Determinants of the nucleolar targeting of protein phosphatase-1

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Abstract The ubiquitously expressed protein Ser/Thr phosphatase-1 isoforms PP1\textsubscript{x}, PP1\textsubscript{b} and PP1\textsubscript{y} are dynamically targeted to distinct, but overlapping cellular compartments by associated proteins. Within the nucleus of HeLa cells, EGFP-tagged PP1\textsubscript{y} and PP1\textsubscript{b} were predominantly targeted to the nucleoli, while PP1\textsubscript{x} showed a more diffuse distribution. Using PP1\textsubscript{a} chimaeras and point mutants we show here that a single N-terminal residue, i.e., Gin20 for PP1\textsubscript{x}, Arg19 for PP1\textsubscript{b} and Arg20 for PP1\textsubscript{y} accounts for their distinct subnuclear distribution. Our data also suggest that the N-terminus of PP1\textsubscript{b} and PP1\textsubscript{y} harbours an interaction site for one or more nucleolar interactors.

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1. Introduction

Protein phosphatase-1 (PP1), a member of the PPP family of protein serine/threonine phosphatases, regulates numerous cellular processes by the dephosphorylation of key proteins [1–3]. Mammalian genomes contain three genes that together encode four isoforms of PP1, namely PP1\textsubscript{x}, PP1\textsubscript{b} (also known as PP1\textsubscript{h}), and the splice variants PP1\textsubscript{y} and PP1\textsubscript{y}\textsubscript{b}. With the exception of PP1\textsubscript{y}, which is mainly expressed in testis, these isoforms appear to be present in all cell types. The PP1 isoforms are more than 90% identical at the protein level and differ mainly in their substrate specificity [1–3]. The interacting polypeptides bind to PP1 via multiple, short-sequence motifs. The best characterized and most common PP1-binding motif is referred to as the RVxF-motif and conforms to the consensus sequence [RK][S]x[0–1][V][P][FW], where X denotes any residue and {P} any residue except Pro [6]. Binding of the RVxF-motif not only tethers PP1 but also promotes the interaction of secondary, lower affinity binding sites, which modulate the activity and substrate specificity of the phosphatase [1–3].

PP1\textsubscript{x}, PP1\textsubscript{b} and PP1\textsubscript{y} display an overlapping but distinct and highly dynamic subcellular localization. Within the nucleus PP1\textsubscript{x} is mainly attached to the nuclear matrix, while PP1\textsubscript{y} is predominantly found in the nucleoli [7–9]. PP1\textsubscript{b} is present in the non-nucleolar chromatin fraction but is also targeted to the nucleoli. The distinct subnuclear localization of endogenous PP1 isoforms can be reproduced with exogenous, EGFP-tagged fusions of PP1 isoforms [7–9]. An obvious explanation for the isoform-specific subcellular localization of PP1 is that these isoforms differ in their affinity for interacting targeting proteins. Consistent with this notion, the C-terminus of PP1\textsubscript{b} was shown to interact with the myosin-targeting subunit Mypt1, explaining why it is predominantly this isoform that dephosphorylates myosin [10].

We have explored why PP1\textsubscript{b} and PP1\textsubscript{y} are enriched in the nucleoli while PP1\textsubscript{x} is not. Using EGFP-tagged PP1 fusions and site-directed mutagenesis, we found that a single residue in the N-terminus of PP1\textsubscript{x} prevents its nucleolar accumulation. Our data also imply that the N-terminus of PP1\textsubscript{b} and PP1\textsubscript{y} contains an interaction site for (a) nucleolar interactor(s).

2. Materials and methods

The full-length sequences of rabbit PP1\textsubscript{x} and PP1\textsubscript{b}, and rat PP1\textsubscript{y} were inserted between the Xhol and BamH\textsubscript{I} sites of the pEGFP-C1 vector (Clontech) to produce expression vectors for EGFP-tagged PP1 isoforms. To construct chimaeric EGFP-PP1\textsubscript{y} with the C-terminus of PP1\textsubscript{x}, a SalI site was first introduced by silent mutation of codons 285–286 of full-length EGFP-PP1\textsubscript{y} and EGFP-PP1\textsubscript{b}. Subsequently, the SalI–Mbol digested PP1\textsubscript{x}(286–330) fragment was ligated to EGFP-PP1\textsubscript{y}(1–285). After the introduction of a SalI site in codons 54–55 of full-length EGFP-PP1\textsubscript{y} and EGFP-PP1\textsubscript{b}, the N-terminal PP1\textsubscript{x}(1–55) fragment was fused to PP1\textsubscript{y}(56–323). Point mutations were introduced by the QuickChange protocol (Stratagene), with the appropriate primers and templates. All constructs and mutants were verified by DNA sequencing.

HeLa cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. 24 h after transfection with the indicated plasmids and FuGENE 6 (Roche Molecular Biochemicals), the green fluorescence was visualized by confocal microscopy. The images were obtained with a Zeiss LSM-510 laser-scanning confocal microscope (Jena, Germany), equipped with a Zeiss Axiovert 100 M objective (Plan Apochromat 40× 1.30 NA oil immersion objective) and the standard fluorescein isothiocyanate filter set. The label was excited with the 488-nm line of an argon laser. The emission from the fluorochrome was detected after 505–530-nm (fluorescein thiocyanate) band-pass filtering.

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Abbreviation: PP1, protein phosphatase-1

3. Results

In accordance with data of Trinkle-Mulcahy et al. [8], we found that transiently expressed EGFP-PP1α, EGFP-PP1β and EGFP-PP1γ1 in living HeLa cells were largely, but not exclusively nuclear (Fig. 1). Within the nucleus EGFP-PP1α showed a diffuse distribution but appeared to be excluded from the nucleoli in some cells (Figs. 1 and 2Aa). In contrast, EGFP-PP1β and EGFP-PP1γ1 were enriched in the nucleoli (Fig. 1). A chimaera of EGFP-PP1γ1 with the C-terminus of PP1α was also nucleolar but an EGFP-PP1γ1 chimaera with the N-terminus of PP1α was not enriched in the nucleoli (Fig. 2A and B). These data indicated that it is the N-terminus of PP1γ that mediates its nucleolar targeting.

An alignment of the first 33 residues of PP1α and PP1c only revealed differences at eight positions (Fig. 3A). To find out which of these residues account for the nucleolar targeting of EGFP-PP1γ, they were substituted for, individually or in group, by the corresponding residues of PP1α (Fig. 3B). Only one of the mutants, namely an EGFP-fusion of PP1γ-R20Q, was not enriched in the nucleoli, indicating that Arg20 is required for the nucleolar localization of PP1γ. PP1β is also nucleolar and has an Arg at the corresponding position (Fig. 4A). Mutation of this residue, as in EGFP-tagged PP1β-R19Q, also abolished the nucleolar targeting of this isoform (Fig. 4B). Conversely, EGFP-PP1α, which is not enriched in the nucleoli, could be directed to the nucleoli by the replacement of Gln20 of PP1α by an Arg (Fig. 4B).

While the above data clearly show that Arg19 for PP1β and Arg20 for PP1γ account for the nucleolar enrichment of these isozymes, they do not provide an insight into the underlying mechanism. Is an Arg per se at this position required for nucleolar targeting or does a Gln at this position, as in PP1α, hamper the nucleolar targeting? To differentiate between these two possibilities, we replaced this N-terminal residue in the three PP1 isoforms by an alanine. In Fig. 5, it is shown that these mutations abolished all detectable differences in the subnuclear localization of the EGFP-fusions. Importantly, all three EGFP-PP1 mutants were enriched in the nucleoli, indicating that an Arg at position 19/20 is not required for the nucleolar targeting but that a Gln at this position opposes the nucleolar enrichment of PP1.

4. Discussion

We have shown here unequivocally that the nucleolar enrichment of PP1β and PP1γ is determined by their N-terminus (Fig. 2). This was a surprising finding since the N-terminus, unlike the less conserved C-terminus, is not known to harbour binding sites for PP1 interactors. Within the N-terminus of PP1α we have mapped a single residue, namely Gln20, that hampered its nucleolar enrichment (Fig. 4). Replacement of this residue by an Arg, which represents the corresponding residue in PP1β or PP1γ, also resulted in a nucleolar enrichment of PP1α. Conversely, PP1β and PP1γ were no longer enriched in the nucleoli when this Arg was replaced by a Gln.
Surprisingly, an Ala at this position abolished all detectable differences in the subnuclear distribution of the PP1 isoforms but still enabled their nucleolar accumulation (Fig. 5). Thus, our data suggest that a Gln at position 19/20 hinders the nucleolar targeting of PP1 but that an Arg at this position does not function as a positive determinant for nucleolar targeting. We therefore hypothesize that the nucleolar enrichment of PP1\(b\) and PP1\(c\) is explained by the interaction of their N-terminus with one or more nucleolar proteins that remain to be identified. This interaction is impeded by the presence of a Gln at position 20, explaining why PP1\(a\) is not enriched in the nucleoli. Our working hypothesis does not rule out the possibility that the putative nucleolar targeting subunit(s) also have additional PP1 binding sites, such as an RVxF-motif, and that the interaction is highly dynamic and subject to regulation.

If Arg19/20 of PP1\(b\) and PP1\(c\), and their flanking sequence, function as an interaction site for nucleolar targeting subunit(s), as we propose here, one expects this sequence to be conserved and solvent accessible. To examine these postulates, we first used sequence similarity searches to identify publicly available metazoan isoforms and aligned them with selected unique fungal sequences. In this manner, we reconstructed the branching sequence of various vertebrate PP1 isoforms (Fig. 6A). The isoforms were grouped in alpha- and beta-subtypes, each forming a branch of the evolutionary tree. The beta-subtype only comprises PP1\(b\) while the alpha-subtype comprises PP1\(a\), PP1\(c\) and a novel fish isozyme that was termed PP1\(e\) (Fig. 6A). An alignment of the N-termini of the PP1 isoforms revealed A-type and B-type specific residues as well as residues that are subtype-specific. Importantly, only PP1\(a\) has a Gln at position 20 while all other isozymes have a basic residue at the corresponding position (Fig. 6B). This indicates that a Gln at position 20, which hampers nucleolar targeting, is a rather recent innovation. We also superimposed the N-termini from the five published crystal structures of mammalian PP1 isoforms\[5,11–13\] by minimal root mean square deviation of the corresponding C-alpha atoms (Fig. 6C). This structural analysis shows that the N-terminus of PP1 is ‘glued’ to the catalytic core with hydrophobic interactions. The hydrophobic residues in the top half of this interface are invariant from ancestral PP1 and are expected to

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rigidly fix the Ca atom of the Gln or Arg at position 20. However, the sidechain of the latter residue is solvens-exposed and flexible, and is therefore accessible for interacting proteins.

In conclusion, we have shown here that a single N-terminal residue determines the extent to which PP1 isoforms accumulate in the nucleoli. Our data also indicate that the N-terminus...
of PPI harbours a binding site for one or more nucleolar targeting subunits.

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