Role of PTP-1B in aortic smooth muscle cell motility and tyrosine phosphorylation of focal adhesion proteins

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HASSID, Aviv, Shile Huang, and Jian Yao. Role of PTP-1B in aortic smooth muscle cell motility and tyrosine phosphorylation of focal adhesion proteins. Am. J. Physiol. 277 (Heart Circ. Physiol. 46): H192–H198, 1999.—Recent studies have focused attention on the role of protein tyrosine kinases in vascular smooth muscle cell biology, but similar information regarding protein tyrosine phosphatases (PTP) is sparse. PTP-1B is a ubiquitous nonreceptor phosphatase with uncertain function and substrates that are mostly unidentified. We used antisense oligodeoxynucleotides (ODN) against PTP-1B to investigate the role of endogenous PTP-1B in motility of primary cultures of rat aortic smooth muscle cells (RASMC). Antisense ODN decreased PTP-1B protein levels and activity in a concentration-dependent fashion, whereas sense, scrambled, or three-base mismatch antisense ODN had little or no effect. Treatment of cells with antisense ODN, but not sense, scrambled, or three-base mismatch antisense ODN, enhanced cell motility and increased tyrosine phosphorylation levels of focal adhesion proteins paxillin, p130cas, and focal adhesion kinase. Our findings indicate that PTP-1B is a negative regulator of RASMC motility via modulation of phosphorytrosine levels in several focal adhesion proteins and suggest the involvement of PTP-1B in events such as atherosclerosis and restenosis, which are associated with increased vascular smooth muscle cell motility.

antisense oligonucleotides; paxillin; focal adhesion kinase

Migration of vascular smooth muscle cells is an essential step in the development of atherosclerosis and restenosis (36). Several polypeptide growth factors, cytokines, and constituents of the extracellular matrix are thought to regulate vascular smooth muscle cell motility in vitro and are present in atherosclerotic lesions (1). However, the intracellular signal transduction pathways involved in vascular smooth muscle cell motility have not been completely identified.

Protein tyrosine phosphorylation has been recognized as a critical regulatory element in signal transduction, including cell proliferation, motility, and differentiation (13, 32, 40). Tyrosine phosphorylation is dynamically regulated by protein tyrosine kinases and protein tyrosine phosphatases (PTP; see Ref. 41). There is recent evidence supporting a role for tyrosine kinases in vascular smooth muscle cell motility (30, 31). On the other hand, there is relatively little information on the role of tyrosine phosphatases in this regard.

PTP can be classified as either membrane bound or cytosolic enzymes involved in a variety of cellular processes (13, 32, 40). Structurally, PTP share one or two highly conserved catalytic domains but differ in their noncatalytic domains. The latter domains are likely to determine subcellular localization and may thus be of critical importance for substrate recognition and regulation of function, either by targeting to specific subcellular compartments or by directly modulating enzymatic activity. For instance, PTP-1D (also known as Syp, SH-PTP-2, SHP2, SHPTP-3, or PTP-2C) is a nonreceptor PTP functioning as a positive modulator of angiotensin-elicited vascular smooth muscle cell proliferation (2). On the other hand, leukocyte common antigen related (LAR), which is a transmembrane PTP, functions as a negative regulator by decreasing hepatoma cell insulin receptor signaling (29). Furthermore, even the same PTP can play different roles in different cellular events. For example, CD45, another transmembrane PTP, activates members of the Src family protein tyrosine kinases in B lymphocyte antigen receptor signal transduction (19) but downregulates cell signaling of growth factor receptor tyrosine kinases in nonhematopoietic cells (35) and inhibits insulin receptor signaling in hematopoietic cells (23). Therefore, the roles of PTP and the mechanisms by which PTP function are complex and deserve further investigation.

PTP-1B was originally purified from the cytosolic fraction of human placenta as a 37-kDa protein (42). Molecular cloning and sequencing later demonstrated that the full-length molecule is composed of 435 amino acids having a molecular mass of ~50 kDa. This enzyme contains a conserved PTP catalytic domain within residues 30–278 and a COOH-terminal noncatalytic segment with potential regulatory function (4, 10, 11, 16). PTP-1B is ubiquitously and abundantly expressed in various eukaryotic cells and is associated with the endoplasmic reticulum (13, 14, 32, 40), although there is also evidence indicating its association with the cell membrane (26). Cysteine at position 215 in the catalytic domain is necessary for its enzymatic activity (3, 18). PTP-1B dephosphorylates a variety of growth factor receptors (24), negatively regulates insulin signal transduction (4, 20), and inhibits transformation of a range of cells, including NIH 3T3 cells, mouse 3T3 fibroblasts, and 3Y1 rat fibroblasts (4, 28, 46). Recent evidence has further shown that PTP-1B binds to and dephosphorylates the focal adhesion protein p130Cas and inhibits integrin signaling in 3Y1 rat fibroblasts (26, 27). However, the function of PTP-1B in vascular smooth muscle cell signaling is unknown. In this study, we used antisense oligonucleotides against PTP-1B to specifically decrease the levels of PTP-1B and found that such treatment enhanced vascular smooth muscle cell motility in association with increased tyrosine phosphorylation of several focal adhe-
sion proteins, including paxillin, focal adhesion kinase (FAK), and p130Cas. Our findings indicate that PTP-1B is a negative regulator of vascular smooth muscle cell motility in vitro.

MATERIALS AND METHODS

Materials. Lactating Sprague-Dawley rats and their pups were purchased from Charles River Laboratories (Wilmington, MA), or pups of the same strain were bred in the University of Tennessee vivarium. Primaria tissue culture plates or dishes were from Falcon/Becton-Dickinson (Oxnard, CA). Type I collagenase, soybean trypsin inhibitor, FBS, and BSA (fraction V) were from Sigma (St. Louis, MO). DMEM-Ham's F-12 (1:1) medium was from Gibco (Grand Island, NY). Porcine pancreatic elastase, insulin, transferrin, and selenious acid were from Collaborative Research (Lexington, MA). Antibodies against phosphotyrosine (RC20-HRP), paxillin, FAK, and p130Cas were purchased from Transduction Laboratories (Lexington, KY), whereas antibodies against PTP-1B were from Upstate Biotechnology (Lake Placid, NY).

All primary antibodies used were monoclonal, except that anti-PTP-1B was rabbit polyclonal antibody that is specific for the NH2-terminal region of human PTP-1B. The transfection reagent N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methysulfate (DOTAP) was supplied by Boehringer Mannheim. Oligodeoxynucleotides (ODNs) were synthesized by Gibco. All other reagents were of the highest quality available and were generally obtained from Sigma (St. Louis, MO) or Baxter (Edison, NJ), unless stated otherwise.

Cell culture. Smooth muscle cells were obtained from the thoracic aortas of newborn Sprague-Dawley rats (6–9 days old) as described previously (12). The cells were seeded in Primaria culture plates or dishes at a density of 1.8–2.3 x 10⁴ cells/cm² and were cultured for the first 2 days in serum-free DMEM-Ham's F-12 (1:1) medium supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), and selenious acid (5 ng/ml) plus 50 µM penicillin and 50 µg/ml streptomycin in a humidified atmosphere of 5% CO₂-95% air. Most cells (∼95%) attached to the culture surface within the first few hours after seeding and were observed to spread after overnight incubation. After the initial 2 days of culture in serum-free medium, FBS was added to a final concentration of 10%. Cells were cultured for an additional 1–2 days in serum-containing medium. Smooth muscle cell origin was confirmed by positive immunostaining for α-smooth muscle actin. All experiments in this study were performed using primary cultures; moreover, each individual experiment represents results from one such cell isolate, generally obtained from two rat litters.

Treatment of cells with ODN. Cultures that were 60–80% confluent were treated with PTP-1B antisense (5'-CCATTTCATGGCGG-3'), sense (5'-CCGGCATTGAAATGG-3'), scrambled (5'-CTAGGACGTCGTAAGC-3'), or three-base mismatch antisense (5'-CCATTATCAGGCGT-3') ODNs in the presence of the transfection reagent DOTAP (1 µM). ODNs and DOTAP were prepared in serum-free DMEM-Ham's F-12 (1:1) containing 50 µM penicillin and 50 µg/ml streptomycin and were incubated with cells for 24–72 h. Treatment with ODNs did not induce cytotoxicity, as assessed by trypan blue dye exclusion (cell viability >90%).

Measurement of PTP-1B activity by immunophosphatase assay. After incubation with ODNs, cells were lysed in denaturing lysis buffer of the following composition: 188 mM Tris·HCl (pH 6.8), 10 mM sodium pyrophosphate, 15% glycerol, 3% SDS, 1 mM sodium vanadate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, and 1 mM EDTA. Cells were agitated on a vortexer for 15 min. The collected lysates were immediately boiled for 5 min and were cleared by microcentrifugation at 16,000 g for 20 min. Protein concentration was determined by the bicinchoninic acid assay using BSA as the standard (Pierce). Equivalent amounts of proteins from different treatment categories were separated on 7.5% SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon PVDF; Millipore). Membranes were blocked with 0.1% Tween 20 and 3% BSA and were probed with monoclonal rabbit anti-PTP-1B antibody (1:2,000), followed by incubation with goat anti-rabbit IgG conjugated with horseradish peroxidase (1:5,000; Sigma). Immune-reactive bands were visualized using Renaissance chemiluminescence reagent (NEN). Measurement of PTP-1B activity via immunophosphatase assay. After treatment with ODNs, cells were lysed in ice-cold RIP buffer containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 µg/ml aprotinin, and 5 µg/ml leupeptin. Immunoprecipitation was carried out by incubating lysis buffer and two times with PTP assay buffer (0.1 M sodium acetate, pH 5.5, containing 1 mM EDTA and 10 mM dithiothreitol). Immunoprecipitated PTP-1B was incubated with 10 µM p-nitrophenyl phosphate as substrate in the above PTP assay buffer for 30 min at 22°C. The reaction was stopped by addition of NaOH to a final concentration of 1 M. Enzyme activity was determined by measuring the absorbance at 405 nm. The values in the absence of enzyme were taken as background and were always subtracted for correction.

Measurement of cell motility. The effect of antisense ODN against PTP-1B, and corresponding control ODN, on the motility of the primary aortic smooth muscle cells of newborn rats (6–9 days old) was evaluated by using a Transwell culture chamber (Costar, Cambridge, MA) in which the upper and lower culture chamber were separated by a collagen-coated polycarbonate filter having 8-µm pores. Transwell plates and their associated filters were preequilibrated with serum-free DMEM-Ham's F-12 (1:1) medium supplemented with insulin (5 µg/ml), transferrin (5 µg/ml), and selenious acid (5 ng/ml) plus 50 µM penicillin and 50 µg/ml streptomycin for at least 1 h at 37°C, according to the manufacturer's instructions. ODN (antisense, sense, scrambled, or 3-base mismatch antisense) plus DOTAP in the above-mentioned medium (600 µl/well) were added to either the lower chamber, the upper chamber, or both. Meanwhile, isolated primary aortic smooth muscle cells (100 µl at a concentration of 0.6 x 10⁵ cells/ml) were seeded in the upper chamber. Substantially similar results were obtained regardless of whether ODNs were placed in the upper or lower chambers, presumably because ODNs rapidly diffused from one chamber to the other. After incubation for 24 h, the filters were removed, rinsed two times with 0.1 M PBS (pH 7.4), fixed in 4% paraformaldehyde (dissolved in 0.1 M PBS, pH 7.4), and stained with hematoxylin. Cells on the upper side of the filter were wiped off with cotton swabs. Migrated cells on the lower side of the filter were determined by counting specified cross-sectional fields on the filters with a light microscope using ×200 magnification.

Immunoprecipitation and immunoblotting for paxillin, FAK, and p130Cas. After incubation with ODNs, cells were lysed in RIP buffer (150 mM NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, and 50 mM Tris, pH 7.2) containing 1 mM sodium vanadate, 1 mM PMSF, 5 µg/ml aprotinin, and 5 µg/ml leupeptin. Immunoprecipitation was performed by incubation of cell lysates (400 µg) with antibodies against
PTP-1B antisense ODN, but not sense, scrambled, or three-base mismatch antisense ODN, decreases PTP-1B protein levels and activity but fails to alter PTP-1D protein levels. Because of the lack of specific inhibitors, it is currently difficult to identify the role of PTP-1B in signal transduction via the use of conventional nonspecific inhibitor pharmacological agents such as vanadate or phenylarsine oxide. In principle, treatment of cells with antisense ODNs provides a useful approach to this problem. We identified an antisense oligonucleotide sequence, chosen according to well-established criteria designed to elicit specific hybridization to the sequence of sense mRNA surrounding the translational initiation site (5). As control sequences, we used sense ODN and a scrambled and three-base mismatch antisense ODN. Initially, primary aortic smooth muscle cell cultures isolated from newborn rats (6–9 days old) were treated with PTP-1B antisense ODN for 24–72 h. As shown in Fig. 1A, 10 µM antisense ODN decreased PTP-1B protein levels by ~60% as measured via Western blot, whereas sense or scrambled ODNs had little or no effect. In a more extensive study, three-base mismatch antisense ODN also inhibited PTP-1B expression, but to a lesser extent than antisense ODN (Fig. 1B). We obtained substantially similar results after either 24 or 72 h of treatment (results not shown). The relatively long-term efficacy of ODNs is presumably due to the culture of cells in serum-free medium because serum is known to have the capacity to degrade ODNs.

To further verify the specificity of antisense ODN treatment, we found that the protein levels of another PTP, PTP-1D, were unaffected by this treatment (Fig. 1C). Moreover, antisense ODN decreased PTP-1B protein levels in a concentration-dependent fashion, as shown in Fig. 2A. To verify that the decrease of protein was also reflected in decreased enzyme activity, we used an immunophosphatase assay for PTP-1B similar to that used by Kiyomoto et al. (21). As shown in Fig. 2B, treatment of cells with antisense ODN decreased the levels of immunoprecipitated PTP activity in a concentration-dependent fashion. We separately verified that >90% of the immunoprecipitated enzyme activity was contributed by PTP-1B, as determined via an in-gel phosphatase assay (data not shown and Ref. 6).

Treatment of rat aortic smooth muscle cells with antisense ODN against PTP-1B increases cell motility. Several recent studies have reported that tyrosine kinases act as positive regulators of vascular smooth muscle motility (15, 30, 31). To determine whether PTP-1B is a negative regulator of rat aortic smooth muscle cell (RASMC) motility, primary cultures were incubated with antisense ODN. ODN could be added in the upper, lower, or both chambers with similar results, presumably because the substance was able to diffuse rapidly from one chamber to another. In comparison with control, 10 µM antisense ODN increased cell motility by about threefold (Fig. 3). On the other hand, sense, scrambled, or three-base mismatch antisense ODNs did not have significant effects on cell motility (Fig. 3), revealing a specific effect of antisense ODN. Moreover, as shown in Fig. 4, antisense ODN, but not three-base mismatch antisense ODN, enhanced cell motility in a concentration-dependent manner. Treatment of RASMC with antisense ODN against PTP-1B, but not control ODN, increases tyrosine phosphorylation levels of focal adhesion proteins. A recent
study has reported that increased cell motility in transformed fibroblasts is associated with reduced tyrosine phosphorylation in several focal adhesion proteins (27). To determine whether increased motility induced by knock down of PTP-1B in RASMC could be attributed to increased tyrosine phosphorylation, we measured the effect of antisense ODN treatment on phosphotyrosine levels in focal adhesion proteins. As shown in Fig. 5, treatment of RASMC with antisense, but not sense, scrambled, or three-base mismatch, ODN increased the levels of phosphotyrosine in three clusters of proteins consisting of 60–85, 120–130, and 180–200 kDa molecular mass. Some of these proteins were identified as paxillin (70–80 kDa), FAK (125 kDa), and p130cas (130 kDa), respectively, by immunoprecipitation, followed by Western blotting. Treatment of RASMC with antisense, but not sense or scrambled, ODN increased the tyrosine phosphorylation level of these immunoprecipitated proteins without altering their respective protein levels (Fig. 6). Probing of immunoprecipitated p130cas with anti-phosphotyrosine antibody detected a doublet (Fig. 6C, top), whereas reprobing with mouse monoclonal anti-p130cas raised against a fragment (amino acids 644–819) of rat p130cas detected a single band (Fig. 6C, bottom). It is likely that the second tyrosine phosphorylated protein unreactive with anti-p130cas represents a coimmunoprecipitating protein. We suspected that this protein might be FAK based on its established molecular mass of 125 kDa. However, reprobing with anti-FAK did not detect an immunoreactive protein, indicating that either the unknown protein is unrelated to FAK or that coimmu-
noprecipitated FAK is insufficiently abundant to allow detection in this fashion.

DISCUSSION

Vascular smooth muscle cell motility is thought to be necessary for developmental growth and the progression of vascular disease (39). A complete understanding of the mechanisms regulating these processes is lacking. Recently, several studies have focused on the role of tyrosine kinases in vascular smooth muscle cell motility (15, 30, 31). To our knowledge, there are no published studies targeting the role of PTP in vascular smooth muscle cell motility. Therefore, the purpose of the current study was to determine the potential importance of a ubiquitous phosphatase, PTP-1B, in regulation of cell motility and to begin investigating some of the underlying mechanisms. We report the new finding that treatment of RASMC with antisense ODN against PTP-1B decreased the levels of PTP-1B protein and activity, whereas treatment with several control ODN had little or no effect. Moreover, antisense ODN, but not several control ODN, including an antisense ODN that has just a three-base mismatch, increased cell motility in association with tyrosine phosphorylation of several focal adhesion proteins, including p130cas, paxillin, and FAK. These results thus indicate that endogenous PTP-1B plays a role in vascular smooth muscle cell motility.

Our results are the first to show that PTP-1B, expressed at normal levels in untransformed and differentiated primary aortic smooth muscle cells, regulates both motility and tyrosine phosphorylation in cytoskeletal proteins in constitutive fashion. These results are consistent with and complement previous studies demonstrating that overexpression of PTP-1B in transformed fibroblasts elicited a reduction of tyrosine phosphorylation of paxillin, p130cas, and FAK, together with decreased cell migration (27).

Molecular cloning has demonstrated that p130cas contains a central domain consisting of a cluster of potential Src homology 2 (SH2)-binding sites and a COOH-terminal domain containing both consensus Src homology 3 (SH3)- and SH2-binding motifs (37). The distinctive structure of p130cas suggests that it may serve as a docking protein, forming a multicomponent complex with other proteins. PTP-1B lacks SH2- or phosphotyrosine-binding domains. However, as indicated above, it has two potential SH3-binding motifs in its COOH-terminus that may allow binding to SH3 domain-containing proteins. Indeed, Liu et al. (26) have shown that p130cas binds to and is dephosphorylated by PTP-1B via interaction of the proline-rich SH3-binding domain of PTP-1B and the SH3 domain of p130cas.

Unlike many other nonreceptor tyrosine kinases, FAK lacks SH2 and SH3 domains. However, its COOH-terminal domain is composed of 159 amino acids and is thought to play an important role in recruiting FAK to focal adhesions and binding to paxillin and talin, respectively.

Fig. 5. Treatment of RASMC with antisense ODN against PTP-1B increases tyrosine phosphorylation levels of clusters of proteins at 60–85, 120–130, and 180–200 kDa molecular mass. Cells were treated with DOTAP alone (control) and SS, Scr, MAS, or AS ODN in the presence of DOTAP followed by lysis and Western blotting with anti-phosphotyrosine antibody. Similar results were obtained in 2 other independent experiments.

Fig. 6. PTP-1B antisense ODN increases tyrosine phosphorylation of focal adhesion proteins in primary RASMC. Cells were treated with AS, SS, or Scr AS ODN in the presence of DOTAP. Cell lysates were immunoprecipitated with antibodies against paxillin, focal adhesion kinase (FAK), and p130cas, respectively. Immunocomplexes were separated by SDS-PAGE and were transferred to polyvinylidene difluoride membranes, followed by immunoblotting with anti-phosphotyrosine (Py)-horseradish peroxidase conjugates. Loading of equivalent amounts of protein was confirmed by stripping the membranes and reprobing them with corresponding primary antibodies against paxillin (A), FAK (B), or p130cas (C). Densitometric values are given as a ratio of anti-Py-to-anti-protein bands with control ratios normalized to a value of 1.00: A: control, 1.00; SS, 0.96; Scr, 0.68; and AS, 2.15. B: control, 1.00; SS, 1.21; Scr, 1.87; and AS, 3.24. C: control, 1.00; SS, 1.15; Scr, 1.17; and AS, 3.53. Similar results were obtained in 2 other independent experiments.
whereas the NH$_2$-terminal domain is necessary for binding to integrins (33). Moreover, FAK contains binding sites for SH2 (residues 397–400) and SH3 (residues 368–376; see Ref. 33) domains. In vitro, FAK forms stable complexes with p130$^{ca}$ via the SH3 domain of p130$^{ca}$ (17, 34). We assume that the increase of FAK phosphotyrosine levels induced by knock down of PTP-1B is the result of an indirect interaction between these molecules, possibly via the use of p130$^{ca}$ as an adapter protein. Moreover, increased phosphorylation of FAK suggests an increase of FAK activity and thus raises the possibility that some of the increased phosphorylation of focal adhesion proteins could be related to increased FAK (or other kinase) activity.

Paxillin is another cytoskeletal protein involved in actin-membrane attachment at sites of cell adhesion to the extracellular membrane (7). A recent study has indicated the preferential localization of phosphorylated paxillin to focal adhesions, consistent with its potential importance in cytoskeletal assembly (9). cDNA cloning has revealed that paxillin has five potential SH2-binding domains and a proline-rich segment indicative of an SH3-binding motif. A region in the NH$_2$-terminus of paxillin provides binding sites for vinculin and FAK (38, 43). Tyrosine phosphorylation of paxillin is accompanied by phosphorylation of FAK (7, 43). Immunoprecipitated FAK phosphorylates paxillin, suggesting that paxillin is a substrate for FAK in vivo (45). Currently, there is no known mechanism for the direct association of PTP-1B and paxillin, and it is therefore likely that such an association is indirect.

Several studies have reported a correlation between cell motility and tyrosine phosphorylation of focal adhesion proteins. Thus platelet-derived growth factor (PDGF) BB-stimulated motility of rabbit RASMC was associated with tyrosine phosphorylation of FAK and paxillin (1). Similarly, angiotensin II-stimulated rat vascular smooth muscle cells and Swiss 3T3 fibroblasts (J. Biol. Chem. 270: 11367–11376, 1995).


ROLE OF PTP-1B IN CELL MOTILITY


