Protein Ser/Thr phosphatase-1 (PP1) is a ubiquitous eukaryotic enzyme that controls numerous cellular processes by the dephosphorylation of key regulatory proteins. PP1 is expressed in various cellular compartments but is most abundant in the nucleus. We have examined the determinants for the nuclear localization of enhanced green fluorescent protein-tagged PP1 in COS1 cells. Our studies show that PP1γI does not contain a functional nuclear localization signal and that its nuclear accumulation does not require Sds22, which has previously been implicated in the nuclear accumulation of PP1 in yeast (Peggie, M. W., MacKelvie, S. H., Blocher, A., Knatko, E. V., Tatchell, K., and Stark, M. J. R. (2002) J. Cell Sci. 115, 195–206). However, the nuclear targeting of PP1 isoforms was alleviated by the mutation of their binding sites for proteins that interact via RXF motifs (6). The RXF motif conforms to the consensus sequence RKX{P}X{P}. The RXF motif (6) is often referred to as the RVXF motif (6). The RXVF motif binds to a hydrophobic channel near the C terminus of PP1 (7). The binding of the RVXF motif not only has a PP1 anchoring function but also promotes the interaction of secondary, lower affinity binding sites, often resulting in an altered activity and/or substrate specificity of PP1 (1).

Mammalian genomes contain three genes that together encode four isoforms of PP1, namely PP1α, PP1β/δ, and the splice variants PP1γI and PP1γII (1, 5). These isoforms are ~90% identical at the protein level and differ mainly in their extremities. With the exception of PP1γII, which is only expressed in the testis and the brain, the mammalian PP1 isoforms are ubiquitously expressed. PP1α, PP1β/δ, and PP1γI show an overlapping but distinct subcellular localization (8). Within the nucleus PP1α is mainly associated with the nuclear matrix, PP1β/δ is enriched in the non-nuclear chromatin fraction, and PP1γI is predominantly targeted to the nucleoli. However, the overexpression of the nuclear interactor NIPP1, which is normally associated mainly with PP1β/δ (9), also results in a re-targeting of other PP1 isoforms to nuclear sites that contain NIPP1 (10), indicating that the steady-state subnuclear localization of PP1 isoforms is at least partially determined by their relative affinities for various targeting proteins. In accordance with this view, it was recently demonstrated that at least some interactors of PP1 contain isoform-specific binding sites (11).

Although PP1 is very abundant in the nucleus, the mechanism underlying its nuclear translocation is unknown. PP1 (36–38 kDa) is sufficiently small to enter the nucleus passively. Yet, this is an unlikely mechanism for its nuclear translocation, because PP1 does not appear to exist in the cell as a free monomer (12). PP1 isoforms contain classical polybasic or bipartite nuclear localization signals (NLSs) that mediate transport by importins (13), but the functionality of these NLSs has never been explored. Another possible mechanism for nuclear translocation is piggyback transport involving the co-transport with an interactor that contains an NLS (14–16). At least four nuclear interactors of PP1 have putative (PNUTS and Sds22) and where the phosphatase acts. Currently, ~70 mammalian genes are already known to encode interactors of PP1 (4, 5). Some of these function as targeting subunits and bring PP1 in close proximity to its substrates. Others (also) modulate the activity and substrate specificity of PP1 or are themselves substrates for associated PP1. The available information suggests that proteins interact with PP1 via multiple short sequence motifs. These PP1-binding motifs can be shared among PP1 interactors, accounting for the ability of PP1 to form stable complexes with a large number of structurally unrelated proteins. The best characterized and most common PP1-binding motif conforms to the consensus sequence RXK{P}X{P}FW in which X denotes any residue and [P] any residue except Pro; it is often referred to as the RVXF motif (6). The RVXF motif binds to a hydrophobic channel near the C terminus of PP1 (7). The binding of the RVXF motif not only has a PP1 anchoring function but also promotes the interaction of secondary, lower affinity binding sites, often resulting in an altered activity and/or substrate specificity of PP1 (1).
with EGFP-tagged PP1 channel. COS1 cells were transfected and assayed with glycogen phosphorylase (22), whereas the pTactac vector encoding human PP1 gen) encoding FLAG-tagged PNUTS was a kind gift of Dr. Y.-G. Kwon.

BamHI site of the pSG5 vector (Stratagene) with a triple FLAG tag XmaI/NotI sites for NIPP1-(143–224). Sds22 was subcloned in the vector the EcoRV sites were used for the cloning of NIPP1 and the subcloned in the pGMEX-T1 vector (Amersham Biosciences) down-

vectors for EGFP-PP1 isoforms. NIPP1 and NIPP1-(143–224) were XhoI and BamHI sites of pEGFP-C1 (Clontech), yielding expression templates. All constructs and mutants were verified by DNA mutagenesis of a putative NLS and interactor binding sites.

mATERIALS AND METHODS

Plasmids and Recombinant Proteins—The full-length sequences of rabbit PP1α and PP1β/δ and rat PP1γ were introduced between the XhoI and BamHI sites of pEGFP-C1 (Clontech), yielding expression vectors for EGFP-PP1 isoforms. NIPP1 and NIPP1-(143–224) were subcloned in the pGMEX-T1 vector (Amersham Biosciences) downstream of the glutathione S-transferase (GST) encoding cassette. In this vector the EcoRI sites were used for the cloning of NIPP1 and the Xmal/NotI sites for NIPP1-(143–224). Sds22 was subcloned in the BamHI site of the pSG5 vector (Stratagene) with a triple FLAG tag cassette inserted in its EcoRI site. The pcDNA1Neo plasmid (Invitro-
gen) encoding FLAG-tagged PNUTS was a kind gift of Dr. Y.-G. Kwon (22), whereas the pTactac vector encoding human PP1γ was kindly provided by Dr. D. Barford (23). The indicated mutants of PP1γ, EGFP-PP1, and GST-NIPP1-(143–224) were made according to the QuikChange protocol (Stratagene) with the appropriate primers and templates. All constructs and mutants were verified by DNA sequencing.

Polyhistidine-tagged NIPP1-(143–224) was expressed in bacteria and purified on Ni²⁺-Sepharose (24). Bacterially expressed human PP1γ and PP1γ-F257A were purified as described by Egloff et al. (23) and assayed with glycojen phosphorylase a as the substrate (24).

Cell Cultures, Immunoprecipitations, and GST Pull-downs—COS1 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum. 48 h after transfection with the indicated plasmids and FuGENE 6 (Roche Molecular Biochemicals), the cells were washed twice with PBS (1.8 mK H₂PO₄, 8.1 mM Na HPO₄, and 150 mM NaCl, pH 7.4) and harvested in a lysis buffer containing 50 mM Tris-HCl, 0.3 mM NaCl, 0.5% Triton X-100, 0.5 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol, 0.5 mM benzamide, and 5 mM leupeptin. Following centrifugation (10 min at 10,000 x g), the supernatants (cell lysates) were used either for the immunoprecipitation of the EGFP-PP1γ fusion proteins with anti-EGFP antibodies (Santa Cruz Biotechnology) and protein A-TSK-Sepharose (Affiland) or for the pull-down of GST-NIPP1-(143–224) with glutathione-agarose (Sigma). Before immuno-

blotting, the EGFP immunoprecipitates were washed once with PBS containing 0.1% Nonidet P-40 and 0.1 mM LiCl and twice with PBS plus 0.1% Nonidet P-40.

Following GST pull-down, the precipitates were analyzed by immuno-

blotting with antibodies against GST (Santa Cruz Biotechnology) or EGFP (Fig. 4). The EGFP immunoprecipitates were used for immuno-

blotting with affinity-purified polyclonal antibodies against PNUTS, NIPP1 (25), and Sds22 (26) (Fig. 2). For the production of anti-PNUTS antibodies, a synthetic peptide (CGDPNQLTRKGRKRKTVTWPEE-GKL) comprising residues 384–407 of PNUTS, and an additional N-terminal cysteine was coupled to keyhole limpet hemocyanin and used to raise antibodies in rabbits. The antibodies were affinity-purified on albumin-coupled peptide linked to CNBr-activated-Sepharose-4B (Am-

ersham Biosciences).

Confocal Microscopy and Immunocytochemistry—24 h after transfe-
sion, the COS1 or HeLa cells were washed twice with PBS and fixed for 10 min with 2% formaldehyde. Cell permeabilization was performed by a 10-min incubation in PBS supplemented with 0.5% Triton X-100. The permeabilized cells were washed three times for 10 min with PBS, pre-incubated for 20 min with PBS containing 3% bovine serum albu-

min, and then incubated for 90 min with either polyclonal antibodies against GST (Santa Cruz Biotechnology) or monoclonal antibodies against the FLAG tag (Stratagene). After three washes of 10 min each with PBS, the cells were incubated for 1 h with secondary anti-rabbit (for GST detection) or anti-mouse antibodies (for FLAG detection) that were labeled with tetramethylrhodamine isothiocyanate (TRITC; Sigma). Finally, the cells were washed three times for 10 min in PBS. Confocal images were obtained with a Zeiss LSM-510 laser-scanning confocal microscope (Jena, Germany) equipped with the Zeiss Axiosvert

or established (NIPP1 and SIPP1) NLSs, making them candidate co-transporters of PP1 (17–20). Also, strains of Saccharo-
ymyces cerevisiae that carried temperature-sensitive sds22 alleles showed a rapid loss of nuclear PP1 under restrictive conditions, providing additional evidence for a role of Sds22 in maintaining the normal nuclear localization of PP1 (21).

We have explored the mechanism of the nuclear transport and nuclear retention of EGFP-tagged PP1γ by site-directed mutagenesis of a putative NLS and interactor binding sites. The results revealed that the nuclear targeting as well as the nuclear retention of PP1γ depends on proteins such as NIPP1 and PNUTS that contain both an NLS and an RVXF-binding motif. Sds22, which lacks an RVXF motif, does not appear to be involved in the nuclear targeting of PP1γ. Consistent with a role for NIPP1 in the nuclear targeting and function of PP1, we also show that nuclear proteins in NIPP1−/− mouse embryos are hyperphosphorylated.

FIG. 1. The nuclear localization of PP1γ requires the RVXF-binding channel. COS1 cells were transfected with EGFP-tagged PP1γ (A), PP1γ-K301A/K303A/K303A (B), PP1γ-(L285–323) (C), or PP1γ-F257A (D). After 24 h the green fluorescence was visualized by confocal microscopy. The white bar in panel A represents 20 μm.
A. Binding of EGFP-PP1γ1 (mutants) to ligands

![Binding of EGFP-PP1γ1 (mutants) to ligands](image)

B. Inhibition of PP1γ1 (-F257A) by NIPP1

![Inhibition of PP1γ1 (-F257A) by NIPP1](image)

**FIG. 2. Mutation of the RVXF-binding channel of PP1γ1 affects its binding to protein interactors.** A, EGFP-tagged PP1γ1, PP1γ1-Δ285–323, and PP1γ1-F257A were immunoprecipitated from COS1 cell lysates. The immunoprecipitates were used for immunoblotting using anti-EGFP, anti-PNUTS, anti-NIPP1, and anti-Sds22 antibodies as detailed under “Materials and Methods.” B, purified, bacterially expressed PP1γ1 (open circles) and PP1γ1-F257A (closed circles) were assayed with glycerogen phosphorylase α as the substrate in the presence of the indicated concentrations of recombinant NIPP1-143–224. The results represent the means ± S.E. (n = 3).

100 μM (Plan Apochromat 40 × 1.30 NA oil immersion objective) using the standard fluorescein isothiocyanate-TRITC filter set. For the simultaneous imaging of EGFP and TRITC fluorescence, both labels were excited in different tracks with the 488-nm line of an argon laser and the 543-nm line of a helium-neon laser, respectively. The emission from each fluorochrome was detected using 505–530-nm (fluorescein thiocyanate) and 560–615-nm (TRITC) band-pass filters.

**Nuclear Retention Assay**—Cells transfected with EGFP-PP1γ1 were washed twice with “transport” buffer containing 20 mM HEPES, 110 mM potassium acetate, 5 mM sodium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 2 mM dithiothreitol (27). Subsequently, the cells were permeabilized for 5 min with digitonin (40 μg/ml) in transport buffer. After a wash with transport buffer, the permeabilized cells were incubated with transport buffer containing 200 μM synthetic peptide NIPP1-191–200 (KNSRVTFSED) or 200 μM variant peptide KNSRATASED. After 15 min, the green fluorescence was visualized by confocal microscopy.

**RESULTS**

The Nuclear Targeting of PP1γ Requires a Functional RVXF-binding Channel—To study the subcellular localization of PP1γ1, we transfected COS1 cells with a construct encoding a fusion of EGFP and rat PP1γ1. In accordance with published data (10), the EGFP-PP1γ1 fusion was largely nuclear and enriched in the nucleoli (Fig. 1A). Two established inactive point mutants of PP1γ1, i.e. EGFP-PP1γ1-D64N and EGFP-PP1γ1-H125A (28), showed the same subcellular distribution (not shown), demonstrating that the protein phosphatase activity of PP1γ1 is not required for its correct localization. The PSORT II program (29) predicts that the C-terminal KKKP sequence (residues 301–304) of PP1γ1 is a candidate NLS. However, the replacement of each of the three lysines in this sequence by an alanine did not affect the nuclear accumulation of EGFP-PP1γ1 (Fig. 1B), indicating that they are not part of a functional NLS. Because PP1γ1 did not appear to contain a functional polybasic or bipartite NLS, we reasoned that PP1γ1 might be piggyback transported to the nucleus by its association with protein interactors that themselves contain one or more functional NLSs. We therefore generated two PP1γ1 variants that are mutated in the RVXF-binding channel, which is involved in the binding of most interactors of PP1 (see Introduction). EGFP-PP1γ1-Δ286–323, which is truncated just before the last β-strand (β4) that lines the RVXF-binding channel (26), was largely cytoplasmic and sometimes showed a speckled distribution in the cytoplasm (Fig. 1C). A more subtle mutation of the RVXF-binding channel, as in EGFP-PP1γ1-F257A, also largely abolished the nuclear accumulation (Fig. 1D). The corresponding mutations in other PP1 isoforms, as in EGFP-PP1o-F257A and EGFP-PP1β-δ-F256A, also resulted in a nuclear exclusion of these fusions (not shown). These data suggest that the RVXF-binding channel is required for the nuclear targeting of all PP1 isoforms. The cytoplasmic localization of the EGFP-PP1 fusions with a mutated RVXF channel also confirms that PP1 has no NLS that enables importin-mediated transport to the nucleus. Identical data as shown in Fig. 1 were also obtained in HeLa cells (not shown).

To examine whether EGFP-PP1γ1-Δ286–323 and EGFP-PP1γ1-F257A indeed display a reduced affinity for RVXF-containing interactors, we performed immunoprecipitations of the EGFP fusions from COS1 cell lysates with anti-EGFP antibodies (Fig. 2A). The immunoprecipitation of EGFP-PP1γ1-Δ286–323 and EGFP-PP1γ1-F257A did not result in the co-immunoprecipitation of two RVXF-containing interactors (NIPP1 and PNUTS) but showed an increased co-immunoprecipitation of Sds22, which does not contain a functional RVXF motif. The increased interaction of EGFP-PP1γ1-Δ286–323 with Sds22 has been reported previously and has been explained by a lack of competition with RVXF-containing interactors of PP1 (26). Our observation that the cytoplasmically retained mutants of EGFP-PP1γ1 show an increased interaction with Sds22 is initial evidence that the nuclear targeting of EGFP-PP1γ1 is not mediated by Sds22.

We have also examined the affinity of bacterially expressed PP1γ1 and PP1γ1-F257A for NIPP1-143–224 (Fig. 2B). The interaction of NIPP1-143–224 with PP1 is entirely dependent on its RVXF motif and can be easily monitored by phosphatase inhibition assays (24). In Fig. 2B it is shown that PP1γ1-F257A indeed showed a reduced sensitivity to inhibition by NIPP1-143–224. Bacterially expressed PP1γ1-Δ286–323 was inactive and could therefore not be tested in this system.

**Nuclear Re-targeting of EGFP-PP1γ1-F257A by the Overexpression of Nuclear Interactors with an RVXF Motif**—The above data are consistent with the view that PP1γ1 is piggyback transported to the nucleus by association with proteins that contain both a PP1-binding RVXF motif and an NLS. In further agreement with this hypothesis we found that the cytoplasmic EGFP-PP1γ1-F257A, which only showed a moder-
ately decreased affinity for RVXF motifs (Fig. 2B), could be re-targeted to the nucleus by the co-expression of a GST fusion of NIPP1-(143–224) (Fig. 3C), a protein with an established NLS as well as a PP1-binding RVXF motif (19). Importantly, the co-expression of GST-NIPP1-(143–224)-V201A/F203A, which has a mutated RVXF motif but still contains a functional NLS, did not affect the cytoplasmic accumulation of EGFP-PP1γ1-H92531-F257A (Fig. 3D). Intriguingly, the expression of GST-NIPP1-(143–224) re-targeted EGFP-PP1γ1-F257A to the nucleus but not to the nucleoli (Fig. 3C). However, wild-type PP1γ1 (EGFP-PP1γ1) also was no longer enriched in the nucleoli following the expression of GST-NIPP1-(143–224) (Fig. 3A). A similar subnuclear redistribution of PP1γ1 has been described following the expression of full-length NIPP1 (10) and can be accounted for by the accumulation of NIPP1 outside the nucleoli. As expected, the expression of GST-NIPP1-(143–224) with a mutated RVXF motif did not affect the subcellular distribution of EGFP-PP1γ1 (Fig. 3B).

GST pull-down experiments from COS1 cell lysates confirmed that GST-NIPP1-(143–224) interacted much better with EGFP-PP1γ1 than with EGFP-PP1γ1-F257A, whereas EGFP-PP1γ1-Δ285–323 had no affinity at all for GST-NIPP1-(143–224) (Fig. 4). These data explain why the overexpression of GST-NIPP1-(143–224) resulted in a nuclear accumulation of EGFP-PP1γ1-F257A (Fig. 3C) but had no clear effect on the subcellular distribution of EGFP-PP1γ1-Δ285–323 (not shown).

We have subsequently compared the ability of the major nuclear interactors of PP1 to re-target EGFP-PP1γ1-F257A to the nucleus. EGFP-PP1γ1-F257A remained cytoplasmic when FLAG-tagged Sds22 was co-expressed despite the fact that this fusion was partially nuclear (Fig. 5C) and bound very tightly to EGFP-PP1γ1-F257A (not shown). These data show that the nuclear targeting of PP1γ1 is likely to be mediated by various nuclear interactors with an RVXF motif. In contrast, PP1 interactors such as Sds22 that are nuclear but lack an RVXF motif do not seem to be capable of co-transporting PP1 to the nucleus.

The Nuclear Retention of PP1γ1 Is Dependent on Interactors with an RVXF Motif—To examine whether the nuclear retention of EGFP-PP1γ1 is also dependent on its association with RVXF-containing interactors, we have added the RVXF-containing peptide KNSRVTFSED, corresponding to NIPP1-(191–224) to the

FIG. 3. Nuclear re-targeting of PP1γ1-F257A by NIPP1-(143–224). COS1 cells were grown for 24 h on glass coverslips and then transfected with EGFP-PP1γ1 (A and B) or EGFP-PP1γ1-F257A (C and D) and either GST-NIPP1-(143–224) (A and C) or GST-NIPP1-(143–224)-V201A/F203A (B and D). After 24 h the cells were fixed and analyzed for the presence of EGFP by green fluorescence microscopy (left column) and the presence of GST by immunocytochemistry with anti-GST antibodies and TRITC-labeled secondary antibodies (middle column). The right column contains the overlay pictures. The white bar in panel A represents 20 μM.
to digitonin-permeabilized cells. Digitonin was added at concentrations (40 μg/ml) known to perforate the plasma membrane without affecting the nuclear envelope or other major intracellular membranes (27). In Fig. 6B it is shown that the addition of KNSRVTFSED, which is expected to compete with endogenous RVXF-containing proteins for binding to PP1, resulted in a complete loss of EGFP-PP1γ₁ from the nucleus. By contrast, a similar peptide with a mutated RVXF motif (KN-SRATASED) did not have any effect on the nuclear localization of EGFP-PP1γ₁ (Fig. 6C). Thus, the nuclear retention of PP1γ₁ also depends on its interaction with RVXF-containing proteins.

A Deficiency of NIPP1 Is Associated with a Hyperphosphorylation of Nuclear Proteins—To examine whether the nuclear targeting and/or retention of endogenous PP1 also depends on proteins with an RVXF motif, we have initially tried to make use of commercially available PP1 antibodies to visualize endogenous PP1 after the overexpression of NIPP1 or PNUTS. However, PP1 antibodies from three different commercial sources could not be used for the immunodetection of endogenous PP1 in intact cells (not shown), in accordance with observations by Trinkle-Mulcahy et al. (30). As an alternative approach, we have therefore examined the consequence of the targeted disruption of the NIPP1 alleles in mice on the phosphorylation level of nuclear proteins, using anti-phosphothreonine antibodies. Indeed, if NIPP1 would be required for the nuclear targeting and/or retention of PP1, one would expect that the substrates of NIPP1-associated PP1 would be hyperphosphorylated in NIPP1−/− cells. The targeted disruption of the NIPP1 alleles is associated with growth retardation and is embryonic lethal at ~7 dpc (31). In Fig. 7 it is shown that the NIPP1−/− embryos at 6.5 dpc displayed a considerably increased nuclear protein phosphorylation level as compared with that of the wild-type embryos at 6.5 dpc. Wild-type embryos at 5.5 dpc, which correspond in size to the NIPP1−/− embryos at 6.5 dpc, did not show a nuclear hyperphosphorylation on threonine (not illustrated). These data are consistent with a role for NIPP1 in the nuclear targeting and/or retention of PP1 and also show that NIPP1-associated PP1 has nuclear substrates. The latter conclusion also fits in nicely with previous observations that the NIPP1-PP1 complex, albeit inactive

---

**Fig. 4.** Reduced affinity of EGFP-PP1γ₁ mutants for NIPP1-(143-224). COS1 cells in 10-cm Petri dishes were transfected as indicated in Fig. 3. The GST fusions were pulled down with glutathione-agarose, and the pellets were analyzed by immunoblotting with anti-GST and anti-EGFP antibodies (Ab).

**Fig. 5.** Nuclear targeting of PP1γ₁-F257A by RVXF-containing nuclear interactors of PP1. COS1 cells were transfected with EGFP-PP1γ₁-F257A and GST-NIPP1 (A), FLAG-PNUTS (B), or FLAG-Sds22 (C). After 24 h the cells were fixed and analyzed for the presence of EGFP by green fluorescence microscopy (left column) and the presence of GST or FLAG by immunocytochemistry with anti-GST/FLAG antibodies and TRITC-labeled secondary antibodies (middle column). The right column contains the overlay pictures. The white bar in panel A represents 20 μm.
when reconstituted in vitro, can become an active protein phosphatase by the phosphorylation of NIPP1 (24).

DISCUSSION

By far, most proteins enter the nucleus either passively or by importin-mediated transport. However, neither of these mechanisms can account for the nuclear accumulation of PP1. Indeed, there is no evidence for the existence of free PP1, and none of the PP1 holoenzymes are small enough to diffuse through the nuclear pores passively. Also, PP1\(^{+/}\) contains a consensus NLS, but this motif is not conserved in other PP1 isoforms (13) and its mutation did not affect the nuclear accumulation of PP1\(^{+/}\) (Fig. 1B). We have obtained data strongly indicating that PP1 is translocated to the nucleus by the association with proteins that contain both an NLS and a PP1-binding RV\(\times\)F motif. First, a deletion or point mutation of the RV\(\times\)F-binding channel of PP1 isoforms abolished the nuclear targeting of the phosphatase. Second, a PP1\(^{+/}\) mutant with a decreased affinity for the RV\(\times\)F motif (PP1\(^{+/}\)F257A) and a cytoplasmic accumulation could be re-targeted to the nucleus by the overexpression of nuclear interactors with a functional RV\(\times\)F motif (Fig. 3). However, PP1\(^{+/}\) (Δ286–323), which did not bind at all to RV\(\times\)F-containing interactors (Fig. 4), could not be re-targeted to the nucleus. Both NIPP1 and PNUTS/R111, which represent the major nuclear interactors of PP1 in mammalian cells (9), were able to translocate PP1\(^{+/}\)F257A to the nucleus, suggesting that they both represent true “nuclear targeting subunits” of PP1. Consistent with this view, we found that the disruption of the NIPP1 genes was associated with a nuclear hyperphosphorylation of proteins on threonine in mouse embryos. Interestingly, the established NLS of NIPP1-(143–224) is just N-terminal to the RV\(\times\)F motif (19) and has also been implicated in the potent inhibition of PP1 by NIPP1 (32). Our finding that NIPP1-(143–224) and PP1\(^{+/}\)F257A are co-transported (Fig. 3) strongly suggests that the binding of importin-α to this NLS and the binding of PP1 to the RV\(\times\)F motif are not mutually exclusive.

*S. cerevisiae* only expresses a single PP1 isoform termed Glc7. In accordance with our data, Glc7-F256A, which corresponds to PP1\(^{+/}\)F257A, also showed a decreased affinity for interactors with an RV\(\times\)F motif but still interacted with Sds22 (33). Glc7-F256A also showed some subcellular localization deficiencies but, at variance with our results, Glc7-F256A was still associated with the nucleoli. We suggest that the cytoplasmic retention of PP1\(^{+/}\)F257A in mammalian cells is accounted for by the competition of other PP1 isoforms for binding to RV\(\times\)F-containing interactors. In contrast, for the lack of competing PP1 isoforms Glc7-F256A can still accumulate in the nucleus in yeast.

PP1\(^{+/}\)F257A could not be re-targeted to the nucleus by the overexpression of Sds22 (Fig. 5), which interacts with a fragment of PP1 that is remote from the RV\(\times\)F-binding channel (26). This was an unexpected finding, because FLAG-Sds22 is
Nuclear Targeting and Retention of PP1

partially nuclear (Fig. 5C), is associated with PP1 in the nucleus (34), and interacts even better with PP1γ1-F257A and PP1γ1(Δ286–323) than with wild-type PP1 (Fig. 2A). Our data therefore suggest that Sds22 migrates to the nucleus either independently of PP1 or as part of a complex that also contains a protein with a PP1-binding RVXF motif.

The (sub)nuclear targeting and retention of EGFP-PP1γ1 also appears to be mediated by RVXF-containing interactors, because virtually no EGFP-PP1γ1 was left in the nucleus after a short incubation of permeabilized cells with an RVXF peptide (Fig. 6B). Whereas some EGFP-PP1γ1 may have leaked out of the permeabilized cells, a considerable fraction remained cytoplasmic. These data indicate that there exist one or more cytoplasmic proteins that can bind PP1 independently of an RVXF motif and that are associated with structures (the cytoskeleton?) that do not leak out of permeabilized cells. Preliminary biochemical assays indeed provide evidence for the existence of a cytoplasmic protein or proteins that bind and inhibit exogenous purified PP1 in the presence of an RVXF peptide (not illustrated). We speculate that such inhibitory proteins keep newly synthesized PP1 from accidentally dephosphorylating proteins until it can form a holoenzyme by association with an RVXF-containing interactor.

Acknowledgments—We thank Nicole Sente for expert technical assistance and Prof. W. Stalmans for continued support.

REFERENCES

1. Bollen, M. (2001) Trends Biochem. Sci. 26, 426–431
2. Bollen, M., and Beullens, M. (2002) Trends Cell Biol. 12, 138–145
3. Ceulemans, H., Stalmans, W., and Bollen M. (2002) BioEssays 24, 371–381
4. Cohen, P. T. W. (2002) J. Cell Sci. 115, 241–256
5. Ceulemans, H., and M. Bollen. (2003) Physiol. Rev. 84, 1–39
6. Waksal, P., Beullens, M., Ceulemans, H., Stalmans, W., and Bollen, M. (2003) J. Biol. Chem. 278, 18817–18823
7. Egloff, M.-P., Johnson, D. F., Moorhead, G., Cohen, P. T. W., Cohen, P., and Barford, D. (1997) EMBO J. 16, 1876–1887
8. Andreasen, P. R., Lacroix, F. B., Villa-Moruzzi, E., and Margolis, R.L. (1998) J. Cell Biol. 141, 1207–1215
9. Jagiello, I., Beullens, M., Stalmans, W., and Bollen, M. (1995) J. Biol. Chem. 270, 17257–17263
10. Trinkle-Mulcahy, L., Sleeman, J. E., and Lamond, A. I. (2001) J. Cell Sci. 114, 2191–2202
11. Carmody, L. C., Bauman, P. A., Bass, M. A., Mavila, N., DePauli-Roach, A. A., and Colbran, R. J. (2004) J. Biol. Chem. 279, 21714–21723
12. Bollen, M., and Stalmans, W. (1992) Crit. Rev. Biochem. Mol. Biol. 27, 227–281
13. Okano, K., Heng, H., Trevisanato, S., Tyers, M., and Varmus, S. (1997) Genomics 45, 211–215
14. Zhao, L. J., and Padmanabhan, R. (1998) Cell 95, 1005–1015
15. Purpur, P., Hay, R. T., and Dargemont, C. (1999) J. Biol. Chem. 274, 6604–6612
16. Mizuno, T., Okamoto, T., Yoko, M., Izumi, M., Kobayashi, A., Hachiya, T., Tamai, K., Inoue, T., and Hanashita, F. (1996) J. Cell Sci. 109, 2627–2636
17. Allen, P. B., Kwon, Y.-G., Nairn, A. C., and Greengard, P. (1996) J. Biol. Chem. 273, 4089–4095
18. Stone, E. M., Yamano, H., Kinoshita, N., and Yanagida, M. (1993) Curr. Biol. 3, 13–28
19. Jagiello, I., Van Eynde, A., Vulteke, V., Beullens, M., Boudrez, A., Keppens, S., Stalmans, W., and Bollen, M. (2000) J. Cell Sci. 113, 3761–3764
20. Lorian, M., Beullens, M., André, I., Ortiz, J.-M., and Bollen, M. (2004) Biochem. J. 378, 229–238
21. Peggie, M. W., MacKelvie, S. H., Bleecker, A., Knatko, E. V., Tatchell, K., and Stark, M. J. R. (2002) J. Cell Sci. 115, 195–206
22. Kim, Y.-M., Watanabe, T., Allen, P. B., Kim, Y.-M., Lee, S.-J., Greengard, P., Nairn, A. C., and Kwon, Y.-G. (2003) J. Biol. Chem. 278, 13819–13828
23. Egloff, M.-P., Cohen, P. T. W., Reizner, P., and Barford, D. (1995) J. Mol. Biol. 254, 942–959
24. Beullens, M., Vulteke, V., Van Eynde, A., Jagiello, I., Stalmans, W., and Bollen, M. (2000) Biochem. J. 352, 651–658
25. Van Eynde, A., Wera, S., Beullens, M., Torrekens, S., Van Leuven, F., Stalmans, W., and Bollen, M. (1995) J. Biol. Chem. 270, 28068–28074
26. Ceulemans H., Vulteke V., De Maeyer M., Tatchell K., Stalmans, W., and Bollen M. (2002) J. Biol. Chem. 277, 47331–47337
27. Adam, S. A., Marr, R. S., and Gerae, L. (1999) J. Cell Biol. 111, 807–816
28. Zhang, J., Zhang, Z., Brew, K., and Lee, E. Y. C. (1996) Biochemistry 35, 6276–6282
29. Nakai, K., and Horton, P. (1999) Trends Biochem. Sci. 24, 34–35
30. Trinkle-Mulcahy, L., Andrews, P. D., Wickraminage, S., Sleeman, J., Prescott, A., Wab Lam, Y., Lyon, C., Swedlow, J. R., and Lamond, A. I. (2003) Mol. Biol. Cell 14, 107–117
31. Van Eynde, A., Nuytten, M., Dewerchin, M., Schoonjans, L., Keppens, S., Beullens, M., Moons, L., Carmeliet, P., Stalmans, W., and Bollen, M. (2004) Mol. Cell. Biol. 24, 5863–5874
32. Beullens, M., Van Eynde, A., Vulteke, V., Connor, J., Shenolikar, S., Stalmans, W., and Bollen, M. (1999) J. Biol. Chem. 274, 14053–14061
33. Wu, X., and Tatchell, K. (2001) Biochemistry 40, 7410–7420
34. Dinischiotu, A., Beullens, M., Stalmans, W., and Bollen M. (1997) FEBS Lett. 402, 141–144
