Regulation of Phosphorylation Level and Distribution of PTP36, a Putative Protein Tyrosine Phosphatase, by Cell-Substrate Adhesion*

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Recently we have cloned a putative protein tyrosine phosphatase, PTP36/PTPD2/pez, which possesses a domain homologous to the N-terminal half of band 4.1 protein. In mouse fibroblasts adhered to substrates, PTP36 was phosphorylated on serine residues. PTP36 was found to make complexes with serine/threonine kinase(s), which phosphorylated PTP36 in vitro. PTP36 was dephosphorylated rapidly when the cell-substrate adhesion was disrupted and it was phosphorylated again along with the reattachment of the cells to fibronectin. Dephosphorylation of PTP36 seemed to depend on actin polymerization since it was inhibited by cytochalasin D. The cell detachment also induced the translocation of PTP36 into the membrane-associated cytoskeletal fraction. Staurosporine and ML-9, which inhibited the phosphorylation of PTP36 in vitro, induced the translocation of PTP36 too. On the contrary, when the dephosphorylation of PTP36 was inhibited by okadaic acid, no translocation of PTP36 was induced by the cell detachment. These results demonstrate that the cell-substrate adhesion and cell spreading regulates the intracellular localization of PTP36 most likely through its phosphorylation and therefore, PTP36 may play important roles in the signal transduction pathway of cell-adhesion.

The family of protein tyrosine phosphatase (PTP)† has been growing rapidly and it is speculated that there are about 500 human PTPs. Among the diverse functions of PTP, lines of evidence are now emerging which suggest roles of PTP in the regulation and/or signal transduction of cell adhesion (2). For example, reduction of the cell-substrate adhesion activates PTPs in the chicken embryo fibroblast (4). It is also reported that PTP1B, a cytoplasmic PTP, affects the cadherin-actin linkage (5) and the integrin-mediated signaling pathways (6). YopH, a bacterial PTP (7), induces the dephosphorylation of FAK and p130Cas, and disrupts the focal adhesions.

Recently, we have cloned a murine putative PTP, PTP36 (8). A human homologue, PTPD2/pez, was also reported (9, 10). PTP36 is expressed in various tissues and cell lines including fibroblasts. PTP36 possesses a domain homologous to the N-terminal half of band 4.1 protein. Several cytoskeletal proteins and PTPs have this band 4.1 homology domain and constitute the band 4.1 superfamily (11). So far, five mammalian PTPs belong to the band 4.1 superfamily and they fall into three subgroups based on their structure. The first subgroup is composed of PTPH1 (12) and PTPMEG (13). The members of the second subgroup are PTPD1/PTP-KL10/PTP2E (9, 14, 15) and PTP36/PTPD2/pez (8–10). The third subgroup has so far only one member, PTP-BAS/hPTP1E/PTPL1/FAP-1 (16–18). PTPs of the first and the second subgroup have one N-terminal band 4.1 homology domain and one C-terminal PTP domain separated by an intervening sequence. The intervening sequence of the first subgroup has a PDZ domain and a potential SH3-binding site. Although there is poor homology, the intervening sequence of the second subgroup also has a potential SH3 or WW binding motif. Unlike these PTPs, PTP-BAS/hPTP1E/PTPL1/FAP-1, a PTP of the third subgroup, has a band 4.1 homology domain in the middle of the molecule. It has five PDZ domains and interacts with a GTPase-activating protein for Rho (PARG1) (19) and the C terminus of human Fas (CD95) (20, 21) through its PDZ domains.

Conservation of the band 4.1 superfamily PTP in Caenorhabditis elegans but not in yeast might suggest its fundamental roles in the multicellular organism. However, the biological roles of the band 4.1 superfamily PTPs are largely unknown. Recently we have found that the overexpression of PTP36 in the HeLa cells induces changes in cytoskeletons, focal adhesions, cell-substrate adhesions, and cell growth (22). It is also reported that COS-7 cells overexpressing PTPMEG grow slower, reach confluence at a lower cell density, and make much fewer colonies in soft agar (23, 24). These results suggest that the band 4.1 superfamily PTP might have a close relationship to the signal transduction of cell adhesion. However, it was not known if cell adhesion had any effect on the band 4.1 superfamily PTP.

Here we report the regulation of PTP36 by cell-substrate adhesion. Cell-substrate adhesion regulates the intracellular localization of PTP36 most likely through its phosphorylation. Therefore, PTP36 could play an important role in the signal transduction pathways of cell-substrate adhesion.
Regulation of PTP36 by Cell-Substrate Adhesion

FIG. 1. In vivo phosphorylation of PTP36 on serine residues. A, in vivo $^{32}$P labeling. 3T3/P36-8 fibroblasts overexpressing PTP36 were labeled with $[^{32}]$Porthophosphate for 3 h. PTP36 was immunoprecipitated with control rat IgG (lane 1) or anti-PTP36 mAb (21-4) (lane 2) and resolved by 8% SDS-PAGE under reducing conditions. The phosphoproteins were detected by autoradiography. Positions of the molecular weight markers are indicated on the right. B, phosphoamino acid analysis of PTP36. The phosphorylated protein of 130 kDa was hydrolyzed and separated in two dimensions on a TLC plate. Circles and open arrowheads show the positions of phosphoserine, phosphothreonine, and phosphotyrosine, respectively. + indicates the sample origin. C, dephosphorylation of PTP36 in vitro. 3T3/P36-8 cells were lysed in RIPA buffer and the phosphorylated PTP36 was immunoprecipitated with 21-4 mAb (lanes 2–4). Aliquots of the sample were incubated with (lane 4) or without (lane 3) calf intestinal alkaline phosphatase (CIAP) and the rest served as a non-incubation control (lane 2). Then, PTP36 was detected by the immunoblotting using 21-4 mAb. The closed and open arrowheads show the positions of phosphorylated and dephosphorylated PTP36, respectively. D, in vitro dephosphorylation by the phosphatase inhibitors. PTP36 was left untreated (lane 1) or dephosphorylated by CIAP as described above in the absence (lane 2) or presence of 5 mM sodium pyrophosphate (lane 3) or sodium pyrophosphate and 25 mM sodium fluoride (lane 4).

EXPERIMENTAL PROCEDURES

Materials—Female Lewis rats (6–7 weeks of age) were obtained from Shizuoka Experimental Animal Laboratory (Hamamatsu, Japan). Enzymes were purchased from Life Technologies, Inc. (Gaithersburg, MD), New England Biolabs (Beverly, MA), Fromega (Madison, WI), and Stratagene (La Jolla, CA). Stauroporine, calphostin C, cytochalasin D, and colchicine were purchased from Sigma, and KN-62, H-89, W-7, ML-9, and ML-7 were purchased from Seikagaku Co. Ltd. (Tokyo, Japan). Purified myosin light chain kinase (MLCK), calmodulin, and a substrate peptide were kind gifts from Dr. Nakanishi, Kyowa Hakko Kogyo Co., Ltd. Anti-actin (AC-40; mouse IgG2a) and anti-vimentin (V9; mouse IgG1) were purchased from Sigma. Anti-MLCK, anti-c-Src (SRC2; rabbit), and anti-caveolin (C13630; rabbit) were from Cedar Lane (Westbury, NY), Santa Cruz Biotechnology (Santa Cruz, CA), and Transduction Laboratories (Lexington, KY), respectively. Radioclonucleotides were purchased from NEN Life Science Products Inc. and Amersham Pharmacia Biotech.

Generation of Anti-PTP36 Monoclonal Antibody (mAb), 21-4—The immunizing antigen was a glutathione S-transferase fusion protein containing amino acids (aa) 399–738 of PTP36 polypeptide (GST-P36/399–738). The fusion protein was expressed and purified as described (25). To detect anti-PTP36 antibody, a fusion protein of maltose-binding protein and aa 399–738 of PTP36 (MBP-P36/399–738) was used. Female adult Lewis rats were immunized with GST-P36/399–738 for several times. Three days after the last immunization, spleen cells were fused with SP2/0 mouse myeloma cells. The supernatant from each well was tested for the reactivity with both GST-P36/399–738 and MBP-P36/399–738. The fusion protein was expressed and purified as described (26). pEFPP36FC and pNYppk-pc, a plasmid possessing a puromycin resistance gene, were linearized with ScaI and co-transformed into mouse 3T3 fibroblast by the calcium phosphate coprecipitation method. After 2 days, cells were selected in DME containing 10% fetal calf serum and 1.5 $\mu$g/ml puromycin. To test the effects of various inhibitors, cells (1 $\times$ 10$^5$ in 1 ml) were seeded in the 24-well tissue culture plate. The next day, cells were incubated with various inhibitors in DME containing 5% fetal calf serum and lysed directly on the wells by 50 $\mu$l of SDS-PAGE sample buffer. For the cell-detachment experiments, cells were washed with phosphate-buffered saline (PBS) containing 5 mM EDTA and detached from the tissue culture dish by scraping. Alternatively, cells were detached by the incubation with trypsin (2.5 mg/ml) in PBS for no longer than 10 min. For the reattachment experiments, the trypsined cells were washed by PBS containing 0.1% bovine serum albumin (BSA) and resuspended in DME containing 0.1% BSA. Then the cells were incubated in the precoated 24-well tissue culture plate at 37 °C for 20 min. Floating cells were recovered by centrifugation, lysed, and mixed with the lystate from the attached cells. Each well of the plate was coated by incubation with 150 $\mu$l of fibronectin (10 $\mu$g/ml) or heat-treated (80°C for 20 min) BSA (5 mg/ml) in PBS for 1 h at room temperature.

Phosphoamino acid analysis and phosphopeptide analysis were performed as described (27). The hydrolyzed phosphoamino acid or the phosphopeptides digested by TPCK-trypsin (Worthington, NJ) was resolved in two dimensions on the TLC plates (catalog number 5716, Merck, Darmstadt) using the Multipher II horizontal electrophoresis system (Amersham Pharmacia Biotech).

Dephosphorylation of PTP36 in vitro—The phosphorylated PTP36 was immunoprecipitated with 21-4 mAb from 3T3/P36-8 cells. The dephosphorylation of PTP36 in vitro was done as described (28). Briefly, the precipitate was treated with or without 20 units of calf intestinal alkaline phosphatase (New England Biolabs Inc.) in 20 $\mu$l of 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% deoxycholate, 0.1% SDS) containing 1 mM methylamylsulfonic acid (PMSF), 5 mM EDTA, 1 mM Na$_2$VO$_4$, 50 mM sodium fluoride, and 5 mM sodium pyrophosphate. The lystate was centrifuged at 15,000 $\times$ g for 15 min at 4 °C and the supernatant was used for the immunoprecipitation assay (22).
mm sodium pyrophosphate. After PTP36 was immunoprecipitated with 21-4 followed by MAR 18.5, the sample was washed once with the kinase buffer (25 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, 1 mM CaCl₂). Before the kinase reaction, kinase buffer was added to 12.5 µl and the reaction was initiated by the addition of 1 µl of 62.5 mM [γ−32P]ATP (5 µCi). One-fifth of the precipitated sample was used for a reaction. The reaction was terminated after incubation at 25 °C for 30 min. In the preliminary experiment, the phosphorylation level of PTP36 increased linearly up to 30 min. For the reprecipitation of 32P-labeled PTP36, the phosphorylated samples were incubated in 50 µl of 0.5% SDS, 50 mM Tris-HCl, pH 8.0, at 95 °C for 3 min, diluted with 350 µl of RIPA buffer and used for the immunoprecipitation with 21-4 mAb.

To test the possibility that MLCK could phosphorylate PTP36 directly, PTP36 was immunoprecipitated from the cell lysate prepared with RIPA buffer and used as a substrate. The immunoprecipitated PTP36 was incubated in 25 µl of the kinase buffer containing 2.8 µM MLCK, 10 nM calmodulin, 100 µM [γ−32P]ATP at 25 °C for 30 min. A weak phosphorylation of PTP36 was detectable without MLCK probably because a small amount of kinase(s) was coprecipitated even in the RIPA buffer. However, there was no increase in the phosphorylation level of PTP36 whereas 22% (145060 cpm) of the total count was incorporated into the MLCK peptide (KRKPKTNSVNY, Peninsula Laboratories, used at 24 µM) in the same assay condition.

In Gel Kinase Assay—Protein kinases were detected in SDS-PAGE gels by a modification of the method of Ferrell and Martin (37). The immunoprecipitates containing kinases were denatured and separated by SDS-PAGE. Following electrophoresis, SDS was removed by soaking the gels in 50 mM Tris-HCl, pH 7.5, 0.1% (w/v) Nonidet P-40, 3 mM dithiothreitol for 1 h at room temperature. For the denaturation, gels were soaked in 50 mM Tris-HCl, pH 8.3, 7 mM guanidine-HCl, 3 mM dithiothreitol, 2 mM EDTA for 1 h at room temperature. The proteins were then allowed to reature by soaking the gel in 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1% (w/v) BSA, 0.1% Nonidet P-40 overnight at 4 °C. In situ phosphorylation was performed by soaking the gel in 50 ml of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 400 µCi of [γ−32P]ATP (3000 Ci/mmol) for 3 h at room temperature. Unincorporated ATP was removed by washing overnight at room temperature in 50 mM Tris-HCl, pH 7.5, containing 20 g of Dowex 2 × 8-50 anion exchange resin, changing the buffer three to four times.

Subcellular Fractionation—3T3/P36-8 cells were fractionated as described (29) with some modifications. Cells were suspended in the hypotonic buffer A containing 10 mM Tris-HCl, pH 7.4, 0.2 mM MgCl₂, 5 mM KCl, 1 mM Na₃VO₄, 5 mM sodium pyrophosphate, 1 mM EDTA, and 1 mM PMSF, and homogenized using Dounce tissue homogenizer until over 90% cells were disrupted, then sucrose was added to 250 mM. The homogenate was centrifuged at 1,000 × g for 5 min at 4 °C yielding the crude nuclear pellet (P1) and the supernatant 1 (S1). S1 was centrifuged at 12,500 × g for 15 min at 4 °C yielding the crude microsomal pellet (P2) and the supernatant 2 (S2). S2 was centrifuged at 100,000 × g for 45 min at 4 °C yielding the crude mitochondrial pellet (P3) and the cytosolic fraction (S3). P2 and P3 were lysed in buffer A containing 250 mM sucrose and 1% Triton X-100, and separated by centrifugation at 100,000 × g for 45 min at 4 °C yielding the pellet (P) and the supernatant (S).

Cytoskeletal fractionation was performed as described (30) with minor modifications. Cells were washed once with PBS and lysed in cytoskeleton stabilizing buffer (CSK-buffer: 10 mM PIPES, pH 6.8, 250 mM sucrose, 3 mM MgCl₂, 120 mM KCl, 1 mM EDTA) containing 0.15% Triton X-100, 1 mM PMSF, 1 mM Na₃VO₄, 25 mM sodium fluoride, 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 and the supernatant. Triton X-100-insoluble microdomains of cellular membranes fraction were separated as described (31).

RESULTS

PTP36 is a 130-kDa Protein Phosphorylated on Serine Residues—We established a stable transfectant of mouse 3T3 fibroblast, 3T3/P36-8, which expressed the PTP36 protein about 5-fold above the endogenous level. 3T3/P36-8 cells were metabolically labeled with [32P]orthophosphate, lysed in the RIPA

FIG. 2. Regulation of the phosphorylation level of PTP36 by cell attachment. A, cell detachment by scraping. 3T3/P36-8 cells were detached by scraping in PBS containing 5 mM EDTA and incubated at 37 °C for the periods indicated. B, cell detachment by trypsinization. 3T3/P36-8 cells were labeled in vivo with [32P]orthophosphate and left untreated (lane 1) or detached by trypsin and incubated at 37 °C for 30 min (lane 2). PTP36 was immunoprecipitated with 21-4, separated by SDS-PAGE, and visualized by autoradiography. C, rephosphorylation of PTP36. 3T3/P36-8 cells were either left untreated (lane 1) or detached by trypsin, washed, and incubated in suspension at 37 °C for 20 min (lane 2). Then, cells in DMEM containing 0.1% BSA were incubated in the wells precoated with either 10 µg/ml fibronectin (lanes 3 and 5) or 1 mg/ml poly-l-lysine (lanes 4 and 6) for 20 min with 0.5% SDS, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 2 mM dithiothreitol, 2 mM EDTA, 1% (w/v) BSA, 0.1% Nonidet P-40 overnight at 4 °C. In situ phosphorylation was performed by soaking the gel in 50 ml of 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 400 µCi of [γ−32P]ATP (3000 Ci/mmol) for 3 h at room temperature. Unincorporated ATP was removed by washing overnight at room temperature in 50 mM Tris-HCl, pH 7.5, containing 20 g of Dowex 2 × 8-50 anion exchange resin, changing the buffer three to four times.

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PTP36 was immunoprecipitated from cell lysates, dephosphorylated with the alkaline phosphatase 

in vitro, and analyzed by immunoblotting. The dephosphorylated PTP36 migrated faster (Fig. 1, C, lanes 2 and 3 and D, lane 1). The change in migration was not observed in the presence of phosphatase inhibitors (Fig. 1D, lanes 3 and 4).

Regulation of the Phosphorylation Level of PTP36 Through Cell-Substrate Adhesion—When the cell-substrate adhesion of 3T3/P36-8 cells was disrupted by scraping, the less phosphorylated and faster migrating form of PTP36 was detectable within 5 min (Fig. 2A). This dephosphorylation was completely blocked by preincubating the cells with okadaic acid, an inhibitor of serine/threonine phosphatases (Fig. 2E, lane 4). Similar results were obtained using wild type 3T3 cells (Fig. 2F). Cell detachment by trypsinization also induced dephosphorylation (Fig. 2B, lane 2). The dephosphorylation was reversible since PTP36 was phosphorylated again along with the reattachment of the cells to the fibronectin-coated wells (Fig. 2C, lanes 3 and 5). The rephosphorylation was induced by the cell spreading on the poly-L-lysine-coated wells but was much slower (Fig. 2C, lane 4 and 5). Neither cell spreading nor rephosphorylation was induced when cells were incubated in the BSA-coated wells (Fig. 2D, lane 3). The phosphorylation was inhibited by cytochalasin D, an inhibitor of actin polymerization (Fig. 2D, lane 5), but not by colchicine, a microtubule-disrupting agent (Fig. 2D, lane 6).

Association of PTP36 with Serine/Threonine Kinases, Which Phosphorylate PTP36—To study the kinases involved in phosphorylation of PTP36, we first examined whether PTP36 makes complexes with serine/threonine kinases. 3T3/P36-8 cells were lysed in a buffer containing 1% Triton X-100. Then, PTP36 was immunoprecipitated with an anti-PTP36 mAb (21-4). The immune complex kinase assay was performed and as shown in Fig. 3A, PTP36 was phosphorylated in vitro by the coprecipitated kinase(s). To confirm that the major 32P-labeled 130-kDa band contained PTP36, PTP36 in the sample was reprecipitated after removing associated molecules by denaturation with SDS (Fig. 3A, lane 4).

To visualize the coprecipitated kinases, in gel kinase assay was performed. The immunoprecipitates were separated by SDS-PAGE, renatured in gel, and then kinases were detected by 32P incorporation. Two kinase bands, 160 and 88 kDa in size, were observed (Fig. 3B, lane 2) and the phosphoamino acid analysis showed that serine residues were phosphorylated in these bands (data not shown).

Phosphorylation sites of PTP36 in vivo and in vitro were compared by the phosphotryptic peptide mapping. Although several spots in vitro were phosphorylated weakly (Fig. 4B, arrowheads), almost all the spots in vivo (Fig. 4A) were detectable in vitro after a prolonged exposure (Fig. 4D).

The Effects of Various Inhibitors on the Phosphorylation of PTP36 in Vitro and in Vivo—To characterize the kinase(s) further, various inhibitors were added to immune complex kinase reactions. Staurosporine, a broad spectrum inhibitor of protein kinases, was found to inhibit phosphorylation of PTP36 in vitro (Fig. 5, A, lane 2, and C). Other inhibitors, including ML-9 (MLCK inhibitor), calphostin C (protein kinase C inhibitor), KN-62 (calcium calmodulin-dependent kinase II inhibitor), H-89 (protein kinase A inhibitor), genistein (tyrosine kinase inhibitor), and W-7 (calmodulin inhibitor), revealed little effect (Fig. 5A).

In vivo, staurosporine also suppressed phosphorylation of PTP36 when it was added to 3T3/P36-8 cells (Fig. 5, B, lane 2, and C). Unexpectedly, ML-9, which did not inhibit the phosphorylation in vitro, suppressed phosphorylation in vivo (Fig. 5B, lanes 8). ML-7 (50 μM), another inhibitor of MLCK, also inhibited the phosphorylation (data not shown). Furthermore, W-7, a calmodulin inhibitor, revealed the inhibitory effect (Fig. 5B, lane 6). This makes sense since MLCK is a Ca2⁺-calmodulin-dependent kinase. However, no direct phosphorylation of PTP36 in vitro was detectable when purified MLCK and calmodulin were added to immunoprecipitated PTP36 as described under “Experimental Procedures.” Thus, MLCK might regulate the phosphorylation level of PTP36 indirectly. It should be noted that a relatively high amount of ML-9 (100 μM) is required to completely inhibit the phosphorylation of PTP36.

 Redistribution of PTP36 Induced by Cell Detachment—To study the effects of the phosphorylation of PTP36, we compared the subcellular distribution of the phosphorylated and dephosphorylated PTP36 in attached and detached 3T3/P36-8 cells. We first performed the subcellular fractionation experiments. 3T3/P36-8 cells grown on the tissue culture dish were either left untreated or detached by scraping and incubated in suspension (Fig. 6A). The membrane fractionation was done as described under “Experimental Procedures.” Cell homogenate was centrifuged at 1,000 × g and separated in the pellet (P1; crude nuclear fraction) and the post-nuclear supernatant (S1). S1 was further separated by the centrifugation at 12,500 × g followed by centrifugation at 100,000 × g yielding the pellets (P2 and P3) and the final supernatant (S3). The phosphorylated PTP36 in the attached cells was found almost exclusively in the S3 cytosolic fraction (Fig. 6A, lane 5) while the dephos-
phorylated PTP36 in the detached cells was relatively enriched in the P3 membrane fraction (Fig. 6A, lane 9). The dephosphorylated PTP36 in the P2 and P3 fractions was resistant to solubilization by Triton X-100 (Fig. 6A, lanes 11 and 13). This suggested the association of the dephosphorylated PTP36 with cytoskeletons. To confirm the association, the cytoskeletal fractionation was performed. Cells were lysed in the cytoskeleton stabilizing buffer (CSK buffer) containing Triton X-100 and separated by centrifugation. In this separation condition, the actin-based microfilaments and the intermediate filaments were stabilized and recovered in the 14,000 g pellet, whereas the microtubules were destabilized and recovered in the supernatant (Fig. 6B). Actin monomers were recovered in the supernatant fraction too. As expected, the phosphorylated PTP36 was found in the cytosolic supernatant fraction (Fig. 6B, lane 3) whereas the dephosphorylated

![Phosphopeptide analysis of PTP36 phosphorylated in vivo and in vitro.](image)

**FIG. 5.** The effects of various inhibitors on the phosphorylation level of PTP36 in vitro and in vivo. A, inhibition of in vitro phosphorylation. Various inhibitors were added to immune complex kinase reactions. Lane 1, none; lane 2, 50 nM staurosporine; lane 3, 100 μM ML-9; lane 4, 1 μM calphostin C; lane 5, 10 μM KN-62; lane 6, 1 μM H-89; lane 7, 100 μM genistein; lane 8, 50 μM W-7. After the kinase reactions were performed at 25 °C for 30 min, samples were separated by SDS-PAGE and visualized by autoradiography. B, inhibition of in vivo phosphorylation. Intact 3T3/P36-8 cells were incubated with various inhibitors at 37 °C for 30 (lane 2) or 60 min (lanes 3–6 and 8). Lanes 1 and 7, none; lane 2, 50 nM staurosporine; lane 3, 1 μM calphostin C; lane 4, 50 μM ML-9; lane 5, 1 μM H-89; lane 6, 50 μM W-7; lane 8, 100 μM ML-9. Then, PTP36 was detected by the immunoblotting using 21-4. The closed and open arrowheads show the phosphorylated and dephosphorylated PTP36, respectively. C, dose-dependent effects of staurosporine. Phosphorylation levels in vitro (open circle) were measured using an imaging plate scanner BAS 2000. The relative phosphorylation levels are shown as (intensity of the band with inhibitors)/intensity of the band without inhibitors. The phosphorylation levels in vivo (closed circle) were measured by immunoblotting and shown as (intensity of the slower migrating band)/intensity of the slower + faster migrating bands.)
PTP36 was enriched in the pellet fraction containing the polymerized actin and vimentin (Fig. 6B, lane 5). Collectively, we conclude that cell detachment induces dephosphorylation and translocation of PTP36 from cytosol to the membrane-associated cytoskeletons. The translocation of the endogenous PTP36 was confirmed in the wild type (untransfected) 3T3 cells (Fig. 6F).

The translocation of PTP36 was further confirmed by the immunofluorescence microscopy experiments. Unfortunately, 21-4 mAb failed to detect PTP36, probably because the epitope was masked in the cells. Thus, the localization of the HA-epitope-tagged PTP36 (PTP36-HA) and the PTP36 fused to green fluorescent protein (PTP36-GFP) (22) was studied. As expected, PTP36-HA and PTP36-GFP distributed homogeneously in the cytoplasm in the attached cells (Fig. 7, b and d), while they were enriched in the region near the cytoplasmic membrane in the detached cells (Fig. 7, a and c).

After centrifugation at 14,000 × g, a substantial amount of dephosphorylated PTP36 was found in the supernatant fraction (Fig. 6B, lane 6). However, most of PTP36 in the supernatant fraction was insoluble in Triton X-100 and was precipitated by centrifugation at 100,000 × g (data not shown). This might suggest that some of the dephosphorylated PTP36 translocated to the microdomains of cellular membranes that are insoluble in Triton X-100 (32). However, PTP36 was not coprecipitated with caveolin, a marker protein for the microdomain (Fig. 6C).

Effects of Various Inhibitors on the Redistribution of PTP36—The results described above suggest that cell detachment induces the translocation of PTP36 through its dephosphorylation. In good accordance with this notion, the reduction of the phosphorylation level of PTP36 by staurosporine and ML-9 resulted in the translocation of PTP36 without cell detachment (Fig. 6D, lanes 7 and 9). Moreover, preincubation of...
The mechanism that regulates the phosphorylation level of PTP36 is largely unknown. The phosphorylation of PTP36 is induced by the cell attachment to fibronectin more efficiently than the cell spreading on the poly-I-l-lysine-coated wells. Thus, the cell adhesion through integrins might not be essential but an important regulation mechanism. We found that ML-9, an inhibitor of MLCK, reduced the phosphorylation level of PTP36 in vivo. It was reported that the inhibitors of MLCK promoted disassembly of actin stress fibers and focal adhesions (35, 36). Moreover, cytochalasin D, an inhibitor of actin polymerization, reduced the rephosphorylation of PTP36. Collectively, we speculate that actin-cytoskeleton might be involved in the regulation.

Based on the subcellular fractionation and the immunofluorescence microscopy experiments, we conclude that the dephosphorylated PTP36 is enriched in the membrane-associated cytoskeletal fraction. This distribution pattern is similar to that of PTPMEG, which is localized primarily to the membrane and the cytoskeletal fractions (24). Not all the localizations of PTP36 are clarified completely. For example, while the dephosphorylated PTP36 is not copurified with Triton X-100-insoluble membrane microdomains, it is still possible that PTP36 may dissociate from the microdomain during the purification process. Further study is necessary to answer such a question.

The role of the phosphorylation in the regulation of PTPH1 and PTPMEG is largely unknown. Little change in the enzymatic activity was observed after the phosphorylation of PTPH1 (33). Intriguingly, it was reported that PTPH1 made a complex with 14-3-3β in a phosphorylation-dependent manner (34). This might suggest that the phosphorylation of PTPH1 regulates its association with 14-3-3β. However, no physiological stimulation that affects the phosphorylation level of PTPH1 has been reported. We demonstrated in this study that cell-substrate adhesion regulates the phosphorylation and the localization of PTP36. The localization seems to be regulated through the phosphorylation level of PTP36 since: 1) okadaic acid, an inhibitor of serine/threonine phosphatase, completely blocks the translocation induced by cell detachment and 2) the reduction of the phosphorylation level by staurosporine and ML-9 is sufficient to induce translocation. Furthermore, dephosphorylation of PTP36 in vitro is accompanied by the translocation. Taken collectively, it is most likely that the phosphorylation level of PTP36 regulates its subcellular localization. For more definitive demonstration, identification and specific inactivation of the kinase and/or identification and mutagenesis of the phosphorylation sites on PTP36 might be necessary.

The physiological functions of PTP36 remain to be determined. Recently, we established a HeLa cell line in which PTP36 overexpression was inducible (22). There was little endogenous PTP36 detectable in the parental HeLa cells. When the overexpression of PTP36 was induced, we observed: 1) morphological changes, 2) a decrease in the actin stress fibers, 3) a decrease in the focal adhesions, 4) a reduction in the cell adhesion to collagen, and 5) a decrease in cell growth (22). Interestingly, unlike in the HeLa transfectants, we failed to detect any convincing effect of the overexpression in the 3T3/P36-8 cells. One fascinating explanation for the apparent discrepancy is that PTP36 overexpressed in 3T3 cells is attenuated by sequestration from its targets. In 3T3 cells adhered to the substrates, PTP36 is phosphorylated and localized to the cytosol. In contrast, PTP36 in the HeLa transfectant is largely in the dephosphorylated form and, as expected, PTP36 is localized near the plasma membrane (22). Collectively, our current working hypothesis is as follows. Detachment from

\[ ^2 \text{ M. Ogata and T. Hamaoka, unpublished observation.} \]
substrates in part of the cell induces the dephosphorylation and translocation of PTP36 from cytosol to cytoskeleton, where it plays roles in the regulation of cytoskeleton, cell adhesion, and cell growth.

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