Nuclear Localization of Non-receptor Protein Tyrosine Phosphatase ε Is Regulated by Its Unique N-Terminal Domain

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Precise subcellular localization is an important factor in regulation of the functions of protein tyrosine phosphatases. The non-receptor form of protein tyrosine phosphatase ε (cyt-PTPε) can be found in cell nuclei, among other cellular locations, while p67 PTPε, a naturally occurring isoform which lacks the 27 N terminal residues of cyt-PTPε, is exclusively cytosolic. Using deletion and scanning mutagenesis we report that the first 10 amino acid residues of cyt-PTPε in particular residues R4, K5, and R9, are critical components for its nuclear localization. We also establish that increased oxidative stress enhances accumulation of cyt-PTPε in cell nuclei. Of the four known protein forms of PTPε, cyt-PTPε is the only one which includes the extreme N-terminal sequence containing R4, K5, and R9. The role of the unique N terminus of cyt-PTPε is therefore to regulate its subcellular localization. The existence of naturally occurring forms of PTPε which lack this sequence and which are generated by translational and posttranslational mechanisms, suggests that nuclear localization of cyt-PTPε can be actively regulated by cells.

Key Words: tyrosine phosphatase; nuclear localization signal; oxidative stress; hydrogen peroxide.

INTRODUCTION

Accurate phosphorylation of tyrosine residues in proteins is well established as a vital mechanism by which protein structure and function are regulated in a reversible manner. Tyrosine dephosphorylation is carried out by members of the protein tyrosine phosphatase (PTP) superfamily, which are genetically, structurally, and functionally distinct from the tyrosine kinases whose activities they counter [1–4]. In recent years it has become evident that subcellular localization of nonmembranal PTPs is crucial in regulating their physiological roles [5, 6]. Examples of this phenomenon include PTPs such as the non-receptor-type PTP1B, PTP-PEST, and STEP, whose proteolytic cleavage in platelets and T-cells alters their subcellular localization patterns and can result in their activation [7–12]. Additional examples are SHP1 and SHP2, whose SH2 domains mediate their recruitment to activated growth factor receptors in whose vicinity they act [13].

The protein tyrosine phosphatase ε (PTPε) subfamily contains four distinct protein species, all products of a single gene [14, 15]. The receptor-type form of PTPε (RPTPε) [16, 17] is an integral membrane protein, which has been linked to assisting with mouse mammary tumorigenesis [17, 18; Gil-Henn and Elson, submitted] and to down-regulation of insulin receptor signaling in cultured cells [19, 20]. The non-receptor form of PTPε (cyt-PTPε), which is expressed from the PTPRε gene by use of an alternative promoter [15, 21, 22], is predominantly cytoplasmic but can also be detected at the cell membrane and within the cell nucleus [23]. The absence of this form from Schwann cells of young PTPε-deficient mice increases phosphorylation and activity of the delayed-rectifier, voltage-gated potassium channels Kv1.5 and Kv2.1 and correlates with myelination defects in sciatic nerves in these mice [24]. p67 PTPε, which is produced by internal initiation of translation from PTPRε mRNAs, and p65 PTPε, which is produced by calpain-mediated proteolytic processing of the larger PTPε forms, are N-terminally truncated forms of PTPε and are exclusively cytoplasmic [23, 25].

The distinct N-termini of the four PTPε proteins dictate their unique subcellular localization patterns and affect their physiological roles; this is exemplified in the significantly reduced ability of the cytosolic p67 and p65 PTPε proteins to dephosphorylate the integral membrane potassium channel Kv2.1 [23, 25]. Other roles for PTPε include suppression of endothelial cell proliferation [26], ensuring proper function of mouse macrophages [27], and inhibition of JAK-STAT signaling in M1 leukemia cells in response to various cytokines [28, 29].

p67 PTPε can be produced by initiation of translation from cyt-PTPε mRNA at an alternative ATG initiation codon located 81 bp downstream of the initiator ATG for full-length cyt-PTPε [23]. As a result, the only structural difference between the cyt-PTPε and p67
PTPε proteins is the absence of a stretch of 27 amino acid residues from the N-terminus of the latter. The absence of this region from p67 correlates with the inability of p67 to associate with cell membranes and with its absence from cell nuclei [23], suggesting that the N-terminal domain of cyt-PTPε regulates the precise subcellular localization of the protein. The present study disrupted the 27 N-terminal residues of cyt-PTPε and identifies residues within it that are required for nuclear localization of cyt-PTPε. Importantly, residues required for this effect reside in the extreme N-terminal region of this domain, which are absolutely unique to cyt-PTPε. The study also links nuclear localization mediated by the above domain of cyt-PTPε with increases in oxidative stress. The existence of p67 and p65 PTPε, which are naturally occurring isoforms of PTPε that lack these sequences, suggests that the ability of cyt-PTPε to enter the nucleus is dynamically regulated within cells.

MATERIALS AND METHODS

Reagents. cDNAs for full-length mouse cyt-PTPε [21] and p67 PTPε [23] cloned in pCDNA3 (Invitrogen) were used in this study. The N-terminal deletion mutants of cyt-PTPε were constructed by PCR, using the following series of recessed 5′ oligos: R4 (CTGGATCCACATAGAAAGAAGCTTCCCCG), S8 (CTGGATCCACATGACCCGCTACCTGGT), T11 (CTGGATCCACATGACCTGGTCCGGGCAAGCAG), and S22 (CTGGATCCACATGACCGAGCAAGAAGAAA) (initiating ATG underlined). pcDNA3 was used as a control vector; the latter vector attaches a GFP molecule at the C terminus of cDNAs were cloned either into pcDNA3 or into pEGFP-N1 (Clontech); the latter vector attaches a GFP molecule at the C terminus of cDNAs that contain signals which are predominantly cytosolic, while cyt-PTPε is predominantly cytosolic but can be found also at the cell membrane and within the cell nucleus [23]. In order to demonstrate this, full-length and p67 PTPε were linked at their C termini to green fluorescent protein (GFP) to enable visualization of their localization in live cells (Fig. 1B).

RESULTS AND DISCUSSION

The 10 Extreme N-Terminal Residues of cyt-PTPε Are Required for Nuclear Localization

As indicated, p67 is identical to cyt-PTPε except that p67 PTPε lacks 27 amino acid residues located at the extreme N terminus of cyt-PTPε (Fig. 1A). This structural difference strongly affects the subcellular localization pattern of p67—this molecule is exclusively cytosolic, while cyt-PTPε is predominantly cytosolic but can be found also at the cell membrane and within the cell nucleus [23]. In order to demonstrate this, images presented in Fig. 1B were acquired by confocal microscopy, indicating that GFP-tagged cyt-PTPε is located inside the cell nucleus and occupies the same focal plane as Hoechst-stained DNA and that the large unstained regions in cells expressing p67-PTPε-GFP are in fact unstained nuclei. Protein blotting of lysates prepared from cells expressing cyt-PTPε-GFP or p67-GFP fusion proteins did not reveal the presence of GFP not linked to cyt-PTPε (not shown), indicating that the nuclear fluorescence in Fig. 1B did not arise from free GFP, which can enter the nucleus on its own. The diffuse pattern of cyt-PTPε-GFP staining vs the punctate pattern of Hoechst-stained DNA within the nucleus suggests that cyt-PTPε is most likely not associated with chromatin. These results indicate that the 27 N-terminal residues of cyt-PTPε contain signals which regulate the subcellular localization of the molecule. Of note, the amino acid composition of this region is atypical. Over half of the residues in this region are serines or threonines (6 residues of 27) or basic amino acids (9/27) (Fig. 1A), suggesting that phosphorylation or the
presence of a basic-type nuclear localization signal [31] may regulate cyt-PTPε localization.

In order to identify regions within the N-terminal domain which regulate nuclear localization of cyt-PTPε we created a series of N-terminal deletion mutants, in which an initiator methionine was inserted upstream of R4, S8, T11, R14, or S22 in cyt-PTPε. These N-terminally truncated proteins were fused to GFP as described. Expression of this series of mutants revealed that R4 cyt-PTPε was expressed throughout the entire cell in a pattern indistinguishable from that of full-length cyt-PTPε. In contrast, T11, R14, and S22 cyt-PTPε mutants were excluded from the nucleus in a manner similar to p67 PTPε, while S8 cyt-PTPε exhibited intermediate levels of nuclear localization (Fig. 2A and data not shown). Lack of nuclear staining of p67 PTPε and of the T11, R14, and S22 deletion mutants was widespread and was detected in virtually all cells expressing these molecules. In order to rule out the possibility that the GFP tag affected nuclear localization we expressed in 293T cells nontagged versions of the same series of deletion mutants and examined their subcellular localization by biochemical fractionation. Results of this study were similar to those obtained with the GFP-tagged PTPε mutants (Fig. 2B).

Note that despite somewhat higher expression levels of S22 and p67 PTPε in the experiment shown these molecules were not detected in the nuclear fraction. We conclude that the region between R4 and L10 in cyt-PTPε contains sequences which participate in regulating the nuclear localization of cyt-PTPε.

In order to determine whether residues located between R4 and L10 were both necessary and sufficient for nuclear localization of cyt-PTPε we fused this region to the p67 PTPε protein (construct ∆11-27, Fig. 2A). Examination of cells expressing this protein revealed significantly reduced nuclear staining, similar in intensity and affecting virtually all transfected cells in a manner like that seen in the T11, R14, and S22 mutants and in the p67 PTPε protein (Fig. 2A). Residues R4–L10 of cyt-PTPε are therefore necessary, although not sufficient, for nuclear localization of cyt-PTPε. Residues R4–L10 are part of the 12 N-terminal residues of cyt-PTPε which are unique to this form of
PTP\(\epsilon\). Residues further downstream, starting with F13, are included in at least one of the three other known isoforms of PTP\(\epsilon\), none of which are found in the nucleus [23, 25]. This indicates that the role of residues R4–L10, which are found only in cyt-PTP\(\epsilon\), is to help regulate subcellular localization of the phosphatase.

R4, K5, and R9 of cyt-PTP\(\epsilon\) Are Part of a Nuclear Localization Signal

In order to further dissect the region between R4 and L10 we prepared a series of point mutants of full-length GFP-tagged cyt-PTP\(\epsilon\), in which residues R4 through R9 were individually replaced with alanine. Expression of these molecules in 293T cells revealed that nuclear localization of R4A-, K5A-, and R9A-cyt-PTP\(\epsilon\) was reduced (Fig. 3A). This effect was detected in all transfected cells expressing the above three constructs, although its intensity was somewhat weaker than that observed in p67 PTP\(\epsilon\)-GFP. In contrast, F7A and S8A mutants were expressed throughout the cells in a manner indistinguishable from full-length cyt-PTP\(\epsilon\). We conclude that R4, K5, and R9 are part of a nuclear localization signal, most likely a basic-type NLS, and are necessary for driving nuclear localization of cyt-PTP\(\epsilon\). This result agrees with the intermediate nuclear localization phenotype of the S8 deletion mutant (Fig. 2A); this mutant includes R9 but not R4 and K5. In agreement with the above findings, all cells

![FIG. 2. Nuclear localization is driven by a sequence between R4 and T11 of cyt-PTP\(\epsilon\). A. Top: Representation of the N-termini of deletion mutants beginning at R4, S8, T11, and S22 of cyt-PTP\(\epsilon\); dashed line denotes region missing from \(\Delta 11-27\) cyt-PTP\(\epsilon\). Bottom: Pattern of subcellular localization of these mutants when expressed in 293T cells as GFP fusion proteins. B. Biochemical fractionation of 293T cells transfected with untagged deletion mutants. Cells were fractionated into nuclear (N) and nonnuclear (C, cytosol plus cell membrane); equal amounts of total protein from each fraction were electrophoresed and blotted with anti-PTP\(\epsilon\) antibodies.](image-url)
expressing the double (R4A/R9A) or triple (R4A/K5A/R9A) mutants of cyt-PTPε exhibited a decrease in nuclear staining that was similar in extent to p67 PTPε (Fig. 3B). This result indicates that these combinations of mutations can recapitulate the effect of deleting the entire N-terminal domain of cyt-PTPε. Similar results were observed when the same constructs were expressed in NIH3T3 and in COS cells (not shown), indicating that this phenomenon was not unique to 293T cells. Of note, R4 and R9 of mouse PTPε used here are conserved in cyt-PTPε from rat and human; K5 is conserved in rat cyt-PTPε and is replaced in a nonconservative manner by a serine residue in human (Fig. 3C). Yet, despite its lack of conservation in human and the fact that the R4A/R9A mutant exhibited the same intensity of nuclear exclusion as the R4A/K5A/R9A mutant, K5 appears to play a role in nuclear localization of mouse cyt-PTPε as the K5A mutant was excluded from the nucleus (Fig. 3A).

Surprisingly, the N6A mutation resulted in a signif-
icant increase in nuclear localization of cyt-PTPε (Fig. 3A). The strength of this phenotype varied among cell types; it was weaker in COS cells and was not detected in NIH3T3 cells (not shown). This suggests that nuclear localization of cyt-PTPε might also be regulated by nuclear export regulated in part by N6; however, as this residue is not conserved in human cyt-PTPε (Fig. 3C) and its effect appears to be variable in the cell types examined, this issue requires further study.

Nuclear localization is regulated also by active export of proteins from the nucleus which, in many cases, relies on the presence of a nuclear export sequence (NES) of the type recognized by Crm1 [32]. Many NESs, such as those of STAT1, cyclin B, MEK, and the HIV accessory protein Rev, are of the form L(X)₂L(X)₃ (33–36), cyt-PTPε contains no sequences which fit this consensus, although several sequences within this protein are somewhat similar (L₁₇₅VM-L₁₇₇, L₁₉₈L₁₉₉Q₁₉₉L₁₉₉, L₁₃₄L₁₃₅S₁₃₅) [33–36]. Note that the N6 residue noted above is not located in any of these sequences. Nonetheless, treatment of NIH3T3 and 293 cells expressing a full-length cyt-PTPε–GFP fusion protein with leptomycin B, an inhibitor of Crm1-mediated nuclear export [37] (5–10 ng/ml, 1–8 h), did not alter cellular localization of cyt-PTPε–GFP as assessed by fluorescence microscopy (not shown). This finding indicates that cyt-PTPε does not contain an NES of the type recognized by Crm1, nor does it appear to associate with and be transported out of the nucleus by another protein which contains an NES of this type. cyt-PTPε is ~70 kDa molecular mass, which is most likely too large to exit the nucleus by passive diffusion; we then conclude that if cyt-PTPε exits the nucleus, this is probably regulated by other, non-Crm1-related mechanisms.

Oxidative Stress Increases Nuclear Localization of cyt-PTPε

In order to better understand the physiological relevance of nuclear cyt-PTPε we searched for stimuli which would result in increased amounts of cyt-PTPε in cell nuclei. Subcellular localization was followed by direct visualization of cyt-PTPε–GFP fusion proteins and by biochemical fractionation in NIH3T3 cells. Oxidative stress in the form of H₂O₂ treatment was recently shown to promote inter- and intramolecular associations of RPTPα [38] and of cyt-PTPε (Toledano-Katchalski et al., submitted). In order to examine the effects the N-terminal domain of cyt-PTPε on subcellular localization of the protein in response to H₂O₂, we expressed cyt-PTPε–GFP fusion proteins in NIH3T3 cells and then treated the cells with 1 mM H₂O₂ as described under Materials and Methods. Upon treatment with H₂O₂, enhancement of nuclear and membranal localization of cyt-PTPε was detected by GFP fluorescence (Fig. 4A); increased nuclear localization was further verified by biochemical fractionation (Fig. 4B). No such changes were observed in cells expressing GFP alone, indicating that these changes were indeed mediated by cyt-PTPε. Interestingly, H₂O₂ treatment of cells expressing the R4A/K5A/R9A cyt-PTPε triple mutant did not result in enhancement of nuclear and membranal localization of the mutant PTPε (Fig. 4A). A similar result was obtained in cells expressing p67 PTPε, where nonfluorescent nuclei were clearly detected in the GFP staining patterns of H₂O₂-treated cells expressing this protein (not shown). We conclude that presence of the nuclear localization domain defined above significantly increased the ability of cyt-PTPε to localize to the nuclear in response to H₂O₂. H₂O₂ treatment has previously been shown to cause redistribution of the 45-kDa nuclear variant of the T-cell tyrosine phosphatase into the cytosol [35], suggesting that cellular redistribution of PTPs may be a common outcome of enhanced oxidative stress. Fluorescence microscopy data presented here suggest that the same sequence region also affects membrane localization, although further studies are required in this respect. It should be noted that cyt-PTPε nuclear localization was not affected by treating cells with inhibitors of serine-threonine or tyrosine phosphatases (oka-diac acid or sodium pervanadate, respectively), UV (50 J/M²) or gamma (1.5 Gy) irradiation, and transfection with various pro-apoptotic gene products, such as Bax, ZIP kinase, or DAP kinase [39–41], at testing to the specificity of the H₂O₂ effect.

Nuclear localization of cyt-PTPε may have two nonmutually-exclusive consequences—gain of function, in which nuclear cyt-PTPε gains access to previously inaccessible substrates, or loss of function, whereby cyt-PTPε is removed from the cytosol and loses access to cytosolic substrates. As the amount of cyt-PTPε found in the nucleus in response to stimuli examined in this study does not deplete cytosolic stores of cyt-PTPε, it is reasonable to believe that nuclear localization provides access to new substrates for PTPε. It is of particular interest to note that the two naturally-occurring, shorter forms of PTPε, p65 and p67, lack the region identified here as essential for nuclear localization of PTPε [23]. As expression of these forms is regulated by translational and postranslational mechanisms of gene expression, it is tempting to speculate that physiological stimuli can regulate nuclear localization of cyt-PTPε by affecting these mechanisms. Coexistence of two mechanisms for removal of the N-terminal NLS of cyt-PTPε may provide cells with flexibility in denying nuclear access to the enzyme. Along these lines, proteolytic processing of full-length cyt-PTPε to p65 PTPε may constitute a mechanism for preventing existing PTPε molecules from entering the nucleus, while a shift in translation initiation toward production of
p67 PTPε may be aimed at preventing synthesis of PTPε molecules which can enter the nucleus. Further studies aimed at isolating nuclear substrates of cyt-PTPε and linking them with stimuli which increase amounts of nuclear cyt-PTPε are expected to help clarify these issues.

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