Nuclear localization of protein phosphatase 5 is dependent on the carboxy-terminal region

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Abstract Endogenous and overexpressed protein phosphatase 5 (PP5) localizes to the nucleus and cytoplasm of HeLa cells, while the overexpressed TPR domain of PP5 is restricted to the cytoplasm. Deletion and mutational analysis of human PP5 demonstrates that the C-terminal amino acids 420–499 are essential for nuclear localization and PP5 activity is not required. Since the phosphatase domain terminates at 473, these studies suggest that the highly conserved section (476–491) with the eukaryotic consensus FXAVPHXØXPXMYAN is required for nuclear localization of PP5. Bacterially expressed PP5 is inhibited by several tumor promoters but not by the anticancer drug fostriecin. © 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.

Key words: Protein phosphatase; Nuclear localization; Tetratricopeptide repeat; Tumor promoter; Cell signalling

1. Introduction

Reversible phosphorylation of proteins on serine and threonine residues regulates most cellular functions. Protein phosphatase 5 (PP5) is a widely distributed member of the PPP family of protein serine/threonine phosphatases that has been reported to regulate several cellular processes including cell growth, signalling and transcription (reviewed in [1,2]). Mammalian PP5 and its probable homolog in lower eukaryotes, Ppt1, comprise a phosphatase catalytic domain preceded by a fused amino-terminal domain that contains three tetratricopeptide (TPR) motifs [3^5]. Removal of the TPR domain by proteolysis leads to a substantial increase in phosphatase activity indicating that the TPR domain suppresses activity probably by shielding the active site [6,7]. Arachidonic acid and compounds containing polyunsaturated fatty acids activate PP5 by binding to the TPR domain [6,8], although it is unknown whether this form of activation is physiologically important. The crystal structure of the TPR domain of PP5 reveals a super-helical structure with an amphipathic groove which provides a site for binding of other TPR domains or unrelated proteins [9]. PP5 has been reported to interact in vitro with the atrial natriuretic peptide/guanylate cyclase receptor [4], hsp90 [10,11], the human blue light photoreceptor CRY2 [12] and with CDC16 and CDC27 which are part of the anaphase promoting complex [13]. A number of studies suggest that PP5 may regulate glucocorticoid receptor function. Overexpression of the TPR domain of PP5, presumed to act in a dominant negative fashion on PP5 function, was reported to inhibit glucocorticoid receptor-mediated activation of transcription [10]. Depletion of PP5 using antisense oligonucleotides was reported to cause glucocorticoid receptor-mediated hyperphosphorylation of p53 and induction of the cyclin-dependent protein kinase inhibitor, p21WAF1/Cip1 with growth arrest [14,15]. In these studies, PP5 depletion was found to enhance dexamethasone-mediated glucocorticoid receptor transcriptional activity ∼10-fold.

PP5 is present in all tissues examined, in both the cytoplasm and the nucleus but with a prominent nuclear localization in some mammalian cell lines which would support its role as a transcriptional regulator [16]. In this article, we show that nuclear localization of PP5 is dependent on a conserved carboxy-terminal region of PP5.

2. Materials and methods

2.1. Construction of PP5 expression vectors

The construct for the expression of full length human PP5 (amino acids M1–M499 [17]) with an HA-tag (YPYDVPDYA) directly following the C-terminal methionine was produced by the polymerase chain reaction (PCR) using human PP5 cDNA [3] as template and oligonucleotide primers encoding the additional amino acids and incorporating EcoRI sites. The PCR fragment was subcloned into the EcoRI site of pCMV5 [18] to yield pCMV5-PP5-HA for expression in mammalian cells. Further PCR and restriction fragment substitutions were performed to produce the C-terminal deletion constructs pCMV5-PP5(nt 1–720)-HA, pCMV5-PP5(nt 1–1005)-HA and pCMV5-PP5(nt 1–1257)-HA expressing PP5(M1–C240)-HA, PP5(M1–F335)-HA and PP5(M1–N419)-HA respectively. pCMV5-PP5(nt 1–942+nt 1260–1497)-HA which expressed the TPR domain (M1–G314) followed by one serine residue fused to the C-terminal 80 amino acids (L420–M499) with an HA-tag (termed PP5ΔF315-N419-HA) was also constructed by PCR and restriction fragment substitution. The construct pEYFP-PP5(nt 1260–1497) was constructed by PCR fragment cleaved with BglII and KpnI from the previous construct into the same sites of the pEYFP-C1 vector (Clontech, Palo Alto, CA, USA). (pCMV5-PP5(304A)-HA in which histidine 304 was substituted by alanine was produced using the Quickchange oligonucleotides was reported to cause glucocorticoid receptor-mediated activation of transcription [10]. Depletion of PP5 using antisense oligonucleotides was reported to cause glucocorticoid receptor-mediated hyperphosphorylation of p53 and induction of the cyclin-dependent protein kinase inhibitor, p21WAF1/Cip1 with growth arrest [14,15]. In these studies, PP5 depletion was found to enhance dexamethasone-mediated glucocorticoid receptor transcriptional activity ∼10-fold.

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For bacterial expression, EcoRI fragments encoding PP5-HA and PP5(304A)-HA were inserted into the EcoRI site of pGEX-4T-3 (Pharmacia, Uppsala, Sweden). After expression in Escherichia coli DH5Δ, the glutathione-S-transferase fusion proteins, GST-PP5-HA and GST-PP5(304A)-HA, were affinity purified on GSH-Sepharose.

2.2. Protein phosphatase assays

Protein phosphatase assays were performed as described in [6] using

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2.3. Cell transfections and immunological analyses

HeLa cells and human embryonic kidney 293 cells were cultured at 37°C in an atmosphere of 5% CO2 in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. Cells were plated onto 12 mm glass cover slips in 10 cm diameter dishes and grown until 70-80% confluent. Each 10 cm diameter plate was transfected with 10 µg DNA by a modified calcium phosphate method [19]. 24 h after transfection, HeLa cells were fixed in 2% paraformaldehyde in phosphate buffered saline (PBS) for 10 min, and then washed in buffer A (50 mM Tris-HCl pH 7.5, 150 mM NaCl and 0.1% Triton X-100). The cells were permeabilized in 1% NP40 in buffer A, processed and examined with anti-TPR antibodies (5 £ 10^(-12) M) as described in [3]. Indirect immunofluorescence staining was visualized with a Bio-Rad MRC 600 Series laser scanning/confocal imaging system in conjunction with a Nikon Microphot-SA microscope or with Openlab digital imaging system (Improvision, Coventry, UK) in conjunction with a Leica DM IRB inverted microscope. 293 cells, 24 h after transfection, were washed twice with PBS and lysed in 50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.1 mM EGTA, 150 mM NaCl, 1% deoxycholate and Complete Protease Inhibitor Cocktail (Boehringer, Mannheim, Germany), with 10 passes in and out of a syringe. After centrifugation at 14,000 g for 20 min at 4°C, proteins in the supernatant were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membranes and probed with anti-TPR antibodies or anti-HA antibodies, followed by secondary antibodies coupled to horseradish peroxidase (HRP). Detection of the immunoreactive proteins was by enhanced chemiluminescence (Amersham Pharmacia Bio-tech, UK). All anti-PP5 antibodies were raised in sheep by the Scottish Antibody Production Unit (Carluke, Lanarkshire, Scotland) and affinity purified against their respective antigens. Anti-PP5 peptide antibodies were produced against peptides AEPPRDEPPADGLKRR and KQPEEDNARTKAE and, corresponding to human PP5 (amino acids 12-27) and Drosophila melanogaster PP5 (amino acids 30-45) respectively, coupled to bovine serum albumin. The anti-peptide antibodies were used in immunoblot analysis of PP5 and PP5 deletion mutants expressed from pCMV5 constructs in 293 cells. Western blots were probed with anti-TPR antibodies (data not shown). In addition, detection of the expression of this protein, PP5 with a carboxy-terminal HA-tag (Fig. 1A and B) transiently overexpressed in HeLa cells was detected in both the nucleus and the cytoplasm with anti-HA antibodies (Fig. 2B). The expression pattern was similar, although not identical, to that of endogenous PP5 detected with anti-PP5 peptide antibodies (Fig. 2A). However, overexpressed PP5-HA showed higher levels in the cytoplasm than in the nucleus, while endogenous PP5 was observed to be fairly equally distributed (Fig. 2A) or have higher levels in the nucleus than the cytoplasm [3]. Transient overexpression of PP5(M1-C240)-HA, encompassing the TPR domain of PP5 but lacking the carboxy-terminal half that includes the phosphatase domain (Fig. 1A and B), revealed a strikingly different localization restricted entirely to the cytoplasm (Fig. 2C). This localization was also observed in human embryonic kidney 293 cells. Expression of PP5(M1-F335)-HA and PP5(M1-N419)-HA was also restricted to the cytoplasm (Fig. 2D and E). This suggests that the carboxy-terminal 80 amino acids of PP5 are responsible for the nuclear localization of PP5. A plasmid was therefore produced that expressed the TPR domain of PP5 fused to the 80 carboxy-terminal amino acids of PP5 and as predicted the expression of this protein, PP5(F315-N419)-HA, was detected in both the nucleus and the cytoplasm (Fig. 2F). These results indicate that all or part of the carboxy-terminal region of PP5 is required for the nuclear localization of PP5. Identical results to those seen with anti-PP5-peptide antibodies (Fig. 2A) and with anti-HA antibodies (Fig. 2B-F) were obtained when PP5 subcellular location was detected with anti-TPR antibodies (data not shown). In addition, detection with anti-TPR antibodies showed that PP5-HA and PP5(M1-C240)-HA were overexpressed in HeLa cells at high levels compared to endogenous PP5.

3. Results

3.1. Nuclear localization of PP5 depends on the extreme C-terminal region

Human PP5 with a carboxy-terminal HA-tag (Fig. 1A and B) transiently overexpressed in HeLa cells was detected in both the nucleus and the cytoplasm with anti-HA antibodies (Fig. 2B). The expression pattern was similar, although not identical, to that of endogenous PP5 detected with anti-PP5 peptide antibodies (Fig. 2A). However, overexpressed PP5-HA showed higher levels in the cytoplasm than in the nucleus, while endogenous PP5 was observed to be fairly equally distributed (Fig. 2A) or have higher levels in the nucleus than the cytoplasm [3]. Transient overexpression of PP5(M1-C240)-HA, encompassing the TPR domain of PP5 but lacking the carboxy-terminal half that includes the phosphatase domain (Fig. 1A and B), revealed a strikingly different localization restricted entirely to the cytoplasm (Fig. 2C). This localization was also observed in human embryonic kidney 293 cells. Expression of PP5(M1-F335)-HA and PP5(M1-N419)-HA was also restricted to the cytoplasm (Fig. 2D and E). This suggests that the carboxy-terminal 80 amino acids of PP5 are responsible for the nuclear localization of PP5. A plasmid was therefore produced that expressed the TPR domain of PP5 fused to the 80 carboxy-terminal amino acids of PP5 and as predicted the expression of this protein, PP5(F315-N419)-HA, was detected in both the nucleus and the cytoplasm (Fig. 2F). These results indicate that all or part of the carboxy-terminal region of PP5 is required for the nuclear localization of PP5. Identical results to those seen with anti-PP5-peptide antibodies (Fig. 2A) and with anti-HA antibodies (Fig. 2B-F) were obtained when PP5 subcellular location was detected with anti-TPR antibodies (data not shown). In addition, detection with anti-TPR antibodies showed that PP5-HA and PP5(M1-C240)-HA were overexpressed in HeLa cells at high levels compared to endogenous PP5.

3.2. Mutation of histidine 304 to alanine in PP5 inactivates the enzyme but does not prevent nuclear localization

Since the protein PP5 with a carboxy-terminal HA-tag (Fig. 1A and B) transiently overexpressed in HeLa cells was detected in both the nucleus and the cytoplasm with anti-HA antibodies (Fig. 2B). The expression pattern was similar, although not identical, to that of endogenous PP5 detected with anti-PP5 peptide antibodies (Fig. 2A). However, overexpressed PP5-HA showed higher levels in the cytoplasm than in the nucleus, while endogenous PP5 was observed to be fairly equally distributed (Fig. 2A) or have higher levels in the nucleus than the cytoplasm [3]. Transient overexpression of PP5(M1-C240)-HA, encompassing the TPR domain of PP5 but lacking the carboxy-terminal half that includes the phosphatase domain (Fig. 1A and B), revealed a strikingly different localization restricted entirely to the cytoplasm (Fig. 2C). This localization was also observed in human embryonic kidney 293 cells. Expression of PP5(M1-F335)-HA and PP5(M1-N419)-HA was also restricted to the cytoplasm (Fig. 2D and E). This suggests that the carboxy-terminal 80 amino acids of PP5 are responsible for the nuclear localization of PP5. A plasmid was therefore produced that expressed the TPR domain of PP5 fused to the 80 carboxy-terminal amino acids of PP5 and as predicted the expression of this protein, PP5(F315-N419)-HA, was detected in both the nucleus and the cytoplasm (Fig. 2F). These results indicate that all or part of the carboxy-terminal region of PP5 is required for the nuclear localization of PP5. Identical results to those seen with anti-PP5-peptide antibodies (Fig. 2A) and with anti-HA antibodies (Fig. 2B-F) were obtained when PP5 subcellular location was detected with anti-TPR antibodies (data not shown). In addition, detection with anti-TPR antibodies showed that PP5-HA and PP5(M1-C240)-HA were overexpressed in HeLa cells at high levels compared to endogenous PP5.

Since the protein PP5(F315-N419)-HA contained part of

![Fig. 1. A: Schematic representation of human PP5 and deletion mutants of PP5 used for expression in HeLa cells. PP5 sequence is indicated by open bars with the three TPR motifs identified by diagonal stripes. The HA-tag is indicated by a filled bar. A single bent line indicates an internal deletion. B: Immunoblot analysis of PP5 and PP5 deletion mutants expressed from pCMV5 constructs in 293 cells. Lysates containing 10 mg protein from transfected 293 cells were subjected to SDS-PAGE. After transfer to nitrocellulose membranes, PP5 variants were detected with anti-HA antibodies, followed by anti-mouse IgG-HRP secondary antibodies. Immunoreactive proteins were visualized by ECL and autoradiography. Expression constructs are indicated above each lane.](image)
the phosphatase domain, it was possible that phosphatase activity was necessary for nuclear localization. However, PP5(H304A)-HA showed similar expression levels and localization to wild-type PP5-HA (Figs. 3A and 4A). PP5(H304A)-HA is mutated at the residue homologous to the histidine in PP1 that is believed to act as a general acid in the proposed catalytic mechanism [20] and loss of which leads to inactivation of PP1 [21]. In order to examine the activity of PP5(H304A)-HA, both wild-type and mutant PP5 were expressed in *E. coli* as GST fusions and affinity purified. Assay of GST-PP5-HA revealed a low activity of 2.8 mU/mg using 32P-labelled casein as substrate in the standard assay. In the presence of 200 μM arachidonic acid, GST-PP5-HA was activated > 25-fold to 77 mU/mg, similarly to the native enzyme and the bacterially expressed PP5 containing no additional tags [6,8]. The activity of GST-PP5-HA was also inhibited by low nanomolar concentrations of the tumor promoters, okadaic acid and microcystin (Table 1) as observed for the native and bacterially expressed PP5. In addition, the toxins cantharidin, calyculin A and tautomycin inhibited GST-PP5-HA in the nanomolar range. The anti-tumor drug fostriecin, that inhibits PP2A and PP4 with an IC50 of 1.5 nM and 3 nM respectively [22], did not inhibit GST-PP5-HA in the nanomolar range, inhibition only being apparent at much higher concentrations (IC50 700 μM). GST-PP5(H304A)-HA was expressed as a soluble protein, demonstrating the H304A mutation does not result in denaturation of the protein. When assayed with 32P-labelled casein in the standard assay GST-PP5(H304A)-HA essentially showed no activity in the absence or presence of arachidonic acid. PP5 phosphatase activity therefore does not appear to be essential for the localization of PP5 in the nucleus.
3.3. The extreme C-terminal region of PP5 alters the localization of yellow fluorescent protein in the nucleus

In order to examine the mechanism by which the C-terminal region of PP5 might influence localization of the phosphatase, a construct expressing the C-terminal 80 amino acids of PP5 fused to the reporter yellow fluorescent protein (YFP-PP5(L420-M499)) was compared with the vector expressing YFP alone (Fig. 3B). In most HeLa cells, YFP localized predominantly, but not exclusively, to the nucleus, usually with a strong nucleolar staining (Fig. 4B and D). In contrast, although YFP-PP5(L420-M499) localized predominantly, but not exclusively, to the nucleus, the fluorescence was fairly evenly distributed throughout the nucleus with no evidence of nucleolar staining (Fig. 4C and E), suggesting that the C-terminal 80 amino acids of PP5 may bind to nucleoplasmonic structures outside the nucleolus. Some cells highly expressing YFP-PP5(L420-M499) 24 h after transfection showed a distorted nuclear shape (Fig. 4F). 48 h after transfection few cells highly expressing YFP-PP5(L420-M499) were evident although many cells highly expressing YFP were visible. These observations suggest that high levels of the C-terminal amino acids of PP5 within the nucleoplasm may be lethal to the cell.

4. Discussion

Nuclear localization of PP5 has been observed in several species including mammals [3], Xenopus laevis [13] and D. melanogaster [17] and the ability of PP5 to enter and/or accumulate in the nucleus is likely to be important for its function. Here we sought to determine which region of PP5 is required for the nuclear localization by examining the ectopic expression of PP5 and PP5 deletion mutants in HeLa cells. These studies demonstrated that the TPR domain of PP5 and PP5 deletion mutants comprising the TPR domain and part or virtually all of the phosphatase domain localized exclusively to the cytoplasm, while the overexpressed full length PP5 and the amino-terminal half fused to the carboxy-terminal 80 amino acids of PP5 were found in both the nucleus and cytoplasm. In light of these results, use of the TPR domain as a dominant negative mutant to analyze the function of PP5 [10] is inappropriate. Sequence similarity of PP5 and Ppt1 to protein serine/threonine phosphatases in other subfamilies exists over amino acids 204–473 of human PP5, defining this region as the phosphatase domain [23] and leaving only amino acids 474–499 within the carboxy-terminal 80 amino acids for other functions. The short section of 16 amino acids at 476–491, lying outwith the phosphatase domain, is very highly conserved throughout the PP5/Ppt1 subfamily (Fig. 5), with the consensus sequence FXAVPHRTKXPMAYAN. In view of the high conservation, the sequence would be predicted to be important for function and from the above studies it appeared that this section may be required for the nuclear localization of PP5.

Sinclair et al. [7] have shown that a truncated mutant of PP5 missing 13 carboxy-terminal residues (amino acids 487–499) is insensitive to arachidonic acid and as active as the full length lipid-stimulated enzyme. However, we have shown phosphatase activity is not essential for nuclear localization, since the inactive PP5(H304A) mutant localizes to the nucleus. Thus the conserved carboxy-terminal motif, and not the activity of PP5, is likely to regulate nuclear localization. The carboxy-terminus may contribute to the structural stability of the inactive conformation of the enzyme [7], but it is not the sole determinant of the enzyme activity state, since the TPR domain has been shown to play a major role in maintaining the enzyme in an inactive conformation and demonstrated to bind the lipid that activates the enzyme [6]. Since the crystal structure of the TPR domain has been obtained [9], it is unlikely that the overexpressed TPR domain is restricted to the cytoplasm by improper folding or aggregation, proper-
ties that would prevent crystallization. We cannot exclude the possibility that the TPR domain and the TPR domain fused to the amino-terminal or the amino-terminal and middle of the phosphatase domain all undergo cytoplasmic retention by virtue of an exposed TPR domain. However, if this is the case then PP5 carrying a deletion of more than 100 amino acids in the phosphatase domain must adopt a very similar conformation to full length PP5 to obscure sites on the TPR domain causing cytoplasmic retention.

Comparison of the localization of endogenous PP5 with overexpressed PP5 in HeLa cells, shows that the ratio of the cytoplasmic to nuclear level is higher for overexpressed PP5 (Fig. 2A and B). Since PP5 is highly overexpressed in both 293 cells (Fig. 3A) and HeLa cells (Section 3.1), this suggests that PP5 does not enter the nucleus by simple diffusion, but is transported by a factor which becomes rate limiting when PP5 is abundantly overexpressed. In the last few years, a wide variety of nuclear protein import signals have been identified that are involved in binding to nuclear receptors or adaptors for receptors that mediate nuclear entry [24]. However, the conserved C-terminal motif of PP5 identified here does not bear any sequence identity to the classical localization signals containing basic residues or to the more recently identified nuclear localization sequences. In addition, the C-terminal 80 amino acids fused to the reporter YFP did not localize exclusively to the nucleus indicating that amino acids 476-491 of PP5 may not act as a typical nuclear localization signal. Nevertheless, the fusion protein did appear to accumulate more highly in the nucleus than the cytoplasm. Its fairly even distribution in the nucleoplasm, in contrast to YFP alone that often showed higher fluorescence in the nucleolus (Fig. 4), suggests that the PP5 C-terminus interacts with components in the nucleoplasm. High accumulation of the YFP-PP5 C-terminal fusion protein within the nucleus also appeared to be detrimental to the cell.

It has been demonstrated that overexpressed PP5 binds via its TPR domain to the TPR acceptor domain of the hsp90 in steroid receptor complexes in cell extracts [25]. However, PP5 is unlikely to enter the nucleus through this interaction, because most of the hsp90 is believed to dissociate from the

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Fig. 4. A: Localization of inactive PP5 in a HeLa cell transfected with the pCMV5-PP5(H304A)-HA construct. Overexpressed PP5(H304A)-HA (white) was detected with the anti-HA antibody and anti-mouse IgG-FITC and visualized with confocal microscopy. B-F: Expression of YFP (B and D) and YFP-PP5(L420-M499) (C, E and F) in HeLa cells fixed with paraformaldehyde. YFP fluorescence (white) has excitation and emission maxima of 513 nm and 527 nm respectively and was visualized directly. Images B and C were captured by confocal microscopy and images D-F using Openlab digital imaging system. Scale bars 5 μm (A, D-F), 2.5 μm (B, C).
glucocorticoid receptor on ligand binding [26] before nuclear entry. In addition, our studies show that the TPR domain of PP5 alone does not localize to the nucleus. Nevertheless, it is possible that dissociation of hsp90 from the glucocorticoid receptor on ligand binding could allow PP5 to interact with some component of the glucocorticoid receptor complexes via the carboxy-terminal domain and thus 'piggy back' into the nucleus. The transcriptional activity of the glucocorticoid receptor bound to its enhancer (GRE) may then be modulated through dephosphorylation by PP5. Alternatively, since PP5 may play a much wider role in the regulation of transcription [16], interaction of the conserved carboxy-terminal region with a protein that translocates to the nucleus could not only facilitate nuclear entry of PP5, but also convert PP5 to an active conformation so that it can modulate transcription independently of binding to steroid receptors or other signalling molecules.

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