Specific Interaction of Protein Tyrosine Phosphatase-MEG2 with Phosphatidylserine*

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Protein tyrosine phosphatase (PTP)-MEG2 is an intracellular tyrosine phosphatase that contains a Sec14 homology domain. We have purified the full-length and truncated forms of the enzyme from recombinant adenovirus-infected human 293 cells. By using lipid-membrane overlay and liposome binding assays, we demonstrated that PTP-MEG2 specifically binds phosphatidyleragine and phospholipid non-symmetrically distributed in the outer layer and inner layer of cell membranes. It has recently been defined as an important ligand for clearance of apoptotic cells. By specifically binding phosphatidyleragine, PTP-MEG2 may play an important role in regulating signaling processes associated with phagocytosis of apoptotic cells.

Protein tyrosine phosphatases (PTPs)\(^{1}\) consist of a highly diverse family of enzyme with crucial roles in cell signaling (1–3). Like their counterpart protein tyrosine kinases, PTPs can be divided into transmembrane receptor-like and intracellular enzymes. The intracellular PTPs contain a single conserved phosphatase domain. Beyond the limit of the catalytic domains are various segments or domains that modulate the activity and/or intracellular localization of the enzymes. Among these are the Sec14 domain found in PTP-MEG2 (4). The Sec14 domain is shared by several lipid-binding proteins such as cellular retinaldehyde-binding protein (CRALBP), \( \alpha \)-tocopherol transfer protein, and yeast Sec14p and by regulators of G proteins including RhoGAPs, RhoGEFs and the RasGEF, and neurofibromin (5). CRALBP is a water-soluble protein found in the retina and pineal gland. It acts as a carrier protein for cis-retinaldehyde or 11-cis-retinal as previously described (14). Homemade lipid strips were prepared by spotting nitrocellulose membranes 1 \( \mu \)l of individual lipid compounds dissolved in chloroform/methanol (1:1 v/v) at a concentration of 0.01–1 mg/ml. The strips were air-dried and kept at 4 °C in the dark. Anti-PTP-MEG2 antibodies 144 and 159 were raised against peptides corresponding to amino acids 575–593 at the C terminus and to amino acids 297–314 in the middle of the enzyme, respectively.

**EXPERIMENTAL PROCEDURES**

**Materials**—Commercial PIP strips spotted with 100 pmol of 16 different phospholipids were purchased from Echelon Research Lab (Salt Lake City, UT). Individual lipid compounds including phosphatidic acid, PC, PE, phosphatidyglycerol, phosphatidylinositol, PS, sphingomyelin, and cholesterol, all from natural sources, were purchased from Avanti Polar Lipids. Arachidonic acid, oleic acid, linoleic acid, all-trans-retinal, and \( \alpha \)-tocopherol were from Sigma. A mixture of retinal isomers was made by photoisomerization of all-trans-retinal as previously described (14). Homemade lipid strips were prepared by spotting on nitrocellulose membranes 1 \( \mu \)l of individual lipid compounds dissolved in chloroform/methanol (1:1 v/v) at a concentration of 0.01–1 mg/ml. The strips were air-dried and kept at 4 °C in the dark. Anti-PTP-MEG2 antibodies 144 and 159 were raised against peptides corresponding to amino acids 575–593 at the C terminus and to amino acids 297–314 in the middle of the enzyme, respectively.

**Expression and Purification of Full-length and Truncated Forms of PTP-MEG2**—Three recombinant PTP-MEG2 proteins were employed in this study (see Fig. 1). They are full-length form PTP-MEG2 (amino acid residues 1–593), N-terminal Sec14 domain-truncated form \( \Delta \)NPTP-MEG2 (amino acid residues 283–593), and C-terminal catalytic domain-truncated form \( \Delta \)CPTP-MEG2 (amino acid residues 1–329). PTP-MEG2 and \( \Delta \)NPTP-MEG2 were purified by using adenosine and Escherichia coli expression systems, respectively, as previously described (14). \( \Delta \)CPTP-MEG2 was also expressed in the adenovirus. It contains a His\(_{6}\) tag at the C terminus without addition of any other extra amino acid residues. To generate the recombinant adenovirus carrying \( \Delta \)CPTP-MEG2, a cDNA fragment encoding amino acids 1–329 of PTP-MEG2 plus a DNA sequence encoding 6 consecutive histidine residues was inserted into adenovirus transfer vector pACCMV.pLpA. Recombinant adenovirus was generated by co-transfection of 293 cells with the pACCMV.pLpA construct and pJM17 adenovirus genome DNA by using FuGENE 6 cell transfection reagent (Roche Applied Science).
The resulting recombinant virus was purified by soft agar plaque assays and then amplified in 293 cells according to standard procedures (15). Positive clones were selected based on expression of ΔCPTP-MEG2 in infected 293 cells as determined by anti-PTP-MEG2 antibody 159. The purified recombinant adenovirus was used to infect 293 cells to express ΔCPTP-MEG2 following the protocol used for expression of the full-length PTP-MEG2 (14). The His6-tagged recombinant protein was purified from the cytosolic extracts of the infected cells by using a nickel-nitrilotriacetic acid column (Qiagen) following the manufacturer’s protocol. The purity of the enzyme was about 90% as judged by Coomassie Blue staining. Western blotting analyses with antibody 159 showed a single band of expected size (~45 kDa).

Lipid-membrane Overlay Assays—Nitrocellulose membrane strips spotted with various lipid compounds were incubated with 0.5 μg/ml full-length or truncated forms of PTP-MEG2 in Buffer A containing 10 mM Tri-s-HCl (pH 8.0), 0.15 mM NaCl, 0.1% Tween 20, and 3% fatty acid-free bovine serum albumin (BSA). After extensive washing with Buffer A, enzymes bound to the membrane strips were probed with appropriate anti-PTP-MEG2 antibodies and then with horseradish peroxidase-conjugated anti-rabbit secondary antibodies. Detection was carried out with 4′-chloronaphthol as a substrate. Nitrocellulose membrane strips were washed with phosphate-buffered saline containing 5 mg/ml BSA as a carrier, which is necessary to reduce the nonspecific signals seen with several other lipids. We further verified the results by using homemade phospholipid strips. In addition to some common phospholipids, we also included fatty acids, cholesterol, α-tocopherol, and retinal isomers. The data shown in Fig. 2C further confirmed the specific interaction of PTP-MEG2 with PS and ruled out binding with other lipid molecules tested. Further experiments as illustrated in Fig. 2D revealed that PTP-MEG2 could easily detect 25 ng of PS on the membrane.

The Sec14 Domain Is Responsible for the Interaction of PTP-MEG2 with PS—The Sec14 domain of PTP-MEG2 is presumed to be involved in the interaction of PTP-MEG2 with PS. To verify this, we analyzed the binding of catalytic domain-truncated and Sec14 domain-truncated enzymes. ΔNPTP-MEG2 was purified from E. coli cells as previously described (14). However, ΔCPTP-MEG2 was largely insoluble when expressed in E. coli cells. We thus employed the adenovirus system for expression of the Sec14 domain as described for the full-length PTP-MEG2. The protein was expressed as a His6-tagged fusion protein. Over 70% of ΔCPTP-MEG2 was partitioned in the cytosolic extracts of recombinant adenovirus-infected 293 cells. By using a single nickel-nitritolatriacetic acid agarose column, we were able to enrich the recombinant protein from the cytosolic extracts to over 90% purity as revealed by Coomassie Blue staining, and Western blotting with anti-serum 159 showed a single protein band (Fig. 3). Fig. 4 shows the results of lipid membrane overlay assays obtained with the truncated enzymes. Although ΔCPTP-MEG2 displayed a specific binding to PS as observed with the full-length form of PTP-MEG2, ΔNPTP-MEG2 did not show any significant interaction with any of the lipids analyzed. These data indicate that the Sec14 domain mediates the interaction of PTP-MEG2 with PS.

PS-containing Liposomes Bind PTP-MEG2 but Do Not Affect Its Enzymatic Activity toward pNPP—The interaction of PS with PTP-MEG2 was also confirmed by performing liposome-binding assays (22, 23). We used PC and PE as carriers for the lipids to be analyzed, and lipid vesicles were formed by sonication. For the recombinant PTP-MEG2 proteins, we employed BSA as a carrier, which is necessary to reduce the nonspecific interactions.
binding of proteins to lipid vesicles. Upon incubation of the full-length and two truncated forms of PTP-MEG2 with the lipid vesicles followed by centrifugation, proteins bound to the lipid vesicles were recovered from the pellets. As shown in Fig. 5A, among the 8 phospholipids analyzed, only PS displayed a specific and strong binding to PTP-MEG2 as indicated by a near total recovery of the enzyme in the lipid vesicle pellet and a near depletion of the enzyme from the supernatant. All the other lipids showed marginal basal binding. As expected, a similar binding with PS was seen with ΔCPTP-MEG2 but not with ΔNPTP-MEG2. These data confirm not only the specific interaction of PTP-MEG2 with PS but also reveal the role of its Sec14 domain in the interaction. We further analyzed the effect of PS on the activity of PT-MEG2 with pNPP as a substrate (Fig. 5B), but we did not find any significant effect of the lipid on the catalytic activity of PTP-MEG2. If anything, it caused a slight inhibition, which was also seen with ΔNPTP-MEG2, which carries only the catalytic domain. Therefore, PS does not affect the activity of PTP-MEG2 at least toward pNPP. These results agree with our previous study in which the assays were performed with lipid micelle containing Triton X-100 (14). However, these data do not rule out the possible effects on PTP-MEG2 activity toward physiological substrates. By using the same procedures, we have analyzed binding and regulatory ability of other lipid molecules as described in Fig. 2. None of them showed any significant binding or regulatory activity (data not shown).

The Sec14 Domain Is Responsible for the Localization of PTP-MEG2 to the Perinuclear Region—We performed immunofluorescent cell staining to determine the intracellular localization of PTP-MEG2 as shown in Fig. 6. Staining of wild type...
HT-1080 cells with antibody 144 revealed a moderate expression of endogenous PTP-MEG2 in the perinuclear region resembling the endoplasmic reticulum and Golgi apparatus. This localization is further confirmed by a much more intensive staining of over-expressed PTP-MEG2 in the same region upon infection of the cells with recombinant adenovirus carrying the full-length form of PTP-MEG2. Furthermore, PTP-MEG2 that contains the Sec14 domain alone displayed exactly the same staining pattern as seen with full-length PTP-MEG2. In contrast, truncation of the Sec14 domain resulted in the distribution of PTP-MEG2 in the entire cytoplasm. Note that PTP-MEG2 corresponds to the catalytic domain of PTP-MEG2. These data indicate that the N-terminal Sec14 domain of PTP-MEG2 is necessary and sufficient for the perinuclear localization of the enzyme. We believe that PS present in the perinuclear region may be responsible for such localization.

**DISCUSSION**

It has been well accepted that phospholipids are not only components of cell membranes but also act as important second messengers (24). A major characteristic of lipid second messengers is that they cannot diffuse freely through the aqueous compartments of cells. Once produced in a particular cell membrane, they stay there and often serve as markers for the membrane compartment until they are enzymatically converted or translocated or the membrane buds off or fuses with another. This membrane confinement makes lipid messengers ideal regulators of subcellular localization. Many proteins are localized to specific cellular compartments through conserved domains that specifically recognize particular membrane-
bound lipids. PH, FYVE, and PX domains are some examples that have attracted much attention in recent years (17–24). These domains specifically bind phospho-derivatives of phosphatidylinositol. In this study, by analyzing over 20 lipid compounds with lipid-membrane overlay and liposome-binding assays, we defined a specific interaction of PS with the Sec14 domain of PTP-MEG2 that has no identifiable sequence similarities to other known PS-binding proteins. We further demonstrated that changing of PS distribution in cells alters localization of PTP-MEG2. Our study suggests that PS may have an important role in controlling function of PTP-MEG2.

Intracellular PTPs contain various domains or segments surrounding the catalytic domain of ~230 amino acids. It has been well accepted that these flanking peptide segments or protein domains play regulatory and/or targeting roles. Recent studies (14, 25) have shown that the N-terminal Sec14 domain-truncated form of PTP-MEG2 has a significantly higher activity than the full-length enzyme. This suggests a regulatory role of the non-catalytic Sec14 domain. We expected that interaction of PS with the Sec14 domain would stimulate the activity of PTP-MEG2. However, with the artificial substrate pNPP we did not see any significant change in the activity of PTP-MEG2 in the presence of PS. Nevertheless, it should be pointed out that this result does not necessarily rule out the possible regulatory role on the activity toward physiological substrates. In fact, anionic phospholipids stimulate the activities of SHP-1 and SHP-2 toward myelin basic protein but inhibit those toward pNPP (26, 27). Although the possible effects on enzymatic activity needs identification of physiological substrates of PTP-MEG2 to clarify this point, the regulatory role of PS in controlling the function of PTP-MEG2 is well represented by PS-mediated translocation of the enzyme. It should be noted that an earlier study with recombinant PTP-MEG2 expressed as a GST fusion protein in E. coli cells demonstrated a significant activation of the enzyme by phosphatidylinositol 4,5-diphosphate and other phospho-derivatives of phosphatidylinositol (25). Furthermore, a screening of cell extracts with matrices carrying the tethered homologs of lipids identified PTP-MEG2 as one of the binding proteins of phosphatidylinositol 3,4,5-trisphosphate (28). However, with PTP-MEG2 purified from adenovirus-infected 293 cells, we did not see a stimulatory effect of phosphatidylinositol 4,5-diphosphate (14), and in this study, we did not observe a significant binding of PTP-MEG2 to phosphatidylinositol 3,4,5-trisphosphate either. Further work is required to clarify this discrepancy.

PS is a relatively abundant membrane lipid and has been recently implicated as an important ligand for clearance of apoptotic cells (10, 11). Like other phospholipids, it is synthesized in the cytosolic leaflet of the endoplasmic reticulum membrane and moves to the plasma membrane through vesicular transport, and it is asymmetrically distributed on endoplasmic reticulum and the plasma membrane with a higher proportion in the inner leaflet of the membrane bilayer (29). When cells undergo apoptosis, however, PS on the plasma membrane is translocated to the outer leaflet by a phospholipid scramblase activated by protein kinase C. This is accompanied by concurrent inactivation of the aminophospholipid scramblase thereby preventing PS returning to the inner leaflet and leaving PS expressed on the surface of apoptotic cells. Exposed PS is then recognized by its receptor-designated PSR and thereby initiates uptake of the apoptotic cells by phagocytes (12, 13). It is well known that uptake and removal of necrotic or lysed cells involves inflammation and an immune response, but clearance of apoptotic cells does not induce either inflammation or immunity (30, 31). The PSR is thought to be the molecular switch that determines the outcome (13). In fact, engagement of PS with PSR causes the production of inflammatory mediators, including transforming growth factor-β, prostaglandin E2, and interleukin-10 (32, 33). However, the exact signaling pathways involved in the apoptotic cell uptake and the anti-inflammation processes have not yet been sorted out. In this study, we have identified a potential intracellular receptor of PS. It is conceivable that when engulfed by phagocytes, a high level of PS in the outer membrane of apoptotic cells may alter the distribution of PTP-MEG2 in the phagosomes. Because PTPs play both positive and negative roles in cell signaling, we postulate that recruitment of PTP-MEG2 by PS accompanying the phagocytosis of apoptotic cells may suppress signal transduction pathways that lead to production of inflammatory factors and/or augment those pathways that stimulate generation of anti-inflammatory mediators. This notion is supported by the recent findings that PTP-MEG2 is localized on phagosomes and secretory vesicles (25, 34). Finally, it should be pointed out that like PSR, PTP-MEG2 appears to be widely expressed, and thus its function may not be limited to phagocytosis. After all, loss of PS asymmetry is not only found in apoptotic cells but also in other activated cells at least transiently, due to activation of the scramblase (13). Studies of PTP-MEG2 should provide a better understanding of this transient redistribution of PS.

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