Interaction of Pyk2 and PTP-PEST with leupaxin in prostate cancer cells

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LEUPAXIN (LPXN), which was originally characterized as a member of the paxillin extended family of multifunctional docking/adaptor phosphoproteins in cells of hematopoietic origin, was further identified as an additional component of the sealing zone in osteoclasts (7, 25, 40, 51). In both hematopoietic cells and the osteoclast, LPXN was found to associate with the nonreceptor protein tyrosine kinase (PTK) Pyk2 and the cytoplasmic protein tyrosine phosphatase-proline-, glutamate-, serine-, and threonine-rich sequence (PTP-PEST) (25, 40, 72). More recently, we have demonstrated that, in osteoclasts, LPXN can also interact with the PTK c-Src (51).

In the osteoclast podosomal/sealing zone signaling complex, which is analogous to focal adhesions in other cells, Pyk2 has been suggested to functionally replace the focal adhesion kinase FAK, which is expressed in low levels (76). In a variety of cell types, both Pyk2 and FAK participate in the integrin-mediated signal transduction to modulate cellular functions such as adhesion, motility, and anchorage independence (26, 44, 45, 59, 82). Pyk2 is functionally expressed in both normal and benign neoplastic prostate tissue. The expression of Pyk2 in the prostate can vary according to the proliferation, differentiation, and malignancy (46, 62, 71). In addition, the involvement of c-Src in regulating growth, cell proliferation, adhesion, and metastasis of prostate cancer cells has been demonstrated through selective c-Src kinase inhibitors (43, 48). In response to various extracellular stimuli including activation of integrins, Pyk2 and c-Src have been shown to form a signaling complex that initiates several downstream effector pathways in a variety of cell types (16, 18, 23, 28). Finally, paxillin, as an adapter protein, has been demonstrated to facilitate the interaction of Pyk2 and c-Src and also to act as a substrate for both of these PTKs (47, 54, 67).

The process of cell migration, which is both spatially and temporally coordinated, is regulated by a turnover of cell adhesion complexes by several PTKs and PTPs (9). PTP-PEST has been implicated in regulating both cell spreading and migration via dephosphorylation of several focal adhesion-associated proteins, including paxillin, p130Cas, Pyk2, and the paxillin kinase linker (PKL) (3, 56). Paxillin, which is also essential for targeting PTP-PEST to focal adhesions, has been shown to associate with several downstream effectors that can regulate focal adhesion turnover and cytoskeletal reorganization (33). Coordination of the activities of Rho and Rac is important for efficient migration (8). Previous studies have demonstrated that both paxillin and PTP-PEST regulate Rho GTPase function by differentially regulating the activity of Rho proteins at both the leading and retracting edges (53, 74). This regulation is partly effected by modulating the activities of Rho GTPase-activating proteins (RhoGAPs), Rho GDP dissociation inhibitors (RhoGDI), and the Vav family of guanine nucleotide exchange factors (GEFs) (1, 29, 53, 78).

We have previously suggested that the association of Pyk2, c-Src, and PTP-PEST with LPXN may form an additional regulator of the signaling complex of the adhesion zone in osteoclasts (25, 51). In the current study, we provide evidence that, in PC-3 cells, a prostate cancer cell line, LPXN also forms a functional complex with Pyk2, c-Src, and PTP-PEST. A recombinant adenoviral-mediated overexpression of LPXN resulted in an increased association with both Pyk2 and c-Src, whereas overexpression of PTP-PEST decreased the levels of LPXN-associated Pyk2 and c-Src. We have hypothesized that a functional consequence of a signaling complex formed by LPXN is to increase cell migration in PC-3 cells, probably by regulation of Rho GTPase activity.
LEUPAXIN IN PROSTATE CANCER CELLS

MATERIALS AND METHODS

Materials. The anti-LPXN (α-LPXN) monoclonal antibody 283C was a gift from ICOS (Bothell, WA) and has been previously characterized (25). An anti-murine LPXN (mLPXN) chicken antibody IgY 4247 was developed and affinity purified with an amino acid sequence specific for mLPXN (CHLDQQSTEESKIPQPTK), as previously characterized (51). Antibodies to Pyk2 and PTP-PEST were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and Sigma-Aldrich (St. Louis, MO), respectively. The antibody PY20 was purchased from Invitrogen (Carlsbad, CA). The AdEasy-XL Adenoviral Vector system was purchased from Stratagene (La Jolla, CA). Cyclo-RGD peptides [catalog no. PCI-3661-P1; cyclo-(Arg-Gly-Asp-O-Phe-Lys)], cRGDK were purchased from Peptides International (Louisville, KY). The fluorescent secondary antibodies Alexa488 and Alexa568 were purchased from Invitrogen. Rhodamine phalloidin was purchased from Sigma (St. Louis, MO). Both UltraLink Immobilized Protein A/G Plus beads and the SuperSignal West Dura Extended Duration Substrate chemiluminescence reagent were purchased from Pierce Biotechnology (Rockford, IL). The siRNA for LPXN was purchased as predesigned siRNA (Silencer; 40 nmol, standard purity, annealed, identity no. 172914) from Ambion (Austin, TX). The open reading frame of the LPXN gene was ligated into pShuttle-IREs-hrGFP2 (AdEasy-XL Adenoviral Vector system, Stratagene), using the Nhel-Xhol multiple cloning sites. Recombinant adeno-lpXN-GFP2 was confirmed by digestion with PacI. High titer (2 × 10^10 pfu/ml) viral stocks were made as described here and according to methods previously published (2, 35, 51, 65). Approximately 15 μg of shuttle plasmid and 5 μg of pVQAD 9.2–100 backbone plasmid were digested with PacI. Both linear fragments were cotransfected into the human embryonic kidney (HEK)293 cell line on a 60-mm plate at ~50% confluence by standard CaCl2 method. The initial viral lysate was amplified in HEK293 cells, and the final viral lysate was purified over two rounds of CsCl gradient ultracentrifugation. Virus particles were dialyzed against 3% sucrose–PBS buffer, diluted to 1 × 10^12 particles/ml, and then frozen at –80°C. We assayed for infectious particle concentration by a cell-based plaque assay on HEK293 cells. The presence of replication competent adenovirus was checked by plaquing on the wild-type permissive cell line A549 for at least 14 days (2). As negative controls, PC-3 cells were separately infected with an adenovirus encoding for enhanced green fluorescent protein (EGFP; rAd-EGFP), as described previously (51). Similar to methods detailed above, a recombinant adenovirus was also constructed for the protein tyrosine phosphatase PTP-PEST. PC-3 cells were infected with adenoviruses or either LPXN or PTP-PEST at a multiplicity of infection (MOI) of 100 for 48–72 h before experiments.

Immunoprecipitation assays. Both endogenous and overexpressed LPXN PC-3 cells were immunoprecipitated as follows. Briefly, PC-3 cells were lysed in a modified radioimmunoprecipitation (RIPA) lysis buffer for 1 h at 4°C (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 0.5% Triton X-100, 30 mM sodium pyrophosphate, 5 mM NaF, 0.1 mM Na3VO4, 5 mM Cl2, and 2 mM phenylmethylsulfonyl fluoride (PMSF)) and further supplemented with a protease inhibitor cocktail (Complete Protease Inhibitor Cocktail; Roche Bioscience, Palo Alto, CA). The lysates were centrifuged at 14,000 rpm for 10 min, and protein measurements were performed on the supernatants. Approximately 500 μg of lysates were preclared with MOPC-21, murine nonimmune serum, for 2 h at 4°C. The preclared lysates were centrifuged at 8,000 rpm at 4°C, and the supernatants were transferred to fresh microfuge tubes. Approximately 4–5 μg of the monoclonal antibody (mAb) 283C against LPXN were added for specific immunoprecipitation. After incubation at 4°C overnight, 50 μl of a 50% suspension of UltraLink Immobilized Protein A/G Plus beads were added to the individual tubes for 2 h at 4°C. The immunoprecipitates were centrifuged at 8,000 rpm for 10 min at 4°C. Separately, PC-3 lysates were incubated with MOPC-21 for nonspecific immunoprecipitation (NS-IP) for 2 h at 4°C, after which ~50 μl of a 50% suspension of UltraLink Immobilized Protein A/G Plus beads were added. The beads were washed at least four times with lysis buffer and one time with PBS (10 min each, at 4°C). After the last wash, the beads were centrifuged at 8,000 rpm for 10 min, the supernatant was discarded, and the immunoprecipitates were eluted by boiling in ~100 μl of 2X Laemmli sample buffer with 2% β-mercaptoethanol. The immunoprecipitates (both specific and nonspecific) were separated on 8% SDS-PAGE gels and subsequently transferred to polyvinylidene fluoride (PVDF) membranes (Pierce), using a semi-dry transfer apparatus (Bio-Rad, Hercules, CA). The PVDF membranes were blocked in 5% nonfat dry milk for at least 2–4 h at room temperature before Western blot analysis was performed with primary antibodies.

Indirect immunofluorescence. PC-3 cells were rinsed twice in ice-cold PBS, fixed with 4% paraformaldehyde, and permeabilized with PBS containing 0.2% Triton X-100. The cells were blocked with PBS containing 3% bovine serum albumin overnight. The cells were incubated for 2 h in a 1:500 dilution of appropriate antibodies (283C for LPXN and a rabbit polyclonal antibody to Pyk2) at room temperature in the blocking solution described above. The primary antibodies were detected with either Alexa488- or Alexa562-conjugated secondary antibodies (1:500 dilutions, Molecular Probes). Actin was labeled with rhodamine phalloidin. Confocal microscopy (Carl Zeiss LSM Meta 510) was performed; all images shown were taken with a ×63 oil immersion objective lens.

Cell migration assay. PC-3 cells (~5 × 10^5), which were cultured in serum-free RPMI 1640 medium before assays, were seeded into the upper chamber of the Transwell (5.5 mm, 3.0 μm pore size; Corning, Corning, NY) following a 48-h infection with rAd-LPXN or rAd-PTP-PEST at an MOI of 100. In experiments involving siRNA treatment, PC-3 cells were transfected with 10 nM, 100 nM, and 1 μg LPXN-siRNA with transfection reagent (Mirus) for 48 h, according to the manufacturer’s instructions. In separate experiments involving the Rho GTPase inhibitor Y27632, cells were treated for 24 h with 1 μm following prior overexpression of LPXN or PTP-PEST (48–72 h). The outside or lower surface of the polycarbonate filter was coated with 10 μg/ml rat tail collagen (Biomedical Technologies, Stoughton, MA). The cyclo-RGD peptides were added at a concentration of 100 ng/ml to the culture medium (in the lower chamber of the Transwell) to serve as a chemottractant. The cells were allowed to migrate overnight (~14 h) in a 5% CO2 incubator at 37°C. Over 16–24 h, cell migration into the lower surface of the polycarbonate filter was assessed as follows: the PC-3 cells from the upper chamber were removed using a cotton swab, and the polycarbonate filter was fixed and stained with Mayer’s hematoxylin for visualization of the migrated cells. The migrated cells were counted using an inverted microscope. Data were processed as detailed in Statistical analyses.

Rho GTPase activity assays. Rho, Rac, and Cdc42 activation assays were performed using commercially available kits (Cytoskeleton, Denver, CO), according to the manufacturer’s directions. Briefly, PC-3 lysates were prepared fresh from cells that were overexpressing either LPXN or PTP-PEST. In separate experiments involving the Rho GTPase inhibitor Y27632, cells were treated for 24 h with 1 μm following prior overexpression of LPXN or PTP-PEST (48–72 h). The lysates were incubated with GST-rodentin-glutathione-Sepharose beads for 1 h at 4°C for Rho activity assays or PAK-glutathione-Sepharose beads (for Rac and Cdc42 activity assays). The agarose conjugates were collected by centrifugation, washed, and resuspended in 2× Laemmli buffer with 2% β-mercaptoethanol, separated by SDS-PAGE, transferred...
to PVDF membranes, and subsequently probed with antibodies against Rho or with antibodies against either Rac or Cdc42.

Statistical analyses. Statistical analyses were performed as follows: the raw data points for Transwell cell migration assays were used to obtain the mean for the control group of PC-3 cells. Each raw data point within that group was divided by the mean and subsequently transformed (multiplying the quotient by 100) to get percentage values for each data point within that group. From these transformed data points, we determined the mean ± SE for the control group, giving values of 100 ± SE. To obtain similar data for the experimental treatment groups (i.e., LPXN- and PTP-PEST-overexpressing PC-3 cells), each raw data point was divided by the original mean of control group; the quotients were similarly transformed into “percentage of control.” Therefore, similar to the control group, means ± SE were obtained for the experimental groups, as described previously (35, 51).

RESULTS

Expression and subcellular distribution of LPXN in PC-3 cells. Previous reports have characterized several mAbs that specifically detect the human ortholog of LPXN (hLPXN) in cells such as osteoclasts, macrophages, and the J77 cell line (25, 51). Among these, the mAb 283C was shown to specifically recognize hLPXN as a 50-kDa protein (25). Whole cell lysates prepared from both murine osteoclasts and PC-3 cells were used to immunoblot for LPXN; as shown in Fig. 1Aa, the mAb 283C specifically recognized LPXN at ~50 kDa only in PC-3 cells (Fig. 1Aa, lane 2) and not in the murine osteoclast (Fig. 1Aa, lane 1). On the other hand, the anti-murine LPXN (α-mLPXN) chicken antibody (IgY 4247) specifically recognized mLPXN in murine osteoclast lysates (Fig. 1Ab, lane 1), as previously characterized (51). However, IgY 4247 did not recognize hLPXN in PC-3 cells (Fig. 1Ab, lane 2); there was probably cross-reactivity of IgY 4247 with paxillin (~68 kDa) in PC-3 cell lysates. Therefore, the mAb 283C was used for all subsequent experiments in PC-3 cells. Next, PC-3 cells were examined for the subcellular distribution and colocalization of LPXN with either actin or Pyk2, as shown in Fig. 1. B and C. First, the distribution of both LPXN (Fig. 1B1) and actin (Fig. 1B2) was diffuse throughout the cytoplasm but colocal-

![Fig. 1. Expression and subcellular distribution of leupaxin (LPXN) in PC-3 cells. Aa: whole cell lysates prepared from both murine osteoclasts (mOC; lane 1) and PC-3 cells (lane 2) were used to immunoblot LPXN with the monoclonal antibody (mAb) 283C (α-hLPXN). Ab: whole cell lysates prepared from both murine osteoclasts (lane 1) and PC-3 cells (lane 2) were used to immunoblot LPXN with the anti-LPXN chicken antibody IgY 4247 (α-mLPXN). hLPXN, human ortholog of LPXN; mLPXN, murine LPXN. Nos. at right of blots are in kDa. B: indirect immunofluorescence to detect subcellular distribution of LPXN (frame 1) and actin (frame 2) in PC-3 cells. PC-3 cells were fixed and immunostained for LPXN with mAb 283C; actin was stained with rhodamine phalloidin. Colocalization of LPXN and actin is shown in frame 3; scale bar = 20 μm. C: subcellular distribution of LPXN (frame 1) and Pyk2 (frame 2) in PC-3 cells. Colocalization of LPXN and Pyk2 is shown in frame 3; scale bar = 20 μm. Data shown are representative of 3–4 separate experiments.](http://ajpcell.physiology.org/)

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LPXN in Prostate Cancer Cells

Overexpression of LPXN and PTP-PEST in PC-3 cells: effects on LPXN-associated Pyk2 and c-Src. LPXN has previously been demonstrated to associate with Pyk2, c-Src, and PTP-PEST (25, 40, 51, 72). Previously, we have demonstrated that a recombinant adenoviral-mediated overexpression of LPXN in osteoclasts resulted in an increased association with Pyk2 (51). PC-3 cells were infected for 72 h with recombinant adenoviruses encoding for either LPXN (rAd-LPXN) or PTP-PEST (rAd-PTP-PEST). As shown in Fig. 2, the overexpression of both LPXN (~50 kDa; Fig. 2Aa) and PTP-PEST (~112 kDa; Fig. 2Ab) was verified by Western blot analyses compared with cells that had been infected with a control adenovirus (rAd-EGFP), as previously described (51). We next examined the possibility that the tyrosine phosphorylation levels of LPXN-associated Pyk2 could be altered following overexpression of either LPXN or PTP-PEST in PC-3 cells. Following overexpression of LPXN or PTP-PEST, LPXN was immunoprecipitated from PC-3 cell lysates, and the LPXN immunoprecipitates were immunoblotted for LPXN-associated Pyk2, Pyk2Y402, and PTP-PEST, as shown in Fig. 2. The levels of LPXN-associated Pyk2Y402 (Fig. 2B1, lane 3), Pyk2 (Fig. 2B2, lane 3), and PTP-PEST (Fig. 2B3, lane 3) were increased in LPXN-overexpressing cells (Fig. 2B4, lane 3) compared with controls (Fig. 2B, lane 2). On the other hand, when PTP-PEST was overexpressed in PC-3 cells, the levels of LPXN-associated Pyk2Y402 and Pyk2 were decreased (Fig. 2B, lane 1 and 2, lane 4). These data indicated that overexpression of LPXN resulted in greater coimmunoprecipitation with functional Pyk2, whereas coimmunoprecipitation decreased following overexpression of PTP-PEST, suggesting that LPXN-associated Pyk2 may be a substrate for PTP-PEST. It should be noted that Pyk2, and paxillin-associated Pyk2, has previously been shown to be a substrate for PTP-PEST in various cell types.

Fig. 2. Overexpression of LPXN and protein tyrosine phosphatase-proline-, glutamate-, serine-, and threonine-rich sequence (PTP-PEST) in PC-3 cells. A, a and b: PC-3 cells were infected for 72 h with recombinant adenoviruses encoding for either LPXN (rAd-LPXN) or PTP-PEST (rAd-PTP-PEST), both at a multiplicity of infection (MOI) of 100. Western blot analyses were performed to verify overexpression of LPXN (~50 kDa, Aa) and PTP-PEST (~112 kDa, Ab) compared with cells that had been infected with a control adenovirus (rAd-EGFP). The levels of β-actin were used for normalization. B: following overexpression of either LPXN or PTP-PEST, LPXN was immunoprecipitated from PC-3 cells, and the LPXN immunoprecipitates were immunoblotted for Pyk2Y402 (frame 1), Pyk2 (frame 2), PTP-PEST (frame 3), and LPXN (frame 4). As negative controls for the immunoprecipitation, mouse IgG MOPC-21 was used for nonspecific immunoprecipitation (NS-IP, lane 1). C: following overexpression of either LPXN or PTP-PEST, LPXN was immunoprecipitated from PC-3 cells, and the LPXN immunoprecipitates were immunoblotted for Pyk2 (frame 1) and c-Src (frame 2). The blots were stripped and immunoblotted for LPXN (frame 3). As negative controls for the immunoprecipitation, mouse IgG MOPC-21 was used for NS-IP (lane 1). Data shown are representative of 3 separate experiments. EGFP, enhanced green fluorescent protein; HC, heavy chain of IgG.
indirectly through association (of c-Src) with Pyk2, since we sized that the interaction of c-Src with LPXN is mediated by sex steroid hormones (5, 10, 12). We had previously hypoth-
eosed that c-Src plays a role in prostate cancer cell function, primarily in response to sex steroid hormones (5, 10, 12). We had previously hypoth-
eosed that the interaction of c-Src with LPXN is mediated indirectly through association (of c-Src) with Pyk2, since c-Src does not possess NH2-terminal SH3 and SH2 domains that can bind directly to c-Src, as characterized for paxillin (51, 68, 69). Our data suggest that c-Src can also associate with LPXN in PC-3 cells (Fig. 2C, lanes 2–4) and that, following overexpression of LPXN (rAd-LPXN; Fig. 2C, lane 3), the association of both Pyk2 (Fig. 2C1, lane 2) and c-Src (Fig. 2C2, lane 2) with LPXN is increased compared with controls (Fig. 2C1, lane 1 and 2, lane 2) and PTP-PEST-overexpressing cells (Fig. 2C2, lane 4). The LPXN immunoprecipitates were immunoblotted with mAb 283C to serve as loading controls for the coimmunoprecipitation of Pyk2 and c-Src with LPXN (Fig. 2C3).

siRNA-mediated inhibition of LPXN and impact on PC-3 cell migration. Previous studies have demonstrated that an inhibition of LPXN expression in osteoclasts results in decreased resorptive activity (25). We asked whether a reduction in the levels of LPXN would impact PC-3 cell migration. For this purpose, PC-3 cells were transfected with siRNA (10–100 nM or 1 μM) for LPXN for 48 h, and the cell lysates were immunoblotted for LPXN. LPXN protein levels were decreased with both 100 nM and 1 μM siRNA, as shown in Fig. 3A, lanes 3 and 4, compared with untreated controls and 10 nM siRNA (Fig. 3A, lanes 1 and 2). In Transwell migration assays, the siRNA (100 nM)-induced decrease in LPXN levels correlated with decreased cell migration, as shown in Fig. 3B.

Overexpression of LPXN and PTP-PEST in PC-3 cells: effects on cell migration. We examined the possibility that overexpression of LPXN and PTP-PEST could effect changes in the subcellular colocalization of Pyk2 with LPXN in PC-3 cells, since an overexpression of PTP-PEST decreased the levels of LPXN-associated Pyk2 (as shown in Fig. 2B). Previously, we demonstrated that overexpression of LPXN results in enhanced colocalization of LPXN with Pyk2 in osteoclasts, partly at or near the sealing zone (51). As shown in Fig. 4A, both Pyk2 and LPXN were diffusely distributed throughout the cytoplasm in control rAd-EGFP-infected cells (Fig. 4Aa, frames 1–3), the colocalization intensity of which was increased in LPXN-overexpressing cells (Fig. 4Ab, frames 1–3), similar to our previous observations in osteoclasts (51). However, following overexpression of PTP-PEST, the average colocalization intensity of Pyk2 and LPXN was apparently decreased to that of control cells (Fig. 4Ac, frames 1–3). These data, although not quantitative, suggested that overexpression of PTP-PEST in PC-3 cells may cause a dissociation between LPXN and Pyk2 in their subcellular distribution. We next examined whether increased expression of either LPXN or PTP-PEST impacted changes in migration of PC-3 cells. Following adenoviral-mediated overexpression of LPXN or PTP-PEST, as detailed previously, overexpression of LPXN (rAd-LPXN) in PC-3 cells resulted in a modest but significant increase in migration (138.62 ± 11.50, P < 0.05) compared with control rAd-EGFP-infected cells (100.0 ± 9.44) as shown in Fig. 4B. On the contrary, the overexpression of PTP-PEST (rAd-PTP-PEST) resulted in significantly decreased migration (65.75 ± 5.95, P < 0.05). These data suggested that overexpression of PTP-PEST results in a profound inhibition of PC-3 cell migration, an effect that could be due to the dephosphor-
ylation of several regulators of motility, including that of LPXN-associated Pyk2. Several studies on various prostate cancer cell lines have shown that increased activity of the Rho GTPases is correlated to either cytoskeletal reorganization, increased spreading, scattering, motility, or invasion of prostate cancer cells (20, 24, 37, 61, 73, 77). Since cell migration was either significantly increased in LPXN-overexpressing cells or decreased in PTP-PEST-overexpressing cells, we analyzed the individual activities of Rho, Cdc42, and Rac GTPases. PC-3 cell lysates were separately prepared for Rho, Rac, and Cdc42 activity assays according to methods detailed by the manufacturer (Cytoskeleton, Denver, CO). In our study, we demonstrated that the activity of Rho GTPase in LPXN-overexpressing PC-3 cells was specifically increased (≈1.5-fold) compared with controls, as shown in Fig. 4C, top, lane 2; the signal for Rho in the total cell lysates (TCL) was used for normalization of the activity of Rho GTPase. Furthermore, the...
activity of Rho GTPase was decreased in PTP-PEST-overexpressing cells (Fig. 4C, top lane 3). However, the activities of neither Rac nor Cdc42 were altered to any appreciable extent in either LPXN- or PTP-PEST-overexpressing cells (data not shown). It should be noted that other studies have reported that a low, persistent level of activity of Rac GTPase exists in PC-3 cells (37). Finally, when PC-3 cells were treated with the Rho GTPase inhibitor Y27632 (1\(\mu\)M) for 24 h after overexpression of either LPXN or PTP-PEST, cell migration was decreased to control levels only in LPXN-overexpressing cells (rAd-LPXN/Y27632), but there was no additional inhibitory effect on control (rAd-EGFP/Y27632) or PTP-PEST on the activity of Rho GTPase in the absence or presence of the Rho GTPase inhibitor Y27632 (1\(\mu\)M). PC-3 cell lysates were prepared for Rho GTPase activity assays, as determined by binding of active Rho to rhotekin-GST-agarose beads; the signal for Rho in the total cell lysates (TCL) was used for normalization of the activity of Rho GTPase. Data shown are representative of 3 separate experiments.

**DISCUSSION**

This study provides the first report of a role for LPXN in prostate cancer cells, unlike paxillin, for which more functional information exists (34, 66). We have provided evidence that LPXN forms a functional complex with at least two PTKs, Pyk2 and c-Src, in the androgen-independent prostate cancer cell line PC-3. In addition, LPXN is associated with the cytosolic protein tyrosine phosphatase PTP-PEST. The functional impact of a decrease in the levels of LPXN was manifested as decreased cell migration. We have also demonstrated that the overexpression of LPXN results in an increased association with Pyk2, Pyk2\(^{Y402}\) and c-Src, and that overexpression of PTP-PEST resulted in a decrease in LPXN-associated Pyk2 levels and dephosphorylation of Pyk2\(^{Y402}\). The overexpression of LPXN resulted in increased cell migration of PC-3 cells and increased activity of Rho GTPase. Treatment of PC-3 cells...
with Y27632, a Rho GTPase inhibitor, selectively decreased cell migration of LPXN-overexpressing cells. Finally, an overexpression of PTP-PEST also resulted in decreased PC-3 cell migration.

As a model of prostate cancer, we have chosen the PC-3 cell line, which is an androgen-independent prostate cancer derived from bone metastasis (57). This cell line also expresses functional integrin receptors, including αvβ3, that are activated by RGD-containing extracellular matrix proteins such as osteopontin (11, 38, 80). Previous reports have documented that androgen-independent PC-3 cells express the αvβ3 integrin, and the response to αvβ3 engagement by an extracellular matrix protein such as vitronectin led to increased tyrosine phosphorylation of paxillin-associated FAK (66). There are at least two other PC-3 sublines, such as PC-3-M, also androgen independent but highly metastatic (63), and PC-3-AR, that were transfected with a functional androgen receptor (21); future studies can be conducted on the latter PC-3 subline (PC-3-AR) to examine the possibility that LPXN can function as an androgen receptor coactivator, similar to paxillin and hic-5 (27, 34).

Although Pyk2 is expressed in normal epithelial prostate tissue, the expression of Pyk2 is apparently inversely correlated to the degree of prostate malignancy (46, 62). A decrease in the expression of Pyk2 was found to result in a MAP kinase-dependent decreased proliferation of prostate cells (71). Recent studies have suggested that loss of Pyk2 kinase (i.e., the K457A Pyk2 kinase-dead mutant) activity in an epithelial nontransformed prostate cell line correlated with increased cell motility and migration (14). We are unable to reconcile these data with our current study; however, we have observed that overexpression of K457A Pyk2 in PC-3 cells resulted in decreased cell migration (unpublished data, A. Gupta et al.). Therefore, more studies are needed to address and clarify this issue of the importance of Pyk2 in regulation of PC-3 cell migration.

The consequences of the interactions between LPXN, Pyk2, and PTP-PEST in prostate carcinoma cells are poorly understood. First, our data do not provide evidence whether LPXN serves as a direct substrate for PTP-PEST; we have been unable to demonstrate that LPXN is dephosphorylated following PTP-PEST overexpression in PC-3 cells, although it has been shown that LPXN can directly bind PTP-PEST (25, 72). Unlike paxillin, which contains several tyrosine phosphorylation sites and is a direct substrate for PTP-PEST (55, 56), LPXN contains only one putative tyrosine phosphorylation site, which is Y20, corresponding to Y31 in paxillin (7). Second, our data suggest that overexpression of PTP-PEST in PC-3 cells decreased the levels of LPXN-associated Pyk2 and Pyk2Y402, which correlated with a decreased association of LPXN with c-Src. Third, although we have demonstrated that LPXN can interact with PTP-PEST, the overexpression of PTP-PEST in PC-3 cells did not change the levels of LPXN-associated PTP-PEST; it is possible that the activity of LPXN-associated PTP-PEST is increased following its overexpression. This possibility could be examined by conducting in-gel phosphatase assays on LPXN immune complexes (3).

In PC-3 cells, members of the c-Src kinase family are known to regulate growth, cell proliferation, adhesion, and metastasis; several studies have demonstrated that inhibitors of both c-Src and FAK are effective in reducing PC-3 cell adhesion and migration (39, 43, 48, 60, 64, 79, 81). Our data do not provide evidence for a direct interaction of c-Src with LPXN; it is possible that, similar to osteoclasts, the interaction of c-Src with LPXN is indirectly through Pyk2 (51). We do not have evidence to show whether changes in the association of c-Src with LPXN following overexpression of either LPXN or PTP-PEST are reflected by changes in tyrosine phosphorylation of c-Src, as determined by immunoblotting the LPXN immunoprecipitates with an anti- phospho-Src (Src \({\text{Y}}^{140} \) ) antibody (3, 13). Our preliminary data suggest that changes in LPXN-associated Pyk2Y402 levels may determine its association with c-Src. Currently, we have been unable to determine whether LPXN-associated Pyk2 serves as a direct substrate for c-Src. An examination of changes in LPXN-associated Pyk2Y402 levels following activation of c-Src in PC-3 cells in response to specific growth factors could answer this question (12, 22, 42, 50).

Several studies have shown that Rho GTPases are highly expressed in PC-3 cells, and that activation of Rho occurs during cell migration (24, 37, 73, 77). In PC-3 cells, Rac, which is apparently present at higher levels than RhoA, was found to be specifically responsible for IGF-I-mediated directed motility; a reciprocal relationship was observed between activation of Rac and Cdc42 (77). The basal levels of phosphorylated FAK and paxillin in PC-3 cells were decreased in PC-3 cells expressing a dominant-negative Rac, leading to an apparent dissociation between FAK, paxillin, and PKL. Previous studies have also indicated increased RhoA expression in highly invasive sublines of PC-3 cells (30, 32, 49). In addition, the expression levels of neither Rac nor Cdc42 were changed in any of the invasion variant cell lines (30). Overexpression of dominant-negative RhoA N19 led to reduced invasion of PC-3 cells, similar to the effect of RhoA biochemical inhibitors (61). In our study, the Rho GTPase activity assays did not specifically distinguish between RhoA and Rac. Two sets of recent studies have suggested that increased RhoA activity is responsible for PC-3 cell invasion, which was further enhanced by a constitutively active form of RhoA (30, 32). Our current study demonstrates that Y27632, a Rho kinase inhibitor, selectively reduced migration only in LPXN-overexpressing cells, which adds credence to previous studies on the important role of Rho GTPases in PC-3 cell migration (30, 32, 77). Finally, in our studies, although we have observed that increased activation of Rho GTPase occurred following overexpression of LPXN, there were no changes in the activities of Rac or Cdc42, even in PTP-PEST-overexpressing cells. Although we should have expected a decreased activity of Rac activity in PTP-PEST-overexpressing cells (52), as mentioned earlier, a basal level of activation of these two GTPases has previously been observed in PC-3 cells (30, 77).

We have not identified the possible mechanism(s) whereby overexpression of LPXN or PTP-PEST impacts RhoA activity. GDIs regulate the activation of GTPases through cellular compartmentalization of GTPases (17). First, it is probable that, as a consequence of the increased association of c-Src and Pyk2 with LPXN, the ability of RhoGDI to sequester active RhoGTPase could be diminished, resulting in an increased RhoA activity (15). On the other hand, we speculate that, as a consequence of dephosphorylation of LPXN-associated c-Src and Pyk2, the ability of RhoGDI to inhibit RhoA activity would be enhanced, resulting in decreased RhoA activity (17).
Second, an additional mechanism whereby PTP-PEST can probably inhibit RhoA activity is through regulation of Vav2 via dephosphorylation (1, 31, 53). We hypothesize that overexpression of PTP-PEST in PC-3 cells may inhibit the activity of Vav2 via dephosphorylation, leading to decreased Rho GTPase activity (53). These possibilities could be examined in future studies.

The precise mechanism(s) by which overexpression of LPXN regulates PC-3 cell migration remains to be determined. At this point, we can speculate that the most likely mechanism is that LPXN binds directly to Pyk2 and/or Pyk2/c-Src; an increased recruitment of Pyk2 and/or Pyk2/c-Src following overexpression of LPXN would activate downstream cytoskeletal reorganization effectors such as RhoA, and, finally, dephosphorylation of LPXN-associated Pyk2 by PTP-PEST overexpression would result in decreased association of Pyk2 (and c-Src) with LPXN, thereby disrupting downstream activation of RhoA.

In conclusion, we provide preliminary data that demonstrate for the first time that LPXN is an additional focal adhesion-associated adaptor protein in PC-3 cells by virtue of its association with Pyk2, c-Src, and PTP-PEST. Future studies can expand further on the role of LPXN in prostate carcinoma cell function and its downstream effectors in the regulation of cell migration.

GRANTS
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