag as indicated. (D) HEK 293 cells stably expressing the empty vector (mock) or the vector encoding full-length Lasp-1 were tested for chemotaxis with or without 10 μg/ml insulin in the lower chamber as indicated in C. Lasp-1 protein was Western blotted to confirm the protein expression. HEK 293 cells show low levels of endogenous Lasp-1 protein, which is not detected in the short exposure time to the film. (E) Deconvolution images of COS-7 cells transfected with either Lasp-1 or LaspΔC fused with the membrane-targeting sequence (myr) along with a GFP reporter construct. Cells were then fixed and examined for membrane blebs 16 h after transfection, and the number of transfected cells with prominent blebs were counted per microscopic field relative to the total number of GFP-positive cells.
N-ethylmaleimide (LaspY171F) prevented Lasp-1 tyrosine phosphorylation and mobility shift by Abl activation (Fig. 5 F). The remaining phosphoprotein band seen in the LaspY171F and HA-Abl cells likely represents endogenous Lasp-1 phosphorylated by Abl. These findings demonstrate the in vivo association of Abl with the COOH-terminal region of Lasp-1, and confirms tyrosine 171 is the site of phosphorylation induced by Abl activation in cells.

Abl specifically phosphorylates Lasp-1 in apoptotic (but not migratory) cells, and this prevents Lasp-1 translocation to focal complexes

Abl is activated by exposure of cells to growth factors and through integrin activation processes that occur during cell spreading on the ECM (Lewis et al., 1996; Plattner et al., 1999). Like Lasp-1, Abl is a dynamic protein that translocates to focal adhesions and membrane ruffles under these conditions (Woodring et al., 2003). Therefore, we investigated whether Lasp-1 was tyrosine phosphorylated in cells treated with growth factors (FBS, EGF, and PDGF-BB) or in cells spreading on the ECM. However, although these stimuli caused Lasp-1 translocation to focal adhesions and membrane ruffles (Figs. 1–3), they did not induce tyrosine phosphorylation of Lasp-1 (unpublished data). Furthermore, STI 571 did not inhibit Lasp-1 translocation to focal complexes under these conditions (see Fig. 7). Together, these findings demonstrate that Abl activation and tyrosine phosphorylation of Lasp-1 by themselves do not play a primary role in Lasp-1 localization to actin structures and focal adhesions under these conditions.

Interestingly, Abl also induces cell apoptosis in response to DNA-damaging agents (cisplatin; Gong et al., 1999) and oxidative stress (H₂O₂; Sun et al., 2000). Although the mechanisms are not yet understood, the death process in-
vovles deregulation of the actin cytoskeleton and focal adhesions during the execution phase of death (Huot et al., 1998; Houle et al., 2003). Indeed, exposure of cells to either \( \text{H}_2\text{O}_2 \) or cisplatin caused strong Lasp-1 tyrosine phosphorylation that required endogenous Abl kinase activity (Fig. 6). Moreover, \( \text{H}_2\text{O}_2 \)-induced Lasp-1 phosphorylation was significantly impaired in embryonic mouse fibroblast cells isolated from \( \text{abl}^{-/-} \text{arg}^{-/-} \) animals, compared with these cells stably reconstituted with Abl (Fig. 6 B). However, at later times (>45 min) a small level of Lasp-1 phosphorylation is detected, suggesting that another kinase(s) may phosphorylate Lasp-1. Similar findings were obtained with cells treated with pervanadate, which strongly (>17-fold) activates Abl (unpublished data; Woodring et al., 2003). These findings

Figure 6. Apoptotic agents induce tyrosine phosphorylation of Lasp-1, and this requires Abl and Arg kinase activity. (A) Cos-7 cells transfected with GST Lasp were treated with 1 mM \( \text{H}_2\text{O}_2 \) for the indicated times in the presence or absence of 5 \( \mu \text{M} \) STI 571. GST Lasp was precipitated and Western blotted using anti-phosphotyrosine or GST antibodies. (B) Embryonic fibroblast cells isolated from \( \text{abl}^{-/-} \text{arg}^{-/-} \) animals or these cells stably reconstituted with Abl were transfected with GST Lasp and were then serum starved and treated with 1 mM \( \text{H}_2\text{O}_2 \) for the indicated times. GST Lasp expression and tyrosine phosphorylation were determined as described above. (C) Cos-7 cells transfected with GST Lasp were incubated with 25 \( \mu \text{M} \) cisplatin for the indicated times in the presence or absence of 2 \( \mu \text{M} \) STI 571. Lasp-1 expression and tyrosine phosphorylation were determined as described above.

Figure 7. Tyrosine phosphorylation Y171 of Lasp-1 prevents its translocation from the cell periphery to focal adhesions in response to growth factor stimulation. Deconvolution images of NIH 3T3 cells expressing GFP Lasp or GFP LaspY171F were treated for 30 min with 1 mM \( \text{H}_2\text{O}_2 \) and for 15 min with FBS. Cells were fixed and stained with anti-vinculin antibodies to visualize focal adhesions as described above. In some cases, GFP Lasp transfected cells were preincubated with 5 \( \mu \text{M} \) STI 571 before \( \text{H}_2\text{O}_2 \) and FBS treatment to block Abl kinase activity as described in Materials and methods. Merged images represent colocalization (yellow) of focal adhesions (red) and GFP Lasp (green). Bars: 28 \( \mu \text{m} \) (right and left panels) and 20 \( \mu \text{m} \) (middle panel).
The top band is a nonspecific protein that served as a loading control. The autoradiographs of PARP, indicating that TSA induced apoptosis in these cells. Under these apoptotic conditions. Asterisk shows the cleaved form of PARP, indicating that these proteins are not phosphorylated and Abl kinase is not activated.

Figure 8. Depletion of Lasp-1 protein increases cell apoptosis induced by H$_2$O$_2$ and cisplatin, but not TSA. COS-7 cells were depleted of endogenous Lasp-1 protein using siRNA specific for Lasp-1 or control siRNA to GL2 luciferase as described in the Materials and methods. Cells were plated onto fibronectin-coated glass coverslips, and were treated with either (A) 25 μM cisplatin, (B) 1 mM H$_2$O$_2$, or (C) 300 ng/ml TSA for 24 h, or were treated with the indicated vehicle. (D) Cells were treated with or without (NT) H$_2$O$_2$ or TSA as described above in the presence or absence of 5 μM STI 571. Apoptotic cells were determined after 24 h by staining with propidium iodide and counting the number of cells per microscopic field with condensed nuclei as described previously (Kain et al., 2003). The bars represent the mean ± SEM of three independent experiments. (E) COS-7 cells were treated with 300 ng/ml TSA or with DMSO for 24 h, and were then lysed in detergent and Western blotted for the indicated proteins. Note that TSA does not induce a mobility shift of c-CrkII and Lasp-1 protein, indicating that these proteins are not phosphorylated and Abl kinase is not activated under these apoptotic conditions. Asterisk shows the cleaved form of PARP, indicating that TSA induced apoptosis in these cells. The top band is a nonspecific protein that served as a loading control.

demonstrate the tyrosine phosphorylation of Lasp-1 by endogenous Abl activation in response to apoptotic agents.

It is intriguing that the exposures of cells to survival factors like serum and PDGF-BB cause Lasp-1 to translocate from the cell periphery to focal adhesions in an unphosphorylated state (Fig. 2). This suggests that translocation of unphosphorylated Lasp-1 to focal adhesions plays a role in mediating survival signals through the cytoskeleton. If this is the case, then phosphorylation of Lasp-1 by apoptotic agents may prevent Lasp-1 localization to focal adhesions and disrupt survival signals from these structures. To investigate this possibility, serum-starved cells expressing GFP Lasp were briefly treated with H$_2$O$_2$ to induce Lasp-1 tyrosine phosphorylation, and were then stimulated with growth factors to induce translocation of Lasp-1 to focal adhesions and ruffles, as shown before (Fig. 2 A). H$_2$O$_2$ strongly blocked GFP Lasp translocation to focal adhesions, but not membrane ruffles, in response to growth factors (Fig. 7). Importantly, the short-term exposure of cells to H$_2$O$_2$ only effected Lasp-1 translocation and did not generally impact vinculin-positive focal adhesions, which were similar to control cells (Fig. 7). Pretreatment of cells with pervanadate also led to increased Lasp-1 tyrosine phosphorylation and prevented Lasp-1 translocation to focal adhesions (unpublished data).

Importantly, phosphorylation of tyrosine 171 and Abl kinase activity were required for the inhibitory response induced by H$_2$O$_2$ because cells expressing GFP LaspY171F or cells treated with STI 571 showed normal Lasp-1 translocation to focal adhesions (Fig. 7). As expected, vehicle-treated cells expressing GFP LaspY171F or cells treated with STI 571 showed normal translocation of Lasp-1 to focal adhesions in response to growth factors, as this process occurs independent of phosphorylation (unpublished data). It is noteworthy that GFP LaspY171F did not constitutively translocate to focal adhesions in the absence of growth factors (Fig. 7). This suggests that basal phosphorylation of Y171 is not a general mechanism used by the cell to regulate focal adhesion targeting of Lasp-1 in healthy cells, but rather is a specific mechanism that operates downstream of apoptotic stimuli and Abl tyrosine kinase activity. Importantly, although Abl-mediated tyrosine phosphorylation blocked focal adhesion targeting of Lasp-1 in apoptotic cells, it did not impact its translocation to membrane ruffles. Indeed, treatment of cells with H$_2$O$_2$ or pervanadate did not prevent Lasp-1 localization to actin-rich membrane ruffles in response to growth factors, indicating that translocation to this subcellular structure is not, per se, regulated by tyrosine phosphorylation (unpublished data). These findings also demonstrate that H$_2$O$_2$ does not globally block growth factor–induced signaling in these cells. It appears, then, that apoptotic stimuli that induce Abl activation promote Lasp-1 phosphorylation, which specifically prevents Lasp-1 localization to focal adhesions, but not ruffles. Conversely, under conditions that promote cell survival and motility, Lasp-1 is not phosphorylated and is strongly localized to focal adhesions as well as ruffles. Most importantly, Lasp-1 directly contributes to H$_2$O$_2$– and cisplatin-induced apoptosis because cells depleted of Lasp-1 protein by siRNA show significantly increased death in response to these apoptotic agents compared with control cells expressing Lasp-1 protein (Fig. 8).

In contrast, apoptosis induced with the deacetylase inhibitor trichostatin A (TSA; Ruefl et al., 2001) occurred independent of Abl and Lasp-1 activity, and did not involve changes in tyrosine phosphorylation of Lasp-1 or c-Crk protein, a known endogenous substrate of Abl (Kain and Klemke, 2001; Fig. 8, C–E). Thus, phosphorylation of Lasp-1 is not a general response in cells undergoing apoptosis, but rather is a specific event related to Abl-dependent apoptosis. In support of this, cells expressing LaspY171F, which cannot be phosphorylated by Abl and readily transits to focal adhesions, show reduced apoptosis in response to cisplatin and H$_2$O$_2$, but not TSA (Fig. 9). Together, our findings indicate that phosphorylation of Lasp-1 at tyrosine

Downloaded from on April 3, 2017
171 prevents localization to focal adhesions and the loss of survival signals and induction of cell death.

Discussion

Cell migration is characterized by morphological polarization with a dominant-leading pseudopodium and a tail region at the rear of the cell (Lauffenburger and Horwitz, 1996; Palecek et al., 1996). This process requires temporal and spatial organization of signal transduction processes that regulate actin polymerization and focal adhesion turnover. In this report, we used a novel method to isolate the leading pseudopodium from the cell body for biochemical analysis. The unique ability to fractionate the pseudopodium combined with recent advances in mass spectrometry to facilitate large-scale protein identification of complex protein samples allowed us to compare the relative protein profiles of these two subcellular compartments (Washburn et al., 2001; Cho and Klemke, 2002). We identified several previously associated pseudopodial proteins including filamin, tropomyosin, caldesmon, coflin, Arp2/3, myosin 1B and C, PI3K, and α-actinin, demonstrating the feasibility of this approach (Conrad et al., 1989; Aizawa et al., 1997; Helfman et al., 1999; Bailly et al., 2001; Laukaitis et al., 2001; Cooper, 2002; Funamoto et al., 2002). Although numerous pseudopodial and cell body proteins were identified by MudPIT, they represent only a small portion of the complex proteome of these structures. Nevertheless, with the rapid advances in mass spectrometry to quantify and monitor complex post-translational modifications of proteins (Washburn et al., 2001), together with fractionation into cholesterol-rich membrane and detergent soluble/insoluble components, will provide more detailed information on the pseudopodial proteome, and will help unravel the complex signal transduction events that control this cellular structure.

We chose to characterize Lasp-1 because its biological function is not known. In this report, we provide several lines of evidence that demonstrate Lasp-1 is a dynamic protein that transits to focal adhesions and is necessary for proper cell migration. First, depletion of Lasp-1 protein from cells strongly inhibits cell migration in response to ECM proteins. The inhibitory effect is specific to migration, as cell attachment and spreading appear normal in cells without Lasp-1 protein. Second, exogenous amplification of Lasp-1 also inhibits cell migration, but not adhesion and spreading. We believe that exogenous expression of Lasp-1 disrupts its normal signaling polarity. Lasp-1 is strongly polarized in migrating cells, where it localizes to the leading edge of the pseudopodium and to nascent focal adhesions in this structure, but not the central body of the cell. That constitutive relocalization of Lasp-1 to the plasma membrane strongly induces membrane blebbing and detachment from the ECM supports this idea. Third, stimulation of nonmigratory cells with growth factors or ECM proteins that induce cell migration cause the rapid relocalization of Lasp-1 from the peripheral membrane to a subset of focal adhesions in the spreading edge of the pseudopodium. Because cells depleted of Lasp-1 still attach to the ECM and form focal adhesions, it would appear that Lasp-1 plays a supportive role in focal adhesion dynamics during cell migration rather than in the actual formation of these structures. That Lasp-1 localizes to focal adhesions at the tips of retracting tails of migrating cells also supports this notion, as these adhesive sites must turnover for proper cell body translocation and tail release (Palecek et al., 1996). Together, these findings demonstrate that Lasp-1 is a dynamic focal adhesion protein necessary for cell migration.

Although the cellular factors responsible for translocation of Lasp-1 to focal adhesions are not yet known, it appears that the COOH-terminal region of Lasp-1 is critical for this response. Our biochemical and time-lapse analyses (unpublished data) of Lasp-1 indicate that translocation occurs rapidly within 1–2 min of growth factor stimulation and is independent of tyrosine phosphorylation. Indeed, repeated attempts to demonstrate changes in tyrosine phosphorylation of Lasp-1, in response to ECM proteins or growth factors, failed to show this response, even though these factors activate several tyrosine kinases including src and Abl (Lewis et al., 1996; Plattner et al., 1999). Furthermore, we did not detect a mobility shift of Lasp-1 in SDS-PAGE in stimulated cells or purified pseudopodia, which is characteristic of phosphorylation by PKA (Chew et al., 1998) as well as tyrosine phosphorylation (Fig. 6, A and B). This suggests that phosphorylation does not regulate focal adhesion targeting of Lasp-1 during cell spreading or pseudopodial extension. However, in some cases PKA may regulate Lasp-1 in focal...
adhesions as well as its association with actin in gastric parietal cells where it localizes to the actin-rich canicular membrane (Chew et al., 2002; Butt et al., 2003). It is also possible that low levels of Abl and PKA activity regulate Lasp-1 dynamics in the microenvironment of the migrating cell, but this activity was below biochemical detection in our analyses.

Our findings indicate that the COOH-terminal portion of Lasp-1 is for targeting to focal adhesions. The SH3 domain of Lasp-1 may play a pivotal role in focal complex targeting, as these structures can direct proteins like p130CAS (Crk-associated substrate) to focal adhesions (Harte et al., 2000). However, a GFP fusion of the Lasp-1 SH3 domain failed to translocate by itself to focal complexes (unpublished data). It is possible that the GFP tag interfered with the normal targeting function of this domain. Additional truncations in the COOH terminus as well as point mutations that disrupt SH3 domain function will be necessary to pinpoint the region of Lasp-1 responsible for translocation to focal complexes and for identification of binding proteins that mediate this process. In any case, the Lim domain does not play a critical role in focal adhesion targeting, as truncation of this domain did not prevent Lasp-1 localization to these structures (unpublished data).

It is intriguing that Abl tyrosine kinase is activated by growth promoting as well as by apoptotic stimuli (Lewis et al., 1996; Gong et al., 1999; Plattner et al., 1999; Sun et al., 2000). This suggests that Abl activity is a complex process tightly regulated by temporal and spatial mechanisms that couple to specific effector molecules. Our findings indicate that growth factor/motility factors like serum, PDGF-BB, EGF, and ECM proteins do not mediate coupling of Abl to Lasp-1. Rather, Abl activation in response to apoptotic stimuli appears to selectively phosphorylate Lasp-1 on tyrosine 171, preventing its translocation into focal complexes. This event is specific to focal complex targeting, as Abl-induced phosphorylation of Lasp-1 does not inhibit its localization to actin-rich structures including membrane ruffles and actin cables. Thus, Abl-dependent tyrosine phosphorylation of Y171 is a distinct signaling event that specifically blocks Lasp-1 translocating to focal complexes in apoptotic cells. Although the mechanism of Lasp-1 translocation is not known, these findings have important implications, as Abl activity is important for the apoptotic response. Recent evidence indicates that cytoskeletal like integrin and growth factor receptor activation typically promote only a transient response (<30 min; Lewis et al., 1996; Gong et al., 1999; Plattner et al., 1999; Sun et al., 2000). Interestingly, recent evidence indicates that integrins and cell adhesion to the ECM is necessary for Abl-mediated death in response to DNA-damaging agents like cisplatin (Lewis et al., 2002; Truong et al., 2003). This suggests that at least part of the Abl death signal requires integrins and focal adhesion structures. Although the kinetics of Abl activity was not addressed in these reports, it seems reasonable that part of the suicide program involves not only localization of Abl to the nucleus where it regulates p53/73 (Lewis et al., 2002; Truong et al., 2003), but also its localization to the cytoplasm where it inactivates specific focal adhesion proteins like Lasp-1 and c-CrkII. In this way, Abl activation would have widespread impact on the apoptotic machineries that operate at multiple compartments within the cell. This widespread insult is likely important in the cell’s decision to commit suicide or to attempt a repair and rescue program. In any case, the current evidence indicates that Abl-mediated death involves a cooperative effort with integrins, cytoskeleton, focal adhesions, and mitochondrial and nuclear proteins. The challenge now is to understand how Abl coordinates these diverse processes and whether these events contribute to cancer development and progression.

**Materials and methods**

**Purification of pseudopodia and cell body fractions and MudPIT analysis**

Purification of pseudopodial proteins was according to published procedures (Cho and Klemke, 2002). In brief, 1–1.5 × 10^6 NIH 3T3 cells were allowed to extend pseudopodia toward an LPA gradient (100 ng/ml) for 60 min. A pseudopodia isolation kit (ECM 660; CHEMICON International) or Costar chambers (tissue culture treated, 6.5-mm diam, 10-μm thickness, 8-μm pores, Transwell®; Costar Corp.) were used for isolation. For Western blotting, cells were rinsed in excess cold PBS and rapidly fixed in 100% ice-cold methanol. Cell bodies on the upper membrane surface were manually removed with a cotton swab and pseudopodia on the undersurface scraped into lysis buffer and detergent. Purification of pseudopodia and cell body fractions were searched against a combined database of human, mouse, and rat proteins using SEQUEST, and the combined database was constructed from the NCBI protein database (August 20, 2002). The results were further analyzed by DTASelect and Contrast software. Peptides with...
nontryptic cleavage sites were not included. All the proteins identified with a minimum of a single peptide were used to make protein lists for the pseudopod and for the cell body. A total of 980 proteins was identified in vivo by MudPIT were included in the list.

**Immunofluorescence analysis of Lasp-1 dynamics**

NIH 3T3 cells were transfected with the appropriate GFP constructs on glass coverslips as described above. Cells were washed 2× with PBS, fixed with 4% PFA in PBS, permeabilized with 0.1% Triton X-100 in PBS for 1 min, and then blocked with 0.5% BSA for 1 h. Anti-vinculin antibodies or TRITC-conjugated phalloidin (Sigma-Aldrich) was diluted in blocking solution and incubated with the fixed cells for 60 min. Anti-vinculin–treated cells were then incubated with Alexa Fluor® 568-conjugated goat anti–mouse IgG antibodies and were washed with PBS and mounted on coverslips using a ProLong® anti-fade kit (Molecular Probes, Inc.). Immunofluorescence microscopy was performed using a microscope (model IX70; Olympus) and data acquisition by a liquid cooled CCD camera (500 KHz, 12 bit, 2MP, KAF1400GI, 1317 × 1035; model CH530L, Photometrics). Image data were deconvolved with DeltaVision softWoRx version 2.5 software (Applied Precision). To examine the role of Abl activation in Lasp-1, image data were deconvolved with DeltaVision softWoRx version 2.5 soft-12 bit, 2MP, KAF1400GI, 1317

**siRNA silencing**

21-nucleotide double-stranded RNAs (Dharmacon Research) were synthesized by targeting human Lasp-1 (5’-AACUCAAGGGCUACGAGAAG-3’; 5’-GGUGACUUGAACGUCAGCAU-3’; corresponding to the coding region 127–147 relative to the first nucleotide of the start codon). Luciferase GL2 duplex (Dharmacon Research) was used as a negative control. The siRNAs were transfected into Cos-7 cells using Oligofectamine™ (Invitrogen) according to the manufacturer’s instructions. Specific depletion of Lasp-1 was confirmed 48 h after transfection by Western blotting using anti-Lasp-1 and anti-actin antibodies.

**Online supplemental material**

A complete list of all pseudopodia and cell body–associated proteins is shown in Tables S1–S4 and Fig. S1. Materials and methods for cell lines, plasmids, constructs, antibodies, and cell-based assays, kinase assays, immunoprecipitation, and Western blotting are shown. Fig. S1 is a comparative schematic of the number of proteins identified in the each of the subcompartments of the cell body and pseudopodial fractions. Online supplemental material available at http://www.jcb.org/cgi/content/full/jcb.200311045/DC1.

We thank Drs. B. Mayer (University of Connecticut Health Center, Farmington, CT), T. Hunter, P. Woodring (The Salk Institute, La Jolla, CA), J.Y. Wang (University of California, San Diego, San Diego, CA); D. Cheresh, D. Stupack, P. Lee, E. Beutler (The Scripps Research Institute), and M. Schwartz (University of Virginia, Charlottesville, VA) for providing agents and advice concerning this project. We also thank Dr. K. Spencer for helpful advice and assistance with confocal microscopy.

Y.H. Lin is supported by California Breast Cancer Research Program grant 7FB-0117. R.L. Klemke is funded by National Institute of Health grant CA097022. This is manuscript no. 16012-IMM from The Scripps Research Institute.

Submitted: 7 November 2003
Accepted: 5 April 2004

**References**


Houle, F., S. Rousseau, N. Morrici, M. Luc, S. Mongrain, C.E. Turner, S.


Murphy-Ullrich, J.E. 2001. The de-adhesive activity of matricellular proteins: is inactivation of protein tyrosine phosphatase 1B to the Src homology 3 domain of p130(Cas).


Materials and methods

**Cell lines, plasmid constructs, and antibodies**

Cos-7 and HEK 293 cells were from the American Type Culture Collection. Mouse NIH 3T3 cells were provided by Drs. Tony Hunter and Pamela Woodring (The Salk Institute, La Jolla, CA). Bcr/Abl-transformed 3T3 cells were from Dr. Miguel Angel Del Pozo (The Scripps Research Institute, La Jolla, CA) and Dr. Jean Wang (University of California, San Diego, San Diego, CA). The stable Abl/Arg (double knock-out) cell line was from Dr. Anthony Koleske (Yale University, New Haven, CT). Reconstitution of Abl/Arg cells was induced through retroviral mediated gene transfer using Bosc cell–generated retrovirus according to published procedures (Kain et al., 2003). The HEK 293 cells stably expressing Lasp-1 were generated by calcium phosphate transfection of Lasp-1 in pcDNA3.1/neo (Invitrogen) and selected by G418 (GIBCO BRL). Full-length LaspΔC (aa 1–145) and Lasp SH3 (aa 196–261) genes were amplified by PCR using primers containing appropriate restriction sites and pCMVLasp-1 (Schreiber et al., 1998) as a template. The fragments were cloned into pcDNA 3.1/neo (Invitrogen), pRK5HA, pEGFP C1 (CLONTECH Laboratories, Inc.), and pGEX 6P-2 (Amersham Biosciences), respectively. AblΔPro was a gift from Dr. Bruce Mayer (Smith et al., 1999). LaspY171F mutants in pcDNA and pEGFP vectors were generated using the QuikChange® site-directed mutagenesis kit (Stratagene) and DNA sequencing was performed to confirm mutations. pMyr-Lasp and pMyr-LaspΔC (myristylated forms) were generated by fusing a PCR-amplified DNA fragment encoding the first 14 amino acids of p60src to the NH2 terminus of Lasp-1 and LaspΔC in their respective expressing vectors. Rabbit polyclonal Lasp-1 antibodies were from CHEMICON International (A68990). Anti-phosphotyrosine (4G10; Upstate Biotechnology), anti-Abl 8E9 (BD Biosciences), anti-HA (Roche), anti-vinculin, anti-actin (Sigma-Aldrich), and Alexa Fluor® 568-conjugated goat anti–mouse IgG antibodies (Molecular Probes, Inc.), goat anti–rabbit and anti–mouse antibodies (Bio-Rad Laboratories), and rabbit antibodies to the 89-kD cleaved/activated form of PARP (Cell Signaling Technology) were purchased from commercial sources as indicated. H2O2, LPA, insulin, and cisplatin were obtained from Sigma-Aldrich. STI 571 was obtained from Novartis and PDGF-BB was purchased from Genzyme. Tri-chostatin A was obtained from Sigma-Aldrich.

**Migration, adhesion, apoptosis, and wound assays**

Migration assays were performed as described previously (Klemke et al., 1998). In brief, Boyden chambers containing polycarbonate membranes (tissue culture treated, 6.5-mm diam, 8-μm pores; Transwell®, Costar Corp. or CHEMICON International) were either coated on the bottom (haptotaxis) or on the top and bottom (chemotaxis) with either rat tail collagen type I (Upstate Biotechnology, Inc.) or human fibronectin (Oxford Biomedical Products) for 2 h at 37°C. Serum-starved cells were allowed to migrate 3–5 h, were fixed with ethanol, and then were stained with crystal violet. In some cases, migratory cells transfected with Lasp-1 constructs were cotransfected along with the reporter vector pCMVSPORT β-galactosidase (Invitrogen) and developed using X-gal as a substrate according to the manufacturer’s recommendation (Promega) and as described previously (Klemke et al., 1998). For cell adhesion assays, an aliquot of cells used in the migration experiments was allowed to attach for the indicated times to fibronectin- or collagen (10 μg/ml-coated cell culture wells at 37°C and was stained with crystal violet or X-gal reagent. For wound migration, GFP Lasp–transfected NIH 3T3 cells were plated at 150,000 cells per well on a glass coverslip in media containing 0.5% FBS. Cells were incubated overnight to form a confluent monolayer of cells. A wound was introduced into the monolayer by scraping with a clean micropipette tip and the detached cells were removed into the wound at 37°C in 0.5% FBS for 18 h, and then were rinsed with PBS and fixed in 4% PFA. Vinculin-positive focal adhesion, GFP Lasp, and the actin cytoskeleton were visualized as described above. Standard apoptosis assays using propidium iodide staining were performed as previously described, except cells were maintained in 0.5% FBS to reduce serum-deprived cell death responses (Kain et al., 2003).

**Transient transfection**

Transient transfection of Cos-7 cells for biochemical and migration assays was performed as described previously (Klemke et al., 1998). For transfections of mouse NIH 3T3 cells and Bcr/Abl-transformed 3T3 cells, Effectene™ (QIAGEN) was used. 300,000 cells were plated per 100-mm dish the night before transfection. 1.5 μg of DNA was used and serum-starved cells (0.5% FBS for 24 h) were lysed 48 h later. Transfection of abl/arg and abl/arg-fibroblast cells (Koleske et al., 1998) was performed as for the Cos-7 cells, except 300,000 cells were plated in a 100-mm plate and then incubated with DNA and LipofectAMINE™ for 4 h.
Targeting of Lasp-1 to plasma membrane

pMyc-Lasp and pMyc-LaspΔC vectors along with GFP reporter construct were transfected into Cos-7 cells. Cells were fixed after 16 h of transfection. Blebbing cells were counted and deconvolution images were taken as described in the Materials and methods section in the manuscript text.

Immunoblotting, GST purification, and immunoprecipitation

Immunoblotting and immunoprecipitation of proteins were conducted according to published procedures (Klemke et al., 1998). In brief, cells were rinsed with PBS and lysed in Triton X-100 buffer (50 mM NaCl, 1 mM EDTA, 50 mM Hepes, 1 mM EGTA, 1% Triton X-100, 2 mM sodium orthovanadate, and 1 mM sodium fluoride, pH 8.0) on ice for 2 h.

In vitro kinase assay

50 U purified Abl (Cell Signaling Technology) was incubated with 10 ng pGEXLasp purified from Escherichia coli in the presence of 200 μM ATP at 30°C for 30 min or with 1 μg purified GST alone. The reaction was stopped by SDS-PAGE sample buffer and analyzed for tyrosine phosphorylation by Western blotting with anti-phosphotyrosine, GST, or Lasp-1 antibodies.

References


