Dynamic targeting of protein phosphatase 1 within the nuclei of living mammalian cells

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SUMMARY

Protein phosphatase 1 (PP1) is expressed in mammalian cells as three closely related isoforms, α, β/δ and $\gamma 1$, which are encoded by separate genes. It has yet to be determined whether the separate isoforms behave in a similar fashion or play distinct roles in vivo. We report here on analyses by fluorescence microscopy of functional and fluorescently tagged PP1 isoforms in live cells. PP1α and PP1γ fluorescent protein fusions show largely complimentary localization patterns, particularly within the nucleus where tagged PP1γ accumulates in the nucleolus, whereas tagged PP1α is primarily found in the nucleoplasm. Overexpression of NIPP1 (nuclear inhibitor of PP1), a PP1 targeting subunit that accumulates at interchromatin granule clusters in the nucleoplasm, results in a retargeting of both isoforms to these structures, indicating that steady-state localization is based, at least in part, on relative affinities for various targeting subunits. Photobleaching analyses show that PP1γ is rapidly exchanging between the nucleolar, nucleoplasmic and cytoplasmic compartments. Fluorescence resonance energy transfer (FRET) analyses indicate that the direct interaction of the two proteins predominantly occurs at or near interchromatin granule clusters. These data indicate that PP1 isoforms are highly mobile in cells and can be dynamically (re)localized through direct interaction with targeting subunits.

Key words: PP1, Isoforms, Fluorescent

INTRODUCTION

Hundreds of protein kinases and protein phosphatases are involved in changing the phosphorylation states of proteins, and they collectively control a wide range of phosphorylation-mediated cellular events, including division, signalling, differentiation and metabolism. These enzymes are organized both spatially and temporally, and they can change their intracellular localization dynamically during cell cycle progression and other processes (Inagaki et al., 1994). In the case of the serine/threonine protein phosphatase 1 (PP1), which is involved in multiple regulatory events throughout the cell, substrate specificity is likely to be determined both by subcellular localization and by affinity for proteins that bring the enzyme in proximity to the substrate. These proteins have been termed 'targeting subunits' (Hubbard and Cohen, 1993), and several reports have confirmed their importance for the regulation of PP1 activity. In many cases, however, it remains to be established whether interactions identified in vitro actually occur within living cells and in which intracellular locations these interactions take place. It is therefore important to find experimental approaches that permit direct analyses of PP1-targeting subunit interactions in live cells in order to understand in detail how the localization and activity of this phosphatase is controlled.

Complexity is further increased as PP1 is found as three isoforms (α, β/δ and $\gamma 1$), each encoded by distinct genes, in the mammalian cell (Sasaki et al., 1990; Barker et al., 1993; Barker et al., 1994). These isoforms are more than 89% identical in amino acid sequence, most of the differences

being at the N- and C-termini. One complication in studying the specific isoforms is that, when expressed in *Escherichia coli* as recombinant proteins, the PP1α and PP1β/δ isoforms show different properties to their native, purified forms (Alessi et al., 1993). Most of the in vitro analyses have therefore focused on PP1γ. The subcellular localization of endogenous PP1 isoforms is not entirely clear. Anti-peptide antibodies have been generated against the divergent C-terminal regions of PP1α, β/δ and γ isoforms (da Cruz e Silva et al., 1995; Villa-Moruzzi et al., 1996), and immunolocalization studies with these anti-peptide antibodies suggest distinct localization patterns for the three PP1 isoforms within the cell. PP1α is diffuse in the cytoplasm and nucleoplasm and accumulates in unidentified nuclear bodies; PP1β/δ is diffusely distributed throughout the nucleus and cytoplasm; and PP1γ is diffuse in the cytoplasm and nucleoplasm and accumulates within nucleoli (Andreassen et al., 1998). Other studies, however, have reported different localization patterns using independently isolated isoform-specific antibodies (Kotani et al., 1998; Haneji et al., 1998) and argue that PP1β/δ, and not PP1γ, is the isoform that accumulates within the nucleolus. Attempts to link a specific PP1 isoform to a particular substrate led to conflicting reports in the literature, one describing the specific association of PP1α with the retinoblastoma gene product (Rb) (Tamrakar and Ludlow, 2000) and others describing the association of PP1β/δ with the same protein, Rb (Puntoni and Villa-Moruzzi, 1997; Puntoni and Villa-Moruzzi, 1999). A potential problem in studying the endogenous PP1 isoforms is the cross-reactivity of some of the available anti-isoform antibodies, which could explain

conflicting reports in the literature, but it cannot be ruled out that differences in cell type and/or experimental conditions play a role. Also, there is now evidence that all three isoforms can interact with Rb, but they may show different site-specific preferences within the substrate (Rubin et al., 2001).

If separate PP1 isoforms indeed vary in their substrate specificity and/or localization, a reasonable assumption would be that this is due to varying affinities for regulatory subunits, which have distinct subcellular distributions themselves. PP1β/δ, for example, appears to preferentially associate with the glycogen- and myosin-targeting subunits in skeletal muscle (Berndt et al., 1987; Dent et al., 1992; Alessi et al., 1992). Although these results could reflect the binding of PP1 isoforms to distinct sets of targeting subunits, they could also be explained by the isoforms sharing similar affinities for all targeting subunits. However, they may be expressed at different levels or compartmentalized in some other way, for example, through their coordinated expression with a particular targeting subunit.

Although several groups have shown that targeting subunits exogenously expressed in mammalian cells can form complexes with endogenous PP1 (Trinkle-Mulcahy et al., 1999; MacMillan et al., 1999; Kim et al., 2000; Jagiello et al., 2000), it not yet clear whether a PP1 molecule that has already formed a complex with one targeting subunit can be recruited in vivo by another targeting subunit. The formation of new complexes may rely on the expression of new protein instead. It is therefore important to determine where in the cell these complexes form. In the case of nuclear targeting subunits, for example, the question arises of whether the proteins come into contact with each other in the cytoplasm as the protein is translated or whether they contact each other at the targeting sites where the proteins localize under steady-state conditions. Alternatively, they may come into contact at another location.

To study the properties of individual PP1 isoforms in living cells, including their localization and interaction with targeting subunits, we have taken advantage of the in vivo approach of fusing PP1 to fluorescent reporter molecules. This method, which effectively 'tags' the intracellular pool of the protein and allows analyses of dynamic properties in living cells, has been successfully used to study the dynamic localization of the *Saccharomyces cerevisiae* homologue of PP1, GLC7 (Bloecher and Tatchell, 2000). The three mammalian PP1 isoforms, α, β/δ and γ1, were expressed in mammalian cells as functional fusion proteins with either enhanced green, enhanced yellow or enhanced cyan fluorescent protein (EGFP/EYFP/ECFP), all of which are chromatic variants of the green fluorescent protein (GFP) derived from the jellyfish *Aequorea victoria* (Ellenberg, 1999).

To study the interaction of the three PP1 isoforms with a targeting subunit, we chose one of the major nuclear proteins with which PP1 interacts – nuclear inhibitor of PP1 (NIPP1). This protein binds tightly to PP1 both in vitro and in vivo, and can prevent it from dephosphorylating a number of radioactively labeled phosphoproteins (Van Eynde et al., 1995; Vulsteke et al., 1997; Trinkle-Mulcahy et al., 1999). When expressed as a fluorescent-protein fusion in mammalian cells, NIPP1 shows a speckled nucleoplasmic distribution, where it is colocalized with several pre-mRNA splicing factors (Trinkle-Mulcahy et al., 1999). Like most PP1-targeting subunits

described to date, NIPP1 contains an Arg/Lys-Val/Ile-Xaa-Phe/Trp motif important for interaction with PP1 (Egloff et al., 1997). Mutation of one or both of the conserved hydrophobic residues disrupts the interaction of NIPP1 with PP1 without affecting the intracellular localization of the protein (Trinkle-Mulcahy et al., 1999).

Here we have used fluorescent-protein-PP1 (FP-PP1) and fluorescent-protein-NIPP1 (FP-NIPP1) fusion proteins to investigate, in vivo, the organization of the separate PP1 isoforms. Importantly, the use of EYFP and ECFP as fluorescent tags not only permits visualization of the localization of the phosphatases and of their targeting subunits in the same cell, but allows monitoring of direct protein-protein interactions via fluorescence resonance energy transfer (FRET) (Pollock and Heim, 1999), and monitoring of protein dynamics using fluorescence recovery after photobleaching (FRAP) and fluorescence loss in photobleaching (FLIP) techniques [for review see White and Stelzer (White and Stelzer, 1999)]. The data show that PP1 isoforms have distinct intracellular localizations, but they can be dynamically retargeted through high affinity interactions with a targeting subunit showing a different localization pattern.

MATERIALS AND METHODS

Materials
Tissue culture reagents were obtained from Life Technologies Inc. (Paisley, UK) and Protein G-Sepharose from Pharmacia (Milton Keynes, UK). Custom oligonucleotides were purchased from Oswel and MWG Biotech. Anti-PP1 was obtained from Transduction Laboratories.

PP1 cloning
PP1β/δ and PP1γ1 were cloned from a human HeLa cell library (Clontech) and PP1α from an expressed sequence tag (Image clone 3503172, HGMP) using specific oligonucleotide primers. The clones were inserted into the enhanced green, enhanced yellow and enhanced cyan fluorescent protein vectors (EGFP-C1, EYFP-C1 and ECFP-C1, Clontech). The DNA constructs were confirmed by restriction analysis and DNA sequencing (performed by The Sequencing Service, University of Dundee, http://www.dnaseq.co.uk). Catalytically inactive PP1γ was generated by PCR mutagenesis (Quickchange, Stratagene) of His residue 125 in the catalytic site of EYFP-PP1γ to an Ala residue. This mutation was shown previously to abolish in vitro phosphatase activity (Helps et al., 2000).

NIPP1 and U1A cloning
Wild-type NIPP1 was obtained as previously described (Trinkle-Mulcahy et al., 1999) and subcloned from the enhanced green fluorescent protein (EGFP-C1) vector into the EYFP-C1 and ECFP-C1 vectors using unique *Eco*RI and *Sal*I restriction sites. For these experiments we employed a NIPP1(V201K/F203K) mutant that behaves in a similar fashion to the NIPP1(V201A/F203A) mutant previously published (Trinkle-Mulcahy et al., 1999). U1A was obtained as described previously (Sleeman et al., 1998) and subcloned into the EYFP-N1 vector using unique *Eco*RI and *Kpn*I restriction sites.

Transient expression of fluorescent-protein-tagged PP1 isoforms in mammalian cells
Plasmids were transiently transfected into 10 cm dishes of HeLa (human cervical carcinoma) cells using Effectene transfection reagent (QIAGEN) and 1 µg DNA/10 cm dish. For double transfections, 0.5

µg of each plasmid was added per 10 cm dish. After 12-16 hours, the cells were examined by microscopy, either live in a heated chamber or paraformaldehyde-fixed and mounted in Mowiol-DABCO. In some experiments, the plasmids were directly microinjected into the cells at a low concentration (7-15 µg/ml), which allowed tighter control of both protein expression levels and time of detection (from two hours onward). The data are not presented here but were in agreement with those obtained by transient transfection. The plasmids were also transiently transfected into human embryonic kidney 293 cells using a modified calcium phosphate-mediated tranfection procedure with 2 µg DNA/10 cm dish. After 12-16 hours of expression, total cell lysates were prepared by lysis in 0.5 ml of ice-cold 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1% (by vol.) Nonidet P-40, 1% (by mass) sodium deoxycholate, 0.1% (by mass) SDS, 2 mM EDTA plus Complete protease inhibitor cocktail (Roche, one tablet per 25 ml). The lysate was passed through a QIAShredder column (QIAGEN) to break up the DNA and then cleared by centrifuging at 4°C for five minutes at 13,000 *g*.

Phosphatase assays

Lysates from HEK 293 cells expressing fluorescent-protein-tagged PP1α, PP1β/δ, PP1γ, PP1γ(H125A) or fluorescent protein alone (0.5 ml, 6 mg/ml protein) were incubated for one hour on a shaking platform with 25 µl Protein G-Sepharose coupled to 10 µg anti-GFP monoclonal antibody (Roche; also recognizes EGFP, EYFP and ECFP). The beads were washed three times with lysis buffer, once with phosphatase assay buffer, and then assayed for phosphorylase a phosphatase activity as described previously (Cohen et al., 1988).

Microscopy

The localization of fluorescently tagged proteins was examined using either a Zeiss 410 confocal microscope, a Zeiss Axioplan microscope with a cooled CCD camera running IP Lab Spectrum imaging software (Digital Pixel) or a Zeiss Axiovert-DeltaVision Restoration Microscope (Applied Precision, Inc.) running SoftWorx collection and imaging software. In the case of ECFP and EYFP co-transfections, specific excitation and emission filters (Chroma) were used to resolve the two signals.

Heterokaryons

HeLa cells expressing fluorescently tagged constructs were trypsinized to lift them off the dish, mixed with cells expressing a different construct and allowed to settle back onto glass coverslips in 6 cm dishes. After four to six hours, the cells were rinsed in PBS and covered with a thin layer of 50% PEG solution (Sigma) with gentle rocking for 90 seconds to allow fusion. The PEG solution was removed by repeated rinses with media and the cells allowed to recover for two to four hours. They were then viewed live in a heated chamber or fixed and mounted as described above.

Photobleaching analysis

EYFP-PP1γ was transiently expressed in HeLa cells and imaged live in a heated chamber on a Zeiss 410 confocal laser scanning microscope. The entire field of view was first scanned at low laser power, and then, for the region of interest selected, a horizontal line was scanned at full laser intensity to photobleach the YFP fluorophore. For FRAP experiments, following this photobleaching, the field of view was then scanned at low laser power every three seconds to monitor recovery of fluorescent signal within the photobleached region. For FLIP experiments, following the initial photobleaching, the field of view was scanned once at low laser power and then the region of interest was photobleached a second time at full laser intensity. This cycle was repeated every five seconds until all the fluorescent signal was lost from the cell.

FRET measurement

The data used for FRET measurement were collected using the
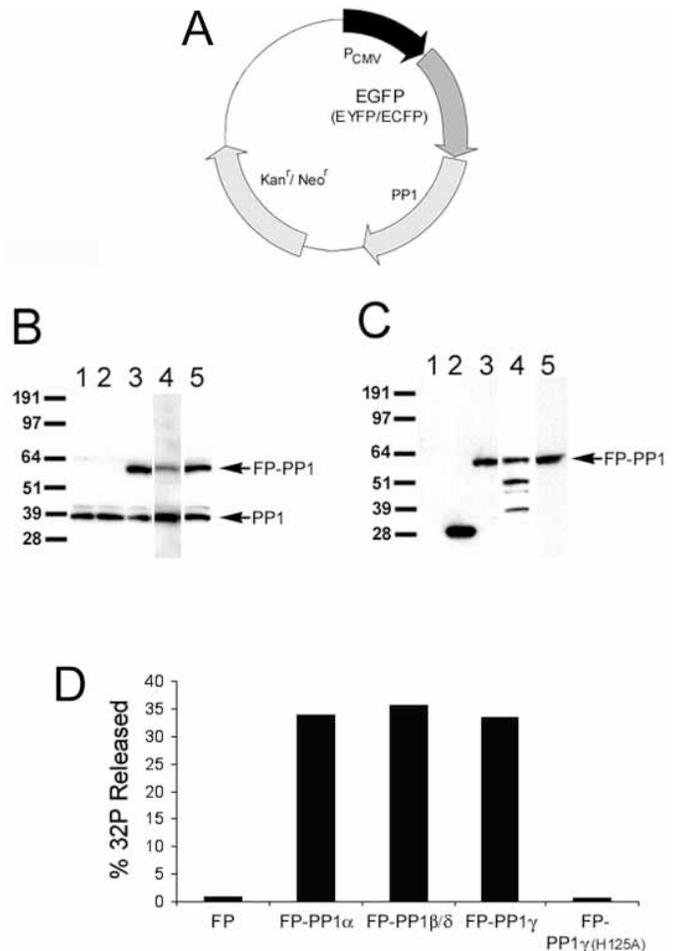
Fig. 1. Expression of FP-tagged PP1 isoforms in transiently transfected mammalian cells. (A) Cartoon depicting the plasmids constructed for expression of EGFP, EYFP or ECFP PP1α, β/δ and γ. (B) Western blot showing the bands detected with anti-PP1 antibodies in lysates (30 µg total protein) from non-transfected HEK293 cells (lane 1), and from cells transiently expressing EYFP alone (lane 2), EYFP-PP1α (lane 3), EGFP-PP1β/δ (lane 4) or EYFP-PP1γ (lane 5). Arrows point to expressed and endogenous PP1. (C) Western blot showing the bands detected with anti-GFP antibodies in the same lysates. (D) In vitro phosphorylase a phosphatase activity associated with EYFP, EYFP-PP1α, EGFP-PP1β/δ, EYFP-PP1γ and EYFP-PP1γ(H125A) immunoprecipitated from these lysates using anti-GFP/YFP antibodies.

DeltaVision system, which allowed independent control of excitation and emission filters. The signals measured in the FRET channel (excitation at 436 nm, emission at 528 nm) were corrected for crosstalk from the cyan (excitation 436 nm, emission 488 nm) and yellow (excitation 517 nm, emission 528 nm) channels using the following equation:

$$\text{Net Energy Transfer} = \text{FRET signal} - \alpha(\text{Cyan Signal}) - \beta(\text{Yellow Signal})$$

For this equation, α and β were determined by measuring the crossover into the FRET channel of the cyan and yellow signals, respectively, in cells expressing each fusion protein on its (Kam et al., 1995). In our system, approximately 67% of the cyan signal and 19% of the yellow signal was detected in the FRET channel.
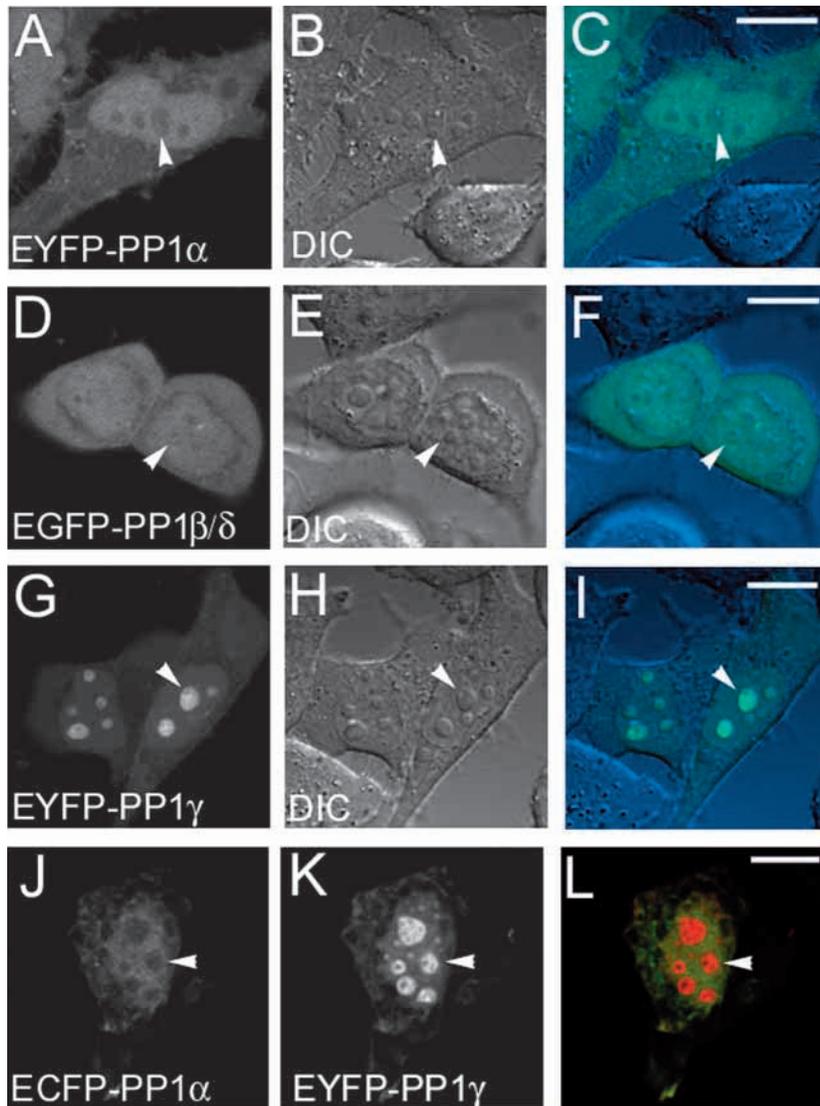
Fig. 2. Localization of FP-tagged PP1 isoforms in transiently transfected HeLa cells. Panel A shows the localization of EYFP-PP1α in live HeLa cells, with an accompanying DIC (differential interference contrast) image in Panel B and the two images merged in Panel C. EGFP-PP1β/δ localization is shown the same way in Panels D-F, and EYFP-PP1γ localization in Panels G-I. PP1α and PP1γ maintain their specific localization patterns when co-expressed in the same cells as ECFP-PP1α (Panel J) and EYFP-PP1γ (Panel K). Panel L shows the two patterns merged, with ECFP-PP1α pseudocolored green and EYFP-PP1γ pseudocolored red. Arrowheads indicate nucleoli. Scale bars are 10 μM.

fusion proteins (63 kDa) were detected by PP1-specific antibodies, along with endogenous PP1 (39 kDa) (Fig. 1B). Lysates from control cells, either non-transfected (lane 1) or expressing FP alone (lane 2), are shown for comparison. The identities of the FP-PP1 fusion proteins were confirmed using GFP-specific antibodies (Fig. 1C). In the case of FP-PP1β/δ, two additional, smaller, GFP-positive bands were detected, suggesting that a subset of the fusion protein may be cleaved or modified within the cell, leading to a loss of the epitope recognized by the PP1 antibody.

To determine whether the fluorescent-protein-tagged phosphatases were catalytically active, both FP alone and the respective FP-PP1 fusion proteins were immunoprecipitated from these lysates under stringent conditions (see Materials and Methods) and assayed for phosphorylase phosphatase activity (Fig. 1D). This shows the high levels of phosphatase activity recovered for EYFP-PP1α, EGFP-PP1β/δ and EYFP-PP1γ constructs and not for EYFP alone. The lack of phosphatase activity with an H125A point mutation of PP1γ, known to disrupt the phosphatase activity of recombinant PP1γ (Helps et al., 2000), served as an additional control to show that the phosphatase activity is not the result of co-immunoprecipitation of endogenous enzyme. Although it is tempting to relate the phosphatase activities in this figure to the activity of endogenous PP1, the heterogenous nature of the transfected cell population prevents this type of quantitative analysis. Nonetheless, the activities measured provide clear evidence that the fusion proteins are functional phosphatases.

RESULTS

Expression of functional fluorescent-protein-tagged PP1α, PP1β/δ and PP1γ in transiently transfected mammalian cells

PP1α and PP1γ were cloned, in frame, downstream of EGFP, EYFP or ECFP in the respective plasmid expression vectors, as depicted in Fig. 1A. In order to accept the fusion proteins as 'tags' for the endogenous pools of PP1 isoforms, it was first necessary to show that they are functional phosphatases recognized by both GFP and PP1 antibodies on a western blot. Both HeLa and HEK-293 cells could be transiently transfected with expression plasmids to permit greater than 90% of the cells to express the fusion proteins, but in all cases fluorescence microscopy revealed a heterogenous population of expression levels. Lysates prepared from these cells represent a range of FP-PP1 expession levels, and we cannot therefore determine the expression level or phosphatase activity of the fusion protein relative to endogenous PP1 in a particular cell.

Following gel electrophoresis and protein blotting of transiently transfected HEK293 cell lysates, all three FP-PP1

### Localization of FP-tagged PP1 isoforms in mammalian cells

Fluorescent-protein-tagged PP1α, PP1β/δ and PP1γ were expressed transiently in mammalian cells, following transfection of their respective expression plasmids, and analysed by fluorescence microscopy. Although all three isoforms were found in both the cytoplasm and in the nucleus, there was a striking difference in their intranuclear distribution (Fig. 2). FP-PP1α was found mainly in a diffuse nuclear pool and largely excluded from the nucleolus (Fig. 2A-C), whereas FP-PP1γ accumulated predominantly within the nucleolus (Fig. 2G-I) in a pattern corresponding to the granular

compartment that contains the bulk of the ribosomal RNA (data not shown). FP-PP1β/δ is found in both the nucleoplasm and the nucleolus (Fig. 2D-F), but does not appear to accumulate within the nucleoli to the same extent as FP-PP1γ. This, along with the localization patterns for FP-PP1α and FP-PP1γ, is in

agreement with immunofluorescence data from a recent study of endogenous PP1 isoforms using anti-peptide antibodies (Andreassen et al., 1998). The possibility that the FP-PP1β/δ
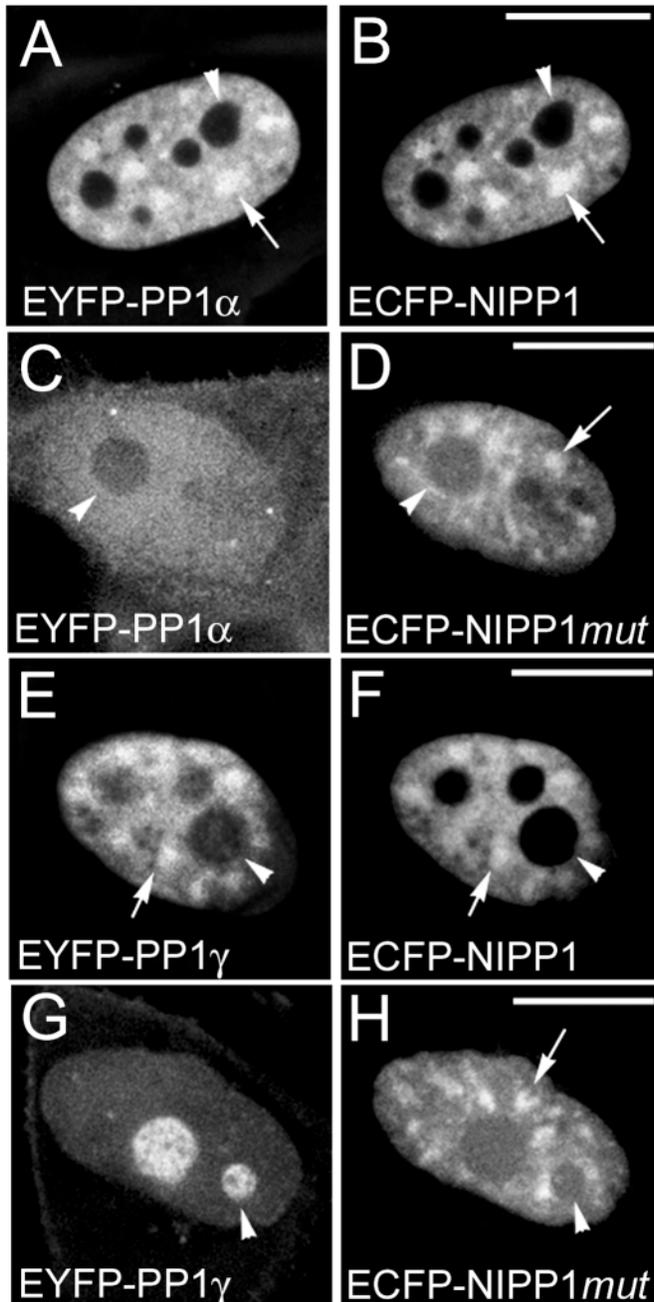
Fig. 3. Relocalization of FP-PP1α and FP-PP1γ by overexpression of a targeting subunit. Co-expression of EYFP-PP1 and ECFP-NIPP1 in transiently transfected HeLa cells leads to retargeting of both the α isoform (A) and the γ isoform (E) to nuclear speckles, where NIPP1 accumulates (B and F). An ECFP fusion of the NIPP1(V201K/F203K) mutant that still accumulates at speckles (D and H), but does not interact strongly with PP1 in vitro (Trinkle-Mulcahy et al., 1999), was not able to retarget either isoform (C and G). Arrows indicate nuclear speckles, whereas arrowheads indicate nucleoli. Scale bars are 10 μM.
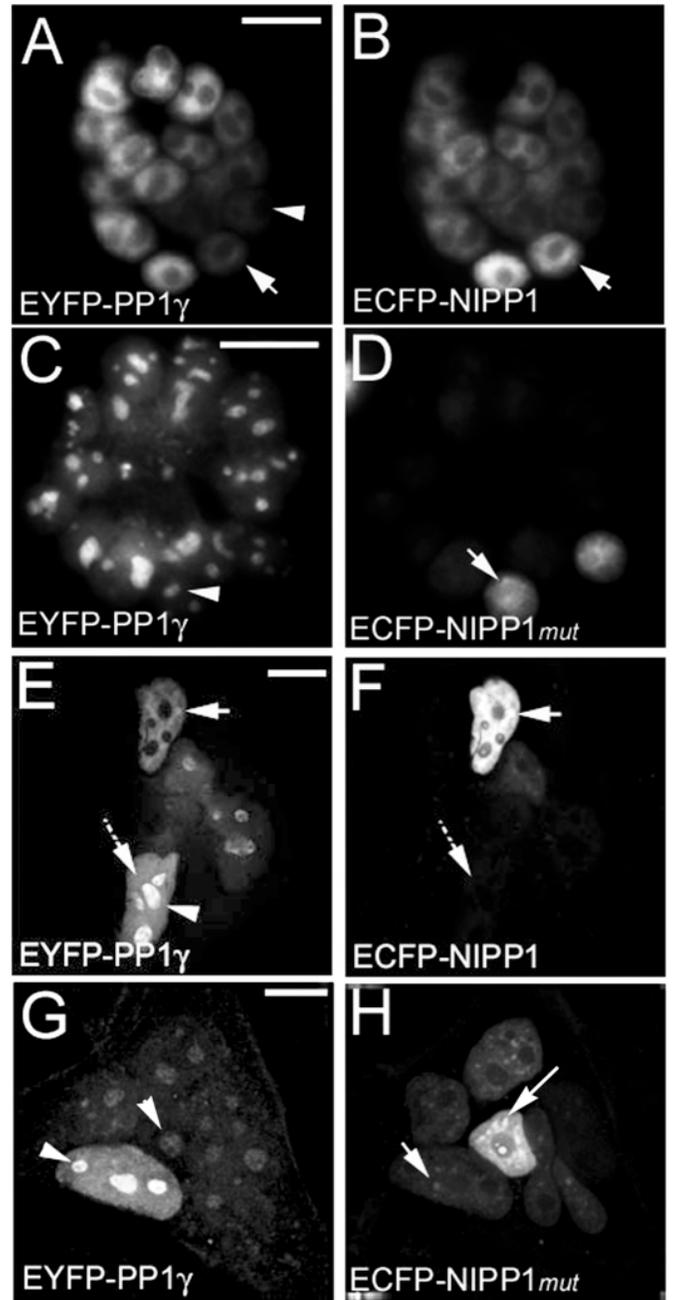
Fig. 4. Dynamic relocalization of PP1γ by a spatially distinct targeting subunit. Heterokaryons created by the fusion of transiently transfected mammalian cells in the presence of cyclohexamide demonstrate that ECFP-NIPP1 can retarget the nucleolar pool of EYFP-PP1γ to nucleoplasmic speckles in both HEK293 (A and B) and HeLa (E and F) cells, whereas the NIPP1(V201K/F203K) mutant cannot (C and D, HEK293; G and H, HeLa). The dashed arrow in Panel E indicates a nucleus within the EYFP-PP1γ/ECFP-NIPP1 HeLa cell heterokaryon that has a lower level of NIPP1 than its neighbors and shows no significant retargeting of PP1γ, demonstrating that this is a concentration-dependent effect. Arrows indicate nuclear speckles, whereas arrowheads indicate nucleoli. Scale bars are 10 μM.

A

B
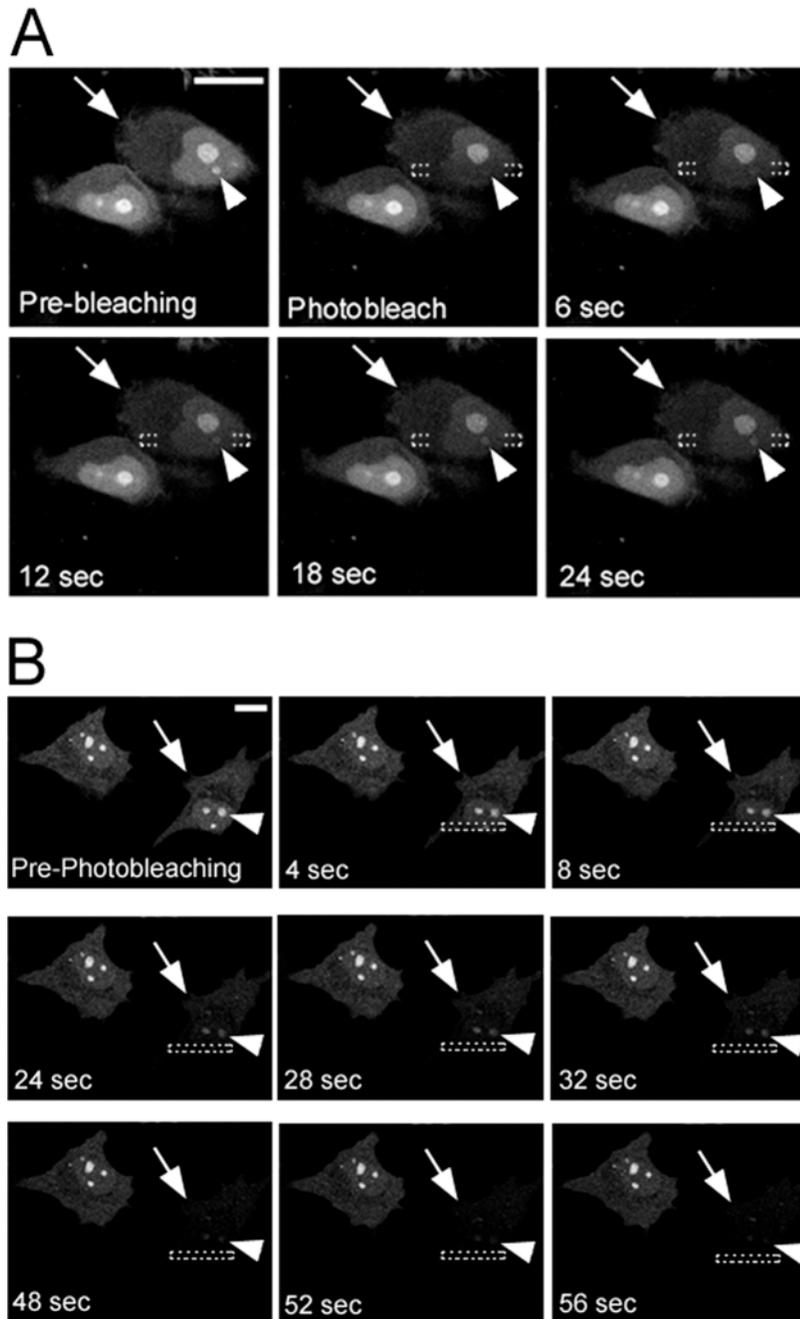
Fig. 5. Photobleaching analyses of EYFP-PP1γ. EYFP-PP1γ was transiently expressed in HeLa cells and imaged live on a confocal laser scanning microscope. Panel A shows a FRAP (fluorescence recovery after photobleaching) experiment in which a nucleolar pool of EYFP-PP1γ in a cell was photobleached at full laser intensity (region indicated in dashed brackets) for a total of four seconds. The recovery of the signal was monitored by repeated scanning of the entire field of view at low laser power. An unbleached neighboring cell is included for comparison. Panel B shows a FLIP (fluorescence loss in photobleaching) experiment in which repeated photobleaching within a defined region (dashed box) was shown to deplete the fluorescent signal throughout the cell. The entire field of view was scanned at low laser power, and the boxed region then bleached with 2 scans at full laser intensity (4 seconds total). This sequence was repeated 15 times, with the bleached cell gradually becoming dimmer while the unbleached control cell remained bright. Times indicated are total photobleaching times at full laser intensity. Arrows indicate photobleached cells whereas arrowheads indicate nucleoli. The scale bar is 10 μM.

several complexes throughout the nucleus, and the patterns detected by microscopy must reflect a combination of these various pools of PP1. What is not yet known is whether these PP1 complexes are relatively stable structures or whether the phosphatase is dynamically exchanged between targeting subunits. In the latter case the steady-state localization pattern for PP1 would represent an equilibrium based on its affinities for the various targeting subunits and would change in response to any alteration in the intracellular levels of different targeting subunits.

In order to test this hypothesis, we chose NIPP1, the major nuclear PP1 binding protein characterized in mammalian cells. This protein localizes primarily to the interchromatin granule clusters known as nuclear speckles (Lamond and Earnshaw, 1998). Neither FP-PP1α nor FP-PP1γ accumulated at nuclear speckles under steady-state conditions, although endogenous NIPP1 was present in these cells. This does not mean that they do not interact with NIPP1, as they both co-immunoprecipitated the endogenous protein from cell lysates (data not shown). Instead, it indicates that the NIPP1-FP-PP1 complex is just one of several pools of FP-PP1 detected in the nucleus.

In order to co-express NIPP1 with the EYFP-PP1 fusion proteins and distinguish between the two signals in the same cell, the NIPP1 cDNA was subcloned into the ECFP plasmid expression vector. ECFP-NIPP1 showed a similar localization pattern to EGFP-NIPP1 (Trinkle-Mulcahy et al., 1999). Expression levels varied from cell to cell, with an average two-fold excess over endogenous levels (data not shown). The protein maintained its speckled localization pattern when co-expressed with either EYFP-PP1α or EYFP-PP1γ (Fig. 3B and F). Under these conditions, however, the nuclear fractions of both PP1 isoforms were redirected to speckles (Fig. 3A and E),

fusion protein is subjected to partial cleavage, however, led us to focus on PP1α and PP1γ for all subsequent analyses. Their complimentary localization patterns also allowed a clear demonstration of the intrinsic nature of this intracellular compartmentalization, as co-expression of both FP-PP1α and FP-PP1γ in the same cell showed that they maintained their distinct intrinsic localization patterns (Fig. 2J-L).
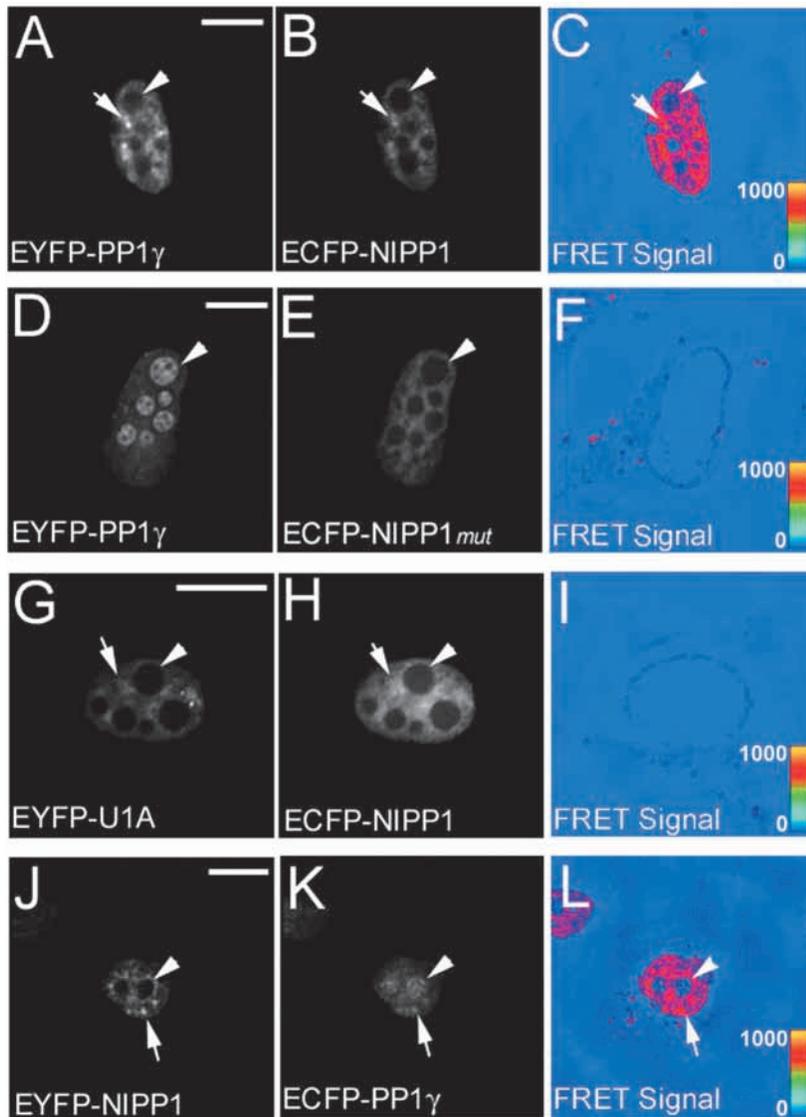
An exogenously expressed PP1 targeting subunit can retarget both PP1α and PP1γ

The intranuclear localization patterns for PP1α and PP1γ, both exogenous (this report) and endogenous (Andreassen et al., 1998), did not correspond to any particular PP1 targeting subunit yet identified. This is not surprising, as PP1 exists in

Fig. 6. Measurement of the direct interaction between PP1 and a targeting subunit. EYFP-PP1γ (A) and ECFP-NIPP1 (B) show a strong FRET signal (C) when co-expressed in transiently transfected HeLa cells, with the phosphatase retargeting from nucleoli to nuclear speckles. Mutant ECFP-NIPP1(V201K/F203K) (E) does not retarget EYFP-PP1γ to speckles (D), and no significant FRET signal is observed (F). Although both EYFP-U1A (G) and ECFP-NIPP1 (H) are found to colocalize at nuclear speckles, the proteins are not known to interact and no FRET signal is observed between them (I). The FRET signal between PP1γ and NIPP1 can also be measured when the fluorophores are exchanged (L), by co-expression of EYFP-NIPP1 (J) and ECFP-PP1γ (K). In the cell shown here, a significant nucleoplasmic pool of EYFP-PP1γ remains to be retargeted, and it can be seen that the FRET signal is found mainly in the nucleoplasm and at the speckles. All EYFP and ECFP signals are shown scaled from 0-4094 pixels, although FRET signals are scaled from 0-1000 pixels and intensity colored as shown. Arrows indicate nuclear speckles, whereas arrowheads indicate nucleoli. Scale bars are 10 μM.

indicating that they had been retargeted by overexpression of exogenous NIPP1. A similar result was obtained for FP-PP1β/δ (data not shown).

Mutation of two conserved hydrophobic residues within the primary PP1 binding site in NIPP1 (shown previously to disrupt interaction of the two proteins) (Trinkle-Mulcahy et al., 1999), prevented PP1 retargeting to speckles, with the isoforms showing their normal localization patterns (Fig. 3C and G), whereas the mutant NIPP1(V201K/F203K) still accumulated at speckles (Fig. 4D and H). Taken together, these results extend the targeting hypothesis to living cells, where PP1 isoforms have localization patterns that are distinct, but can be altered by overexpression of a specific targeting subunit. Mutation of the classic 'Arg/Lys-Val/Ile-Xaa-Phe/Trp' motif in NIPP1 confirmed it as the primary site of interaction with PP1 in vivo, although other regions of NIPP1 play a role in stabilizing the complex (Beullens et al., 1999; Jagiello et al., 2000).

Analysis of the PP1-targeting subunit interaction

Retargeting of the normally predominantly nucleolar EYFP-PP1γ isoform by overexpression of spatially distinct ECFP-NIPP1 permits investigation of both the nature and the location of this interaction. It is possible that both proteins are co-expressed in the cytoplasm, bind with high affinity, enter the nucleus as a complex and localize to nuclear speckles. However, an alternative possibility is that both can reach their nuclear targets independently and that retargeting occurs through the dynamic flux of one or both of them through the cell. To answer this question, HEK293 or HeLa cells were transfected independently with either EYFP-PP1γ or ECFP-NIPP1 and then fused to create heterokaryons (Fig. 4). The cells were left to equilibrate for two to four hours post-fusion in the presence of cycloheximide to block protein synthesis.

When ECFP-NIPP1 was present in a nucleus, EYFP-PP1γ relocalized from the nucleolus to nuclear speckles (Fig. 4A and E). The retargeting was concentration-dependent, in that cells showing lower levels of ECFP-NIPP1 relative to EYFP-PP1γ (as determined by relative fluorescence intensity) maintained the nucleolar localization of the phosphatase (see Fig. 4E, hashed arrow). However, cells with higher levels of ECFP-NIPP1 showed a loss of EYFP-PP1γ from the nucleolus and a corresponding accumulation at nuclear speckles (see Fig. 4E, arrow). The specificity of NIPP1-mediated relocalization of PP1γ in heterokaryons was demonstrated by the fusion of cells expressing EYFP-PP1γ to cells expressing the ECFP-NIPP1(V201K/F203K) mutant that is defective in binding PP1. The presence of high levels of this mutant protein caused little or no retargeting of EYFP-PP1γ (Fig. 4C-D and G-H). The results suggest that the retargeting of PP1γ by NIPP1 is due not only to the interaction of newly synthesized proteins before, or just after, they enter the nucleus, but also to a dynamic process that can occur even after PP1 has been targeted to its original location.

Photobleaching analyses of the dynamic localization of EYFP-PP1γ

Evidence supporting the idea that the nucleolar pool of EYFP-PP1γ is dynamic was provided by photobleaching experiments performed on live cells. FRAP experiments involved photobleaching an area of the cell to destroy the fluorophore on the EYFP-PP1γ fusion protein, and then monitoring the recovery of fluorescence over time as the bleached fusion protein exchanged with fluorescent fusion protein from another area of the cell (White, 1999). Fig. 5A shows the rapid recovery (half-time less than 30 seconds) of the nucleolar pool of EYFP-PP1γ following photobleaching of this region. This recovery did not occur when the same experiment was performed on fixed cells (data not shown), indicating that this is a dynamic process. In a different photobleaching approach, termed FLIP, photobleaching of one area of the cell is used to monitor the loss of fluorescence signal from other areas of the cell that will only occur if they equilibrate with the protein in the bleached region. Fig. 5B shows that repeated photobleaching of a small area of the nucleus in a cell expressing EYFP-PP1γ led to the complete loss of fluorescent signal from the cell. Taken together, these data indicate that the nucleolar pool of PP1γ is dynamic and in equilibrium with the nucleoplasmic and cytoplasmic pools.

FRET analysis shows a localized interaction of PP1 with NIPP1 in vivo

Having shown that both ECFP-NIPP1 and EYFP-PP1γ accumulated in nuclear speckles when co-expressed in the same cell, we next took advantage of their respective fluorophores to demonstrate and map the location(s) where the fusion proteins directly interact. EYFP and ECFP function as a donor-acceptor pair for FRET, with excitation of the donor (ECFP) molecule leading to emission from the acceptor (EYFP) molecule, provided that the fluorophores are in the appropriate relative orientation and close enough (<10 nm) to allow this energy transfer to occur (Pollok and Heim, 1999). A strong FRET signal was observed within the nucleus (strongest at speckles) when ECFP-NIPP1 and EYFP- PP1γ were co-expressed in transiently transfected HeLa cells, confirming that there is a direct interaction between the two proteins in vivo (Fig. 6A-C). No detectable FRET signal was measured when the NIPP1(V201K/F203K) mutant that is defective in PP1 binding was co-expressed with PP1γ (Fig. 6D-F). The NIPP1 mutant did not relocalize a significant amount of the phosphatase to speckles and the FRET data indicated that this protein did not interact strongly with PP1 in vivo. As a negative control, ECFP-NIPP1 was co-expressed with EYFP-U1A, a splicing factor that also accumulates at nuclear speckles but which does not appear to interact directly with NIPP1 (Trinkle-Mulcahy et al., 1999). Under these conditions, the proteins colocalized to the resolution of a light microscope (~200 nm) but showed no sign of a direct interaction (within 10 nm) via FRET analysis (Fig. 6G-I).

FRET interaction between NIPP1 and PP1γ was also shown when the fluorophores were exchanged, so that ECFP-PP1γ was the donor and EYFP-NIPP1 the acceptor (Fig. 6J-L). In this particular cell, some of the PP1γ has not yet been retargeted and remains in the nucleolus, yet the strong FRET signal is restricted to the nucleoplasm and is not seen within the nucleolus (Fig. 6J-L, arrowhead). Therefore, we infer that

a direct, high affinity PP1-NIPP1 interaction predominantly occurs within a restricted region of the nucleus.

DISCUSSION

In this study we analyzed separate PP1 isoforms by expressing them as fusions with fluorescent reporter molecules. To the best of our knowledge, this is the first report of the expression of fluorescent-protein-tagged PP1 in mammalian cells, and this approach opens the possibility of combining microscopy and biochemistry to analyze the properties of PP1 isoforms in both live and fixed cells. We show that the tagged PP1 isoforms each retain phosphatase activity when expressed in vivo. The fact that the cells are able to tolerate exogenous expression of a functional protein phosphatase for any length of time is somewhat unexpected and supports the idea that there are systems in place within the cell to cope with changes in the levels of phosphatase activity.

Fluorescence microscopy revealed specific and distinct in vivo localization patterns for FP-PP1α, FP-PP1β/δ and FP-PP1γ, in agreement with immunolocalization data shown for the endogenous isoforms using anti-peptide antibodies (Andreassen et al., 1998). These data argue that localization is an inherent property of each isoform, either by direct targeting or via interaction with regulatory subunits for which they have differing affinities. The fact that we show here that FP-PP1α and FP-PP1γ maintain their distinct localization patterns even when co-expressed in the same cell (Fig. 2) supports the idea that systems are in place to localize each isoform.

Having shown the distinct localization of fluorescent-protein-tagged PP1 isoforms, we exploited this expression system to analyze how changes in the level of a known PP1-targeting subunit, which localizes to a different region of the nucleus to both PP1α and PP1γ, can affect localization of these isoforms in living cells. NIPP1 is one of the major nuclear targeting subunits for PP1, and the interaction between the two proteins has been demonstrated using such techniques as far western assays, in vitro phosphatase assays and co-immunoprecipitation of the two proteins from cell lysates (Van Eynde et al., 1995; Trinkle-Mulcahy et al., 1999; Jagiello et al., 2000). We demonstrate here the interaction of PP1 with NIPP1 in live cells using fusions of the proteins to EYFP and ECFP. As shown in Fig. 3, NIPP1, when overexpressed in cells, can interact with both PP1α and PP1γ and redirect these isoforms to nuclear speckles. The ability of NIPP1 to interact with both PP1α and PP1γ is in agreement with an earlier study showing that immunoprecipitation of transiently expressed Flag-tagged NIPP1 co-precipitated all three isoforms of PP1 with similar efficiency (Kim et al., 2000). However, we note that targeting of PP1γ to the nucleolus must result predominantly from an interaction with an as yet unidentified nucleolar protein for which it has a high affinity, relative to that for NIPP1, as neither endogenous nor expressed NIPP1 accumulates in any significant amount in the nucleolus.

Although neither FP-PP1α nor FP-PP1γ showed an accumulation in nuclear speckles in the absence of FP-NIPP1, the presence of diffuse nucleoplasmic pools for both isoforms suggests that one or both of them could nonetheless interact transiently with speckles under steady-state conditions. Many

pre-mRNA splicing factors accumulate at these speckles, and in vitro evidence suggests a regulatory role for both NIPP1 and PP1 in the regulation of pre-mRNA splicing (Mermoud et al., 1992; Cardinali et al., 1994; Mermoud et al., 1994; Misteli and Spector, 1996; Trinkle-Mulcahy et al., 1999; Boudrez et al., 2000). A previous report of endogenous localization patterns for the three PP1 isoforms using anti-peptide antibodies showed PP1α accumulating in some type of nuclear speckle or spot (Andreassen et al., 1998), but as the cells were not counterstained with markers for subnuclear bodies it is not clear which structures were detected. We also see expressed FP-PP1α accumulating in nuclear foci, some of which counterstain with an antibody to p80 coilin, a marker for the Cajal body (Andrade et al., 1991) (data not shown), but this FP-tagged isoform does not accumulate at speckles unless it is co-expressed with exogenous NIPP1.

The observed relocalization of the predominantly nucleolar EYFP-PP1γ by nucleoplasmic ECFP-NIPP1 suggests that the proteins can interact, at least transiently, even though their steady-state localization patterns might suggest that they are predominantly segregated. A likely interpretation of these data is that the protein distribution is dynamic and the typical localization pattern represents a steady-state accumulation and not a static localization. This view was further supported by photobleaching experiments that demonstrated the dynamic exchange of EYFP-PP1γ between nucleolar, nucleoplasmic and cytoplasmic pools. Energy transfer between the ECFP- and EYFP-tagged proteins suggests that they contact each other mainly in the nucleoplasm, as PP1γ shuttles in and out of the nucleolus. Alternatively, PP1-NIPP1 interactions may form in the nucleolus but result in extremely rapid exit of the complex from the nucleolus.

The present data offer an explanation for previous discrepancies in studies reporting either localization or association of endogenous PP1 isoforms detected using separate isoform-specific antibodies. We show here, using live cells, that the localization of a particular PP1 isoform can change according to the level of expression of interacting subunits. There is clearly an equilibrium for these complexes within the cell, and this equilibrium may vary from cell type to cell type, and may even vary within the same cell type under different conditions. In conclusion, this study directly validates the targeting hypothesis for PP1 in live cells and further shows the dynamic behavior and distinct specificity of the separate PP1 isoforms.

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