Effects of Overexpression of PTP36, a Putative Protein Tyrosine Phosphatase, on Cell Adhesion, Cell Growth, and Cytoskeletons in HeLa Cells*

(Received for publication, December 9, 1998, and in revised form, January 27, 1999)

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Non-receptor-type putative protein tyrosine phosphatase-36 (PTP36), also known as PTPD2/Pez, possesses a domain homologous to the N-terminal half of band 4.1 protein. To gain insight into the biological function of PTP36, we established a HeLa cell line, HtTA/P36-9, in which the overexpression of PTP36 was inducible. PTP36 expressed in HeLa cells was enriched in the cytoskeleton near the plasma membrane. There was little endogenous PTP36 detectable in uninduced HtTA/P36-9 cells or in the parental HeLa cells. Upon induction of PTP36 overexpression, HtTA/P36-9 cells spread less well, grew more slowly, and adhered to the extracellular matrix proteins less well than uninduced cells. Moreover, decreases in the actin stress fibers and the number of focal adhesions were observed. The tyrosine phosphorylation of the focal adhesion kinase induced by lysophosphatidic acid was suppressed in the HtTA/P36-9 cells overexpressing PTP36. These results indicate that PTP36 affects cytoskeletons, cell adhesion, and cell growth, thus suggesting that PTP36 is involved in their regulatory processes.

Among the diverse functions of protein tyrosine phosphatases (PTPs),1 their roles in the regulation of cell adhesion and motility are now emerging (1). It has been reported that CD45, a transmembrane PTP (2), and YopH, a bacterial PTP (3), negatively regulate the integrin-mediated cell adhesion or its signal transduction. PTPIB, a cytoplasmic PTP, has been reported to affect cadherin-actin linkage (4) and integrin-mediated signaling pathways (5).

Recently, we have cloned a murine putative PTP, PTP36 (6). A human homolog was also cloned and called PTPD2/Pez (7, 8). PTP36 is expressed in various tissues and cell lines including 3T3 fibroblasts. Besides a putative PTP domain, PTP36 has a band 4.1 homology domain that has been found in various cytoskeletal proteins. The band 4.1 homology domains are responsible for the targeting of such cytoskeletal proteins to the membrane-cytoskeleton interfaces. So far, five mammalian PTPs with the band 4.1 homology domain have been reported. These include PTPH1 (9), PTPMPEG (10), PTPD1/PTP-RL10/rPTP2E (7, 11, 12), PTP36/PTPD2/Pez (6–8), and PTP-BAS/ hPTP1E/PTPL1/FAP-1 (13–15).

The presence of the band 4.1 homology domain might imply the involvement of these PTPs in the regulation of cytoskeletons and cell adhesion. It was reported that, in COS-7 cells, overexpression of PTPMPEG inhibited cell proliferation, reduced the saturation density, and blocked cell growth in soft agar (16). Therefore, it is possible that PTPMPEG is involved in the signal transduction of the cell adhesion that regulates the cell growth. There is no evidence, however, that PTPMPEG has any effect on the cell adhesion signaling pathways.

To study the biological functions of PTP36, we established a HeLa cell line in which the overexpression of PTP36 was inducible. PTP36 expressed in HeLa cells was enriched in the cytoskeleton near the plasma membrane. When the overexpression of PTP36 was induced, we observed morphological change (17), reduced cell growth and cell adhesion, decreases in actin stress fibers and focal adhesions, and reduced phosphorylation of focal adhesion kinase (FAK) induced by lysophosphatidic acid (LPA). Our results suggest that PTP36 is involved in the regulation of cytoskeletons, cell adhesion, and cell growth.

EXPERIMENTAL PROCEDURES

Materials—Enzymes were purchased from Life Technologies, Inc., New England Biolabs (Beverly, MA), Promega (Madison, WI), and Stratagene (La Jolla, CA). Anti-actin (AC-40, mouse IgG2a), anti-vimentin (V9, mouse IgG1), anti-vin-culin (hVIN-1, mouse IgG1), phal-lodin-tetramethylrhodamine isothiocyanate (PITC, 151), doxycycline (D-9891), puromycin (P-7255), and lysophosphatidic acid (L-7260) were purchased from Sigma. Anti-FAK (66-367, rabbit polyclonal IgG), anti-a-tubulin (DM1A, mouse IgG1), and PY20 (mouse IgG2b) were from Upstate Biotechnology Inc. (Lake Placid, NY), Cedarlane Laboratories (Westbury, NY), and Transduction Laboratories (Lexington, KY), respectively. The rat anti-PT-P6 monoclonal antibody, 21-4, was established in our laboratory using a glutathione S-transferase fusion protein containing amino acids 399–739 of the PTP36 polypeptide (GST-399–739) as an antigen.

Construction of Expression Plasmids and Transfection—The expression construct pEP36HA, which encoded influenza virus hemagglutinin (HA)-tagged PTP36 (PTP36HA) was created as follows. The stop codon of the PTP36 cDNA was replaced by a sequence encoding a tandem repeat of the HA epitope. The cDNA encoding HA-tagged PTP36 was inserted into the ClaI/XbaI site of the mFas-Fc plasmid (18).
Alternatively, the cDNA was subcloned into pUHD10-3, which contained a tet operator sequence (19). The resulting plasmid, pTREP36HA, and pNYpgk-pc, a plasmid possessing a puromycin resistance gene, were introduced into HeLa cells expressing a tetracycline-controlled transactivator (HtTA) using TfX207™ reagent (Promega). Stable positive clones were selected in DMEM containing 10% fetal calf serum, 0.4 μg/ml puromycin, 0.1 mg/ml geneticin, and 5 ng/ml doxycycline.

Immunoblotting Analysis and Immunoprecipitation—After SDS-polyacrylamide gel electrophoresis, proteins were electroblotted onto polyvinylidene fluoride membranes. The membranes were blocked in T-TBS (25 mM Tris-HCl, pH 7.4, 137 mM NaCl, 5 mM KCl, 0.1% Tween 20) containing 2% casein for 1 h at room temperature or overnight at 4 °C and incubated with the primary antibodies. To detect the primary antibodies, horseradish peroxidase-conjugated sheep anti-mouse IgG, or protein A, and the ECL system (Amersham Pharmacia Biotech) were used. To detect tyrosine-phosphorylated proteins using PY20, the low salt buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 0.1% Tween 20) was used for incubation and washing, and 5% bovine serum albumin (BSA) was used for the blocking.

For immunoprecipitation, cells were lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 1 mM NaVO₄, 50 mM NaF, and 5 mM sodium pyrophosphate. The lysate was centrifuged at 15,000 × g for 10 min at 4 °C. The supernatant was precleared by the addition of 10 μl (pellet volume) of protein A-Sepharose (Amersham Pharmacia Biotech) followed by incubation at 4 °C for 30 min. Then, the lysate was incubated with 10 μl (pellet volume) of protein A-Sepharose preloaded with 2 μg of the rabbit anti-FAK antibody for 1 h.

Immunofluorescence Study—Immunofluorescence microscopy was performed as described (20). Briefly, cells on a 34-mm tissue culture dish were washed with the cytoskeleton buffer (100 mM MES, pH 6.5, 1 mM MgCl₂, 1 mM EGTA, and 4% polyethylene glycol 8000) and fixed with 3.7% formaldehyde in the cytoskeleton buffer for 10 min at room temperature. After permeabilization by 0.1% Triton X-100, cells were incubated with 0.1% Triton X-100, each well was filled with DMEM, and the plate was incubated at 37 °C for 1 h, followed by washing. The labeled cells (1.5 × 10⁶) in 100 μl of DMEM containing 5 μg/ml goat globulin. For the detection of the primary antibodies, 25 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG (catalog no. 115-095-068, Jackson ImmunoResearch) was used. The polymerized actin was visualized using 0.2 μg/ml phalloidin-tetramethylrhodamine isothiocyanate in PBS.

Cell Culture and Cell Adhesion Analysis—HtTA/PTP36-9 and HtTA/PTP36-18 cells were maintained in DMEM containing 10% fetal calf serum, 0.3 μg/ml puromycin, 0.1 mg/ml geneticin, and 5 ng/ml doxycycline. Overexpression of PTP36 was induced by incubating the cells in the culture medium without doxycycline. After 5 days, cells were collected by trypsinization and incubated in fresh culture medium overnight. Then cells were detached by incubating them in PBS containing 5 mM EDTA and 0.1% BSA. After washing, cells were used for the adhesion assay as described (21). Briefly, cells were labeled by incubation in serum-free DMEM containing 5 μg 5-carboxyfluorescein diacetoxymethyl ester (BCECF-AM) (catalog no. FM-0011-50, Dojin) at 37 °C for 1 h, followed by washing. The labeled cells (1.5 × 10⁵) in 100 μl of DMEM containing 0.1% BSA were incubated in 96-well plates precoated with various extracellular matrix proteins. After a 20-min incubation at 37 °C, each well was filled with DMEM, and the plate was centrifuged upside down at 100 × g for 10 s. Cells attached to the plate were lysed in PBS containing 1% Nonidet P-40, and the fluorescence intensity (530 nm) was measured (excitation wavelength at 490 nm). The wells of 96-well microtiter plates (catalog no. 25860, Corning Glass) were coated with various concentrations of extracellular matrix proteins in 50 μl of PBS at 37 °C for 1 h. The total protein concentrations were adjusted to 10 μg/ml by the addition of BSA. The plates were blocked with PBS containing 0.5% heat-treated (80 °C for 15 min) BSA at 37 °C for 1 h.

Cytoskeletal Fractionation—The cytoskeletal fractions were prepared as described (22). Cells were lysed in the cytoskeleton stabilizing buffer (10 mM Pipes, pH 6.8, 250 mM sucrose, 5 mM MgCl₂, 120 mM KCl, 1 mM EGTA) containing 0.15% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM NaVO₄, 25 mM NaF, and 5 mM sodium pyrophosphate for 5 min at 4 °C (total lysate). The total lysate was centrifuged at 14,000 × g for 10 min at 4 °C yielding the pellet, which contained the polymerized actin and the intermediate filaments, and the supernatant, which contained the depolymerized actin and tubulin.

RESULTS

Conditional Overexpression of PTP36 in HeLa Cells—to investigate the biochemical functions of PTP36, we established a cell line in which the expression level of PTP36 was regulatable. A HeLa cell line, HtTA, that expressed the tetracycline-controlled transactivator was transfected with an inducible construct containing the cDNA of PTP36 tagged with the HA epitope. Two cell lines, HtTA/PTP36-9 and HtTA/PTP36-18, were established, and similar results were obtained with them.

In the absence (lane 2) or cells transfected with a plasmid encoding PTP36 were lysed in the cytoskeleton stabilizing buffer at 4 °C and centrifuged at 14,000 × g yielding the pellet (P) and the supernatant (S). The equal cell equivalent of each fraction was analyzed. Actin-based microfilaments and intermediate filaments containing vimentin were recovered in the cytoskeletal P fraction, whereas actin monomers were recovered in the cytosolic S fractions. PTP36, actin, and vimentin in each fraction were detected by immunoblotting analysis.

![Fig. 1. Conditional overexpression and subcellular localization of PTP36 in HeLa cells.](image-url)
parental HtTA cells, PTP36 was undetectable in the presence or absence of doxycycline (data not shown).

Then the subcellular distribution of PTP36 in HeLa cells was analyzed. PTP36 was found in the cytoskeletal fraction (P) containing actin and vimentin (Fig. 1B, lane 1). The immunofluorescence study revealed that PTP36 and PTP36 fused to green fluorescent protein (PTP36-GFP) were enriched in the region near the cytoplasmic membrane (Fig. 2, a–c and e–g). Thus, we concluded that PTP36 in HeLa cells was enriched in the cytoskeleton near the plasma membrane.

Morphology and Growth of HtTA/P36-9 Cells Induced to Overexpress PTP36—HtTA/P36-9 cells and the parental HeLa cells (HtTA) were cultured in the presence (off, the noninducing condition) or absence (on, the inducing condition) of doxycycline. The morphology of the uninduced HtTA/P36-9 cells did not differ significantly from that of the parental HtTA cells (Fig. 3, upper panels). After 3 days of induction, however, HtTA/P36-9 cells became more rounded and spread less well than the uninduced cells (Fig. 3, lower panels). Similar results were obtained with HtTA/P36-18 cells (data not shown). No change in the morphology was detectable when the parental HtTA cells were cultured with or without doxycycline. In addition, HtTA/P36-9 cells (Fig. 4, A and B) and HtTA/P36-18 cells (data not shown) induced to express PTP36 grew slower than the uninduced cells. The growth inhibition was mild but very reproducible. In six independent experiments, the cell number was reduced to 66.3 ± 7.3% (50.5–72.9%) of that of control (uninduced) cells. No difference in the viability was observed using the dye exclusion method. Doxycycline revealed no effect on the growth of the parental HtTA cells (Fig. 4A).

Effects of PTP36 Overexpression on the Adhesion of HtTA/P36-9 Cells to Extracellular Matrix Proteins—The HtTA/P36-9 cells induced to overexpress PTP36 seemed to attach loosely to the culture dish. Therefore, we investigated the adhesiveness of HtTA/P36-9 cells to various extracellular matrix proteins. A representative result from four independent experiments is shown in Fig. 5. The HtTA/P36-9 cells induced to overexpress PTP36 adhered to type IV collagen (Fig. 5A) and laminin (Fig. 5B) less well than uninduced cells. No difference was observed when the parental HtTA cells were cultured in the presence or absence of doxycycline. As shown in Fig. 5C, the adhesion of HtTA/P36-9 cells to these matrix proteins was blocked almost completely by an anti-β1 integrin antibody, SG/19 (23). The expression levels of β1 integrin subunits on the surface of the induced and uninduced HtTA/P36-9 cells were almost identical (Fig. 5D).

Cytoskeletons and Focal Adhesions in HtTA/P36-9 Cells Induced to Overexpress PTP36—The cytoskeletons could have profound effects on the cell shape and the cell adhesion. Thus, we examined the cytoskeletons in the HtTA/P36-9 cells induced (on) or not induced (off) to overexpress PTP36. There was no difference found in the microtubules and the intermediate filaments containing vimentin (Fig. 6, i–l). In contrast, the actin stress fibers were greatly decreased in the induced HtTA/P36-9 cells (Fig. 6b). Furthermore, the number of focal adhesions was reduced (Fig. 6f). In the parental HtTA cells, doxycycline revealed little effect on the actin stress fibers and focal adhesions (Fig. 6, c, d, g, and h).

Effects of PTP36 Overexpression on the Phosphorylation of FAK—Several sources of stimulation are known to induce the formation of focal adhesions or actin stress fibers. The integrin-mediated adhesion of cells to extracellular matrix proteins induces the formation of focal adhesions and the phosphorylation of various proteins including FAK. It is also known that LPA activates the small GTPase, Rho, to trigger the formation of focal adhesions and actin stress fibers as well as the phosphorylation of FAK. Defects in these signal transduction cascades might result in the reduction of actin stress fibers and focal adhesions. Thus, we investigated the phosphorylation of FAK induced by the integrin engagement or LPA. Induction of PTP36 overexpression had little effect on the tyrosine phosphorylation of FAK induced by cell adhesion to fibronectin (Fig. 7A, lanes 2 and 4). On the contrary, the LPA-induced phosphorylation of FAK was suppressed in the cells induced to overexpress PTP36 (Fig. 7B, lanes 2 and 4).

DISCUSSION

The presence of a band 4.1 domain in the band 4.1 superfamily PTPs may suggest their involvement in the regulation of
Procedures. The data shown are the mean values concentrations of type IV collagen. After a 20-min incubation at 37 °C, cells attached to the plate were measured as described under “Experimental Procedures.” The data shown are the mean values ± S.E.

B, adhesion to laminin was measured as described in A. C, effects of anti-β1 integrin antibody (Ab) on the adhesion. During the assay of adhesion to type IV collagen (0.8 μg/ml-coated wells) or laminin (0.63 μg/ml-coated wells), cells were incubated with no antibody, 4 μg/ml mouse IgG, or an anti-β1 integrin antibody (SG/19). D, immunofluorescence staining of β1 integrin subunits. The HtTA/P36-9 cells cultured in the presence (off) or absence (on) of doxycycline were stained with SG/19 followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG and analyzed by fluorescence-activated cell sorter. The sample stained without the primary antibody was shown as a negative control (c).

Effects of PTP36 Overexpression in HeLa Cells

The cytoskeletons and focal adhesions in HtTA/P36-9 cells induced to overexpress PTP36 are shown (right panel) or absence (on) of doxycycline. The expression level of PTP36 in the induced HtTA/P36-9 cells was only 2–5-fold higher than the level of endogenous PTP36 in the 3T3 cells. In addition, no increase in the total phosphatase activity was detectable (data not shown).

These results might support the possibility that the effects of PTP36 observed are not artifacts of overexpression.

In HeLa cells, PTP36 is enriched in the membrane-associated cytoskeletal fraction. This distribution pattern is similar to that of PTPMEG, which localizes primarily to the membrane and cytoskeletal fractions (24).

Although we have found various effects of overexpression, this does not necessarily mean that multiple target molecules of PTP36 are present. Focal adhesions are the sites at which cells adhere to the extracellular matrix via integrin family receptors. At their cytoplasmic face, focal adhesions are linked to actin stress fibers. In addition, various signal transducing molecules have been identified in the focal adhesions (25, 26). Because focal adhesions are involved in both cell adhesion and signal transductions, their changes might affect cell morphology, adhesiveness to extracellular matrix proteins, and cell growth. The target molecule of PTP36 remains to be determined.

Stimulation through integrins, growth factor receptors, and G protein-coupled receptors leads to the formation of focal adhesions, which are the sites of cell adhesion to extracellular matrix proteins.
adhesions and stress fibers (25–27). The tyrosine phosphorylation of proteins including FAK seems to be the essential step for these responses (25, 26, 28–31). The overexpression of PTP36 inhibits the phosphorylation of FAK induced by LPA. LPA stimulates a G protein-coupled receptor and induces the formation of focal adhesions and stress fibers through the activation of Rho, a small GTPase (32). Thus, it is possible that the target molecule of PTP36 might be in the signaling cascade involving Rho. On the contrary, the overexpression of PTP36 reveals no inhibitory effect on the phosphorylation of FAK induced by the cell adhesion to fibronectin. It was reported that the early, but not late, phosphorylation of FAK induced by integrin engagement occurred independently of Rho family GTPases (31). This result suggests that PTP36 might not inhibit the early integrin signals leading to the FAK phosphorylation. Clearly, further study is necessary to elucidate the mechanism of the inhibition.

So far, there is no evidence that PTP36 plays the role of a PTP in HeLa cells. Among the band 4.1 superfamily PTPs, PTP36/PTPDP2/Pez (6–8) and PTP1/D/PTP-RL10/rPTP2E (7, 11, 12) share a high sequence similarity. The amino acid sequence in the PTP domain of PTP1/D/PTP-RL10/rPTP2E has a substitution of glutamic acid for the conserved aspartic acid. This aspartic acid acts as a general acid in the catalytic reaction (33). This substitution was reported to reduce the $k_{cat}$ of a PTP about 600-fold (34). There is a substitution of histidine for the invariant glutamic acid in murine PTP36 but not in human PTPD2/Pez. To test the enzymatic activity, PTP domains of PTP36 and PTPD2/Pez were expressed as glutathione S-transferase fusion proteins, and the catalytic activities were measured using p-nitrophenyl phosphate as a substrate. No significant, or very marginal, activity was detected for PTP36 and PTPD2/Pez, respectively.2 These observations raise the possibility that some members of band 4.1 superfamily PTP, including PTP36, might have noncatalytic functions. In line with this notion, it was reported that the catalytic activity of PTPMEG, a member of band 4.1 superfamily PTP, was not essential for the function of PTPMEG (10, 16, 24).

Nonetheless, the PTP domain might be important for the function of PTP36. A deletion mutant of PTP36 (lacking amino acids 803–1184) was expressed in HeLa cells. This mutant possessed the band 4.1 domain but not the phosphatase domain and revealed little inhibitory effect on the adhesion of the cells to laminin.2 It was reported that the mutation of the invariant aspartic acid converted PTP into a catalytically inactive form, which could make a stable complex with tyrosine-phosphorylated substrates (34). It is possible that PTP36 might interact with signaling molecules through its multiple domains, including PTP and the band 4.1 domains, and function as an adaptor molecule.

Acknowledgment—We thank Drs. H. Bujard and S. Takeda for kindly providing pUHD10-3 plasmid and for helpful information.

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