NOM1 Targets Protein Phosphatase I to the Nucleolus*

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Protein phosphatase I (PP1) is an essential eukaryotic serine/threonine phosphatase required for many cellular processes, including cell division, signaling, and metabolism. In mammalian cells there are three major isoforms of the PP1 catalytic subunit (PP1α, PP1β, and PP1γ) that are over 90% identical. Despite this high degree of identity, the PP1 catalytic subunits show distinct localization patterns in interphase cells; PP1α is primarily nuclear and largely excluded from nucleoli, whereas PP1γ and to a lesser extent PP1β concentrate in the nucleoli. The subcellular localization and the substrate specificity of PP1 catalytic subunits are determined by their interaction with targeting subunits, most of which bind PP1 through a so-called “RVXF” sequence. Although PP1 targeting subunits have been identified that direct PP1 to a number of subcellular locations and/or substrates, no targeting subunit has been identified that localizes PP1 to the nucleolus. Identification of nucleolar PP1 targeting subunit(s) is important because all three PP1 isoforms are included in the nucleolar proteome, enzymatically active PP1 is present in nucleoli, and PP1γ is highly concentrated in nucleoli of interphase cells. In this study, we identify NOM1 (nucleolar protein with MIF4G domain 1) as a PP1-interacting protein and further identify the NOM1 RVXF motif required for its binding to PP1. We also define the NOM1 nucleolar localization sequence. Finally, we demonstrate that NOM1 can target PP1 to the nucleolus and show that a specific NOM1 RV sequence is required for targeting activity. We therefore conclude that NOM1 is a PP1 nucleolar targeting subunit, the first identified in eukaryotic cells.

PP1 is an essential eukaryotic serine/threonine phosphatase required for many cellular processes from transcription and cell cycle control to metabolism (reviewed in Refs. 1–3). Mammals contain three genes that encode four PP1 catalytic subunits called PP1α, PP1β, and the splice variants PP1γ1 and PP1γ2; PP1γ2 is found mainly in testis and will not be discussed further here. The PP1 catalytic subunits are over 90% identical at the amino acid level, differing primarily in sequences at their amino and carboxyl termini. Despite this high degree of identity, PP1α, PP1β, and PP1γ show distinct localization patterns in interphase cells (4–8); PP1α is primarily nuclear and largely excluded from nucleoli; PP1γ is highly concentrated in nucleoli, and PP1β is more uniformly distributed, with detectable nucleolar concentration in some cell types. Although these localization patterns of PP1 catalytic subunits are typical for interphase cells, their localization is dynamic and can change during the cell cycle or in response to different growth conditions. For example, elegant experiments by Trinkle-Mulcahy et al. (6) have followed the location of PP1γ during the cell cycle and demonstrated that although it is highly concentrated in the nucleoli of interphase cells, it localizes at kinetochores early in mitosis and is then recruited to mitotic chromatin during anaphase. These data, as well as others (9 for example), indicate that localization of PP1 is not determined by the catalytic subunits themselves. Instead, it has been demonstrated that the localization, activity, and substrate specificity of PP1 catalytic subunits are determined by their interaction with one of many different targeting subunits (1–3). Over 50 different PP1 targeting subunits have been identified, and this family of proteins continues to grow. Examples include Repo-Man that targets PP1γ to chromatin (10, 11), MYPT1 that targets PP1β to myosin (12) as well as to HDAC7 (13), and PNUTS that targets PP1 to the nucleus (14). The importance of PP1 targeting subunits is further demonstrated by the fact that their overexpression can re-localize PP1 within the cell. For example, overexpression of Repo-Man targets not only PP1γ but also PP1α to chromatin (10), whereas overexpression of PNUTS can relocalize PP1γ from nucleoli to the nucleus (9). Most PP1 targeting subunits contain at least one copy of a so-called RVXF motif that interacts with the hydrophobic RVXF binding groove at the carboxyl termini of all PP1 isoforms whose composition is conserved from yeast to humans (15–17).

Although targeting subunits have been identified that direct one or more of the PP1 catalytic subunits to various locations or substrates, including to the nucleus, to chromatin, to myosin, or to glycolgen (reviewed in Refs. 1–3), no nucleolar-specific targeting subunit has been identified. One recent report (18) did describe co-localization of inhibitor-3 (Inh3) and PP1 in nucleoli, although no evidence was provided that Inh3 played a role in targeting PP1 to this subcellular location. It is unusual that no nucleolar targeting subunit has been identified, because PP1γ and to a lesser extent PP1β are highly concentrated in the nucleoli of interphase cells. In addition, all three PP1 isoforms are included in the nucleolar proteome, and purified nucleoli

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* The abbreviations used are: PP1, protein phosphatase I; NoLS, nucleolar localization sequence; co-IP, co-immunoprecipitation; eGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline.

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contain enzymatically active PP1 (6). Together, these data suggest an important role for PP1 in the nucleolus.

The nucleolus is best known as the site of ribosome biogenesis. In recent years however, it has become clear that nucleoli play critical roles in other cellular processes, including stress response, assembly of ribonucleoprotein complexes, and regulation of mitosis and proliferation (reviewed in Ref. 19). In addition, it has been shown that the nucleolar localization and activity of several important cellular proteins, including the retinoblastoma protein (20–22) and nucleophosmin (23), are regulated by serine-threonine phosphorylation and that at least the retinoblastoma protein is a well established substrate of PP1 (24–29). It is therefore important to identify targeting subunits that direct PP1 to the nucleolus and that modulate its activity in this critical subnuclear region.

We identified NOM1 (nucleolar protein with MIF4G domain 1) several years ago based on its location within the common breakpoint region on human chromosome 7 targeted in children with acute myeloid leukemia that carry 7q36;12p13 rearrangements (30, 31). As part of an effort to define the function of NOM1, we conducted a yeast two-hybrid screen and identified PP1 as a NOM1-interacting protein. Studies in this report verify the NOM1–PP1 interaction in mammalian cells, identify the NOM1 RVXF motif required for interaction with PP1, and further define the NOM1 nucleolar localization sequence (NoLS) required for NOM1 nucleolar localization. Finally, we demonstrate that NOM1 can target PP1 to the nucleolus and show that a specific NOM1 RVXF motif and the NOM1 NoLS are required for this targeting activity. We therefore conclude that NOM1 is the first PP1 nucleolar targeting subunit identified in eukaryotes.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid—**NOM1 sequences that encode amino acids 247–860 of the full-length protein were inserted into the yeast GBKT7 expression vector (Clontech) and the resultant plasmid introduced into the yeast AH109 strain. After NOM1 expression was verified by Western blot (data not shown), AH109 NOM1-expressing cells were mated to yeast cells transformed with a HeLa MATCHMAKER cDNA library (Clontech), and colonies encoding candidate NOM1-interacting proteins were identified and processed following the manufacturer’s recommended protocol.

**Plasmids—**Full-length (amino acids 1–860) and deletion (see below) NOM1 cDNAs shown in Fig. 1 were inserted into the BamHI-Xbal restriction sites of the lentivirus-derived CSII-CDF-EF-3xFLAG expression vector (32) kindly provided by Dr. N. Somia. This vector expresses 3xFLAG-tagged inserts under the control of the EF1α promoter (33). To generate NOM1 deletion constructs, primers were synthesized and used to amplify regions of NOM1 that encode amino acids 1–300 (NOM1-(1–300)), 1–350 (NOM1-(1–350)), 1–561 (NOM1-(1–561)), and 247–860 (NOM1-(247–860)). Each of these fragments was inserted in-frame into the BamHI-Xbal site of the CSII-CDF-EF-3xFLAG vector.

The putative RVXF PP1-binding motif at 307 of NOM1 was mutated (m307) by changing Val-308 and Phe-310 residues to alanines using standard PCR-based site-directed mutagenesis. This mutation was inserted into NOM1-(1–860), NOM1-(1–561), and NOM1-(1–350).

Each of the NOM1 inserts described above was also expressed as an mCherry fusion protein. This was accomplished by multiplying the mCherry coding region (34) as a HindIII–BamHI fragment and cloning this fragment in place of the HindIII–BamHI fragment in the pcDNA6/His vector (Invitrogen) polylinker. NOM1 inserts were then introduced in-frame into this modified vector as BamHI–Xbal fragments to generate NOM1-mCherry fusion proteins with mCherry at the amino terminus.

Several NOM1-eGFP fusion constructs were also generated using the eGFPN2 vector (Invitrogen). These included NOM1-(1–860)-eGFP, NOM1-(247–860)-eGFP, and NOM1-(1–269)-eGFP. As above, the numbering refers to NOM1 amino acids included in the construct. Human PP1α and PP1γ cDNAs were generated by RT–PCR and cloned in-frame into the EcoRI site of the pcDNA6/His (Invitrogen) expression vector, which includes His6 and Xpress epitope tags at the amino terminus of sequences inserted into the multiple cloning site.

**Cell Culture and Transfections—**Human embryonic kidney 293 (HEK 293) cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. HeLa cells stably expressing eGFP-tagged PP1α or PP1γ (HeLaEGFP-PP1α and HeLaEGFP-PP1γ) were generously provided by Dr. Laura Trinkle-Mulcahy and were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 400 μg/ml G418. All cells were maintained at 37 °C in humidified air with 5% CO2.

**Transfection and Microscopy—**HeLaEGFP-PP1α cells were plated on 12-mm cover glasses in 12-well tissue culture dishes and transfected the next day with 1 μg of plasmid DNA using Lipofectamine 2000 (Invitrogen). After 4–6 h, cover glasses were collected and incubated for 10 min in 3.7% formaldehyde (Fluka) diluted in 1× PBS and then washed three times for 5 min in 1× PBS. After a brief water rinse, cover glasses were mounted on glass slides with Vectashield (Vector Laboratories) and sealed with nail polish.

Images were acquired using a Nikon Eclipse TE200 inverted microscope equipped with the confocal imaging system (PerkinElmer Life Sciences) and an Orca-ER digital camera (Hamamatsu). Cells were imaged with a ×60 plan apo (NA 1.4) objective, 5–8 1-micron optical Z-sections and 2 × 2 binning.
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A settle frame was inserted between acquisition of each wavelength to allow time for potential residual signal to be cleared from the camera chip.

Transfection, Protein Extraction, Western Blotting, and Co-immunoprecipitation (Co-IP)—HEK 293 cells plated in 60-mm plates were transfected with 10 μg of DNA using 30 μg of polyethylenimine (M, 25,000; Polysciences, Inc.). The polyethylenimine was prepared by dissolving the powder in 80 °C water, adjusting the pH to 7.0 with 1 N HCl, and filtering through a 0.2-μm filter. The solution was aliquoted and stored at −80 °C. After 48 h, cells were rinsed twice with 1× PBS and lysed by the addition of lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100) containing the manufacturer’s recommended amounts of Complete Protease Inhibitor Mixture (Roche Applied Science) and Phosphatase Inhibitor Mixture 1 and 2 (Sigma). Lysates were sonicated on ice and cleared by centrifugation at 14,000 rpm for 10 min at 4 °C. Protein concentration was determined using the DC Protein Assay (Bio-Rad).

For co-IPs, cell lysates containing 400 μg of protein were rotated overnight at 4 °C with 40 μl of ANTI-FLAG® M2-agarose affinity beads (Sigma) that had been prepared according to the manufacturer’s recommendations. The beads were then washed four times with lysis buffer containing protease inhibitors and phosphatase inhibitors. Pellets were then resuspended in sample buffer (0.05 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.1 M dithiothreitol, 0.1% bromophenol blue), denatured, and fractionated by 10% SDS-PAGE. Fractionated proteins were transferred to Immobilon (Millipore) membranes and incubated with the appropriate antibodies in 1× TBS (0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Tween 20) containing 5% milk. Antibody conjugates were visualized using the ECL Plus™ Kit (GE Healthcare).

RESULTS

Identification of NOM1 as a PP1-interacting Protein in a Yeast Two-hybrid Screen—to identify proteins that interact with NOM1, we conducted a yeast two-hybrid screen with the Matchmaker Two-Hybrid System 3 and a HeLa cDNA library (Clontech) using NOM1 as bait as described under “Experimental Procedures.” Sequencing of candidate NOM1 interaction partners obtained in this screen identified three independent procedures. Subtracting of candidate NOM1 interaction partners identified in this screen identified three independent interactions. The interactions that encode the catalytic subunit of protein phosphatase 1α (PP1α), making PP1α the most frequent cDNA identified.

NOM1 Interacts with PP1 in Mammalian Cells—to determine whether NOM1 interacts with PP1 in mammalian cells, we conducted co-IP assays monitoring NOM1 binding to both endogenous and recombinant forms of PP1.

To this end, we generated Xpress-tagged PP1α and PP1γ expression clones by inserting the relevant PP1 coding sequences into the pcDNA6HisC vector (Invitrogen). We chose to analyze both the α and γ isoforms because they differ dramatically in their subcellular localization in interphase cells; PP1α localizes primarily to the nucleus and is largely excluded from the nucleolus, whereas PP1γ concentrates in nucleoli (4, 8). The PP1α- and PP1γ-Xpress clones were transfected into HEK 293 cells either alone or in combination with the NOM1-3xFLAG expression vector. Protein extracts were then prepared, and expression of NOM1-3xFLAG and PP1α- and PP1γ-Xpress was verified in Western blots (Fig. 2A, INPUT). Aliquots of each sample were then immunoprecipitated with ANTI-FLAG® M2-agarose affinity beads and Western blotted with an anti-Xpress antibody (Invitrogen) (Fig. 2B, WB: α-Xpress). As shown, the recombinant PP1α- and PP1γ-Xpress proteins were present only in co-IPs from samples also transfected with NOM1-3xFLAG (Fig. 2B, compare lanes 3 and 4 with lanes 5 and 6). These results verify the NOM1-PP1 interaction in mammalian cells.

FIGURE 2. NOM1 interacts with overexpressed and endogenous PP1. HEK 293 cells were transfected with the indicated plasmids and extracts analyzed for protein expression by Western blot using the anti-FLAG antibody to detect NOM1-FLAG expression or with anti-Xpress antibody to detect PP1- Xpress expression (A). Extracts were then immunoprecipitated (IP) with anti-FLAG antibody and Western blotted (WB) with antibody against either the Xpress epitope or with anti-PP1 antibody (B). Extracts were also immunoprecipitated with anti-PP1 antibody and then Western blotted with anti-FLAG antibody (C). In this experiment and that shown in Fig. 3 we estimate that ~1% of the input PP1 co-immunoprecipitates with NOM1.

NOM1 Interacts with Endogenous PP1—to determine whether NOM1 also interacts with endogenous PP1, we stripped the blot shown in the upper portion of Fig. 2B and reprobed it with an antibody developed against PP1α (Calbiochem). Data are shown in the lower portion of Fig. 2B (WB: α-PP1). Examination of this blot revealed that endogenous PP1, which runs slightly below the Xpress-tagged protein, was detected in Fig. 2B, lane 2, which represents a sample transfected with the NOM1-3xFLAG construct alone. We interpret this result to indicate that overexpressed NOM1 interacts with and co-IPs endogenous PP1. This is also evident in Fig. 2B, lanes 5 and 6, which were transfected with NOM1-3xFLAG and...
either PP1α-Xpress (lane 5) or PP1γ-Xpress (lane 6). Note that the anti-PP1 antibody, which is supposed to be specific for PP1α, also detected the PP1γ-Xpress protein (Fig. 2B, upper band in lane 6 and data not shown). This is a common problem reported for some “isofrom-specific” PP1 antibodies (9, 10). In either case, these data indicate that NOM1 can interact with overexpressed PP1α and PP1γ and with at least one isofrom of endogenously expressed PP1.

To further validate the NOM1-PP1 interaction, we immunoprecipitated samples with the anti-PP1α antibody and were able to co-immunoprecipitate NOM1-3xFLAG both from samples that included overexpressed PP1α (Fig. 2C, lane 4) or only endogenous PP1 (Fig. 2C, lane 2). We have also confirmed an interaction between PP1α and NOM1 in glutathione S-transferase pulldown assays using bacterially expressed proteins (data not shown). We interpret these data to indicate that the NOM1-PP1 association detected in the yeast two-hybrid assay also occurs in mammalian cells and that this interaction is not dependent on overexpression of at least the α isofrom of PP1.

Identification of a Critical RVXF PP1-binding Site in NOM1—Most proteins that interact with PP1 contain at least one copy of the “RVXF” PP1-binding motif that is defined as [RK]-X(0–1)-[VI]-[P]-[FW], where [P] stands for any amino acid other than proline, where X is most commonly a histidine or arginine, and where the motif is preceded by 2–5 basic residues and is followed by at least one acidic residue (15–17). The predicted NOM1 amino acid sequence includes five candidate RVXF motifs (Fig. 1) beginning at residues 307 (RVRF), 507 (KNVGE), 594 (RVSW), 766 (KVVEF), and 777 (RVRF). Of the five candidate sites in NOM1, the 307 sequence is the only one flanked by the favorable basic and acidic residues.

To define NOM1 sequences required for PP1 binding, a series of NOM1 deletion constructs and mutants were generated and used in co-IP experiments with PP1α- and PP1γ-Xpress. As shown in Fig. 1, NOM1 deletions were generated that removed the three carboxyl-terminal candidate RVXF motifs (NOM1(1–561)), an additional motif at 507 (NOM1(1–350)), and the final motif at 307 (NOM1(1–300)). An aminoterminal deletion was also generated that removed the first 246 amino acids of NOM1 (NOM1(247–860)). Because the RVXF motif at 307 was the best match to the consensus PP1-binding motif or other sequences that can contribute modestly to the NOM1-PP1 interaction.

To determine whether this basic region is required for efficient binding to PP1, we performed pulldown assays using bacterially expressed proteins. As shown in Fig. 2C, lane 2, and in duplicate experiments, each co-IP blot was also probed with the anti-FLAG antibody to verify that the mutant and wild type forms of NOM1 were immunoprecipitated equivalently and that the lack of PP1-Xpress in co-IPs was not because of inefficient IP of the FLAG-tagged PP1α mutants.

Together, we interpret these data to indicate the following: 1) that the NOM1 307 RVXF motif is the major PP1 interaction domain and that it is absolutely required for NOM1-PP1 interaction in the absence of NOM1 sequences downstream of residue 561; 2) that, because the NOM1(1–561) m307 mutant failed to immunoprecipitate PP1, the NOM1 RVXF motif at 507 is not a functional PP1 interaction site; and 3) that sequences downstream of 561 include either another weakly active RVXF motif or other sequences that can contribute modestly to the NOM1-PP1 interaction.

Identification of NOM1 Nucleolar Localization Sequences—When expressed either as a FLAG-tagged or an eGFP fusion protein in 293, HeLa, or NIH3T3 cells, NOM1 concentrates in nucleoli, with some protein also present in the nucleus (31) (Fig. 4A and data not shown). These data are in agreement with the nucleolar localization of Sgd1p, the putative NOM1 homolog in yeast. The predicted NOM1 protein contains a highly basic amino terminus, characteristic of nucleolar localization sequences. To determine whether this basic region is required for NOM1 nucleolar localization, we deleted the first 246 amino acids of NOM1 to generate NOM1-(247–860) (Fig. 1) and fused this protein to eGFP. In contrast to the nucleolar localization of NOM1(1–860)-eGFP (Fig. 4A), NOM1-(247–860)-eGFP was found in the nucleus and cytoplasm but was largely excluded from nucleoli (Fig. 4B). From these data we conclude that the amino terminus of NOM1 is necessary for its
nucleolar localization. To determine whether the amino terminus of NOM1 is sufficient to direct nucleolar localization of a heterologous protein, we fused the amino terminus of NOM1 (residues 1–269) to eGFP and were able to show that in contrast to the normal distribution of eGFP (Fig. 4C), the NOM1-(1–269)-eGFP fusion protein accumulated in nucleoli (Fig. 4D). Together, these data demonstrate that NOM1 contains sequences that are both necessary and sufficient for nucleolar targeting and that these sequences are located between amino acids 1 and 269.

NOM1 Can Target PP1α-eGFP to the Nucleolus—PP1 isoforms show distinct localization patterns in interphase cells that are defined by their association with individual targeting subunits (4–8). However, it has been demonstrated that overexpression of a specific targeting subunit, including PNUTS and NIPP1 (9) and Repo-Man (10), can lead to re-localization of PP1 isoforms within the cell, demonstrating the importance of PP1 targeting subunits in defining the subcellular location of PP1.

Based on the activity of other PP1 targeting subunits, we chose to investigate whether NOM1 could re-localize PP1 within the cell. For these experiments, we obtained HeLa cell lines developed by Dr. Trinkle-Mulcahy that stably express eGFP-tagged PP1 isoforms. These lines have been extensively characterized, and it has been shown that the eGFP-tagged proteins are enzymatically active, have normal localization patterns, and are expressed at near-endogenous levels (8). Fluorescent images of the PP1α- and PP1γ-eGFP HeLa lines are shown in Fig. 5, A and B, respectively, and demonstrate their distinctive subcellular localization. To determine whether NOM1 affects the subcellular localization of PP1 and whether this activity is dependent on NOM1-PP1 interaction, we transfected the HeLaEGFP-PP1α line with wild type and mutant forms of NOM1 shown in Fig. 1 that were fused to the red fluorescent protein mCherry (34). As shown in Fig. 5, C and E, cells transfected NOM1-(1–860)-mCherry and NOM1-(1–561)-mCherry, both of which include the wild type 307 RVXF motif, showed dramatic accumulation of PP1α-eGFP in nucleoli, in clear contrast to the parental cells, in which PP1α-eGFP is excluded from nucleoli (Fig. 5A). Merged images of Fig. 5, C and...
TABLE 1

Percent of HeLaEGFP-PP1α cells with nucleolar PP1α

| % of cells with nucleolar PP1 | Untransfected | mCherry | 1–860 | 1–860m307 | 1–561 | 1–561m307 | 1–350 | 1–300 | 247–860 |
|-----------------------------|--------------|---------|-------|-----------|-------|-----------|-------|-------|--------|
|                            | 0–5          | 0       | 86    | 14        | 93    | 25        | 74    | 17    | 18     |

E, demonstrate co-localization (yellow) of PP1α-eGFP (green) and NOM1-(1–860)- and NOM1-(1–561)-mCherry (red). Targeting of PP1α-eGFP from the nucleus to nucleolus was also seen with NOM1-(1–350) that includes the 307 RVXF motif (merged image shown in Fig. 5G). In contrast, introduction of the m307 mutation into NOM1-(1–860)-mCherry (Fig. 5D) or NOM1-(1–561)-mCherry (Fig. 5F) abrogated the ability of NOM1 to re-localize PP1α-eGFP to the nucleolus, although this mutation did not disrupt nucleolar localization of NOM1 itself. Absence of targeting activity was also seen with the NOM1-(1–300) (merged image shown in Fig. 5H) that lacks the 307 RVXF site, although again, this mutant NOM1 protein still localized to the nucleolus. Also shown in Fig. 5I are results obtained with the NOM1-(247–860) mutant that contains all of the NOM1 RVXF motifs but that is missing the NoLS and therefore does not localize to the nucleolus. As shown, expression of this protein did not affect localization of PP1α-eGFP, demonstrating that overexpression of NOM1 that includes the 307 RVXF motif does not indirectly lead to nucleolar localization of PP1. In several experiments, we counted the number of NOM1-mCherry transfected cells that looked like the parental cells (i.e. maintained nucleolar exclusion of PP1α-eGFP) versus those that showed nucleolar inclusion of PP1α-eGFP; data from representative experiments are included in Table 1. As shown, whereas expression of mCherry alone did not affect the subcellular localization of PP1α, expression of mCherry fused to NOM1-(1–860), NOM1-(1–561), and NOM1-(1–350), all of which include the wild type RVXF motif at 307, increased the percent of cells with nucleolar PP1 from 0 to 5% up to 74 to 93%. When the 307 RVXF was mutated in either the NOM1-(1–860) or NOM1-(1–561), constructs, the percent of cells with nucleolar PP1α decreased dramatically down to 14–25% of cells. Together, these data demonstrate that NOM1 can target PP1 to the nucleolus and that this activity requires an intact 307 RVXF motif and the NOM1 NoLS.

**DISCUSSION**

Distinct populations of PP1 are dynamically targeted to different locations and substrates within the cell. For example, Trinkle-Mulcahy et al. (6) have demonstrated that the location of PP1γ changes during the cell cycle; it is highly concentrated in nucleoli of interphase cells, localizes at kinetochores early in mitosis, and is then recruited to mitotic chromatin during anaphase. Several lines of evidence demonstrate that association of PP1 catalytic subunits with distinct targeting subunits is primarily responsible for determining both the localization and substrate specificity of PP1, most frequently through interaction of a so-called RVXF sequence on the targeting subunit and the hydrophobic RVXF binding groove on PP1. The importance of PP1 targeting subunits is indicated by the finding that some mutations of the RVXF binding groove in yeast PP1 are lethal (35), indicating that interaction with targeting subunits is essential for proper PP1 function. Similarly, Bollen and co-workers (9) found that mutation of the PP1γ RVXF binding groove resulted in its cytoplasmic accumulation, supporting the hypothesis that association with targeting subunits via RVXF-mediated interaction is required for normal nucleolar localization of PP1γ. There have also been a number of studies demonstrating that overexpression of some PP1 targeting subunits in vivo, including Repo-Man (10), NIPPI (9), and PNUTS (9), can alter the location of PP1 in the cell. These data demonstrate the importance of PP1 targeting subunits in defining the normal subcellular location of PP1 isoforms and furthermore that overexpression of PP1 targeting subunits can aberrantly affect localization of catalytic subunits.

Studies in this report demonstrate a specific interaction between PP1 and the nucleolar protein NOM1 as defined in yeast two-hybrid and mammalian co-IP experiments and further identify a single RVXF motif beginning at amino acid 307 in NOM1 that is primarily responsible for PP1 binding. The NOM1-PP1 interaction is further supported by data in the Biomolecular Interaction Network Database that reports interaction between PP1 and c7orf3, a NOM1 EST that includes the 307 RVXF site. These data are consistent with the hypothesis that NOM1, as has been found for most PP1 targeting subunits, interacts with the RVXF hydrophobic binding groove located within the carboxyl terminus of PP1 catalytic subunits, a possibility that we are currently testing directly.

PP1α was the only PP1 isoform identified as a NOM1-interacting protein in the yeast two-hybrid screen; no clones were identified that encoded PP1γ or PP1β. This could indicate that NOM1 interacts preferentially with the α isoform of PP1. However, the α isoform reportedly is the most abundant form of PP1, at least at the protein level (36). The identification of only PP1α in the two-hybrid screen might therefore reflect its higher representation in the HeLa cDNA library used in the screen and not a preferential interaction with NOM1. In fact, we were able to show efficient binding of NOM1 to overexpressed PP1γ in mammalian co-IP experiments and did not see an obvious preference for binding to the α isoform in these analyses. Studies are planned to quantify the relative binding affinity of NOM1 for the α, β, and γ isoforms of PP1. This will be an important point to determine as it may have important implications regarding the role of NOM1 in nucleolar targeting of PP1 isoforms in vivo (see below).

Through the use of NOM1 deletion mutants, we have also identified sequences within the amino-terminal 269 amino acids of NOM1 required for its nucleolar localization, and we further demonstrate that fusion of these sequences to GFP
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results in nucleolar localization of GFP. Although sequences required for nucleolar localization are not well defined, they are generally composed of a high percentage of basic amino acids, which is consistent with the composition of the amino terminus of NOM1. Nucleolar localization has also been described for the putative NOM1 yeast homolog (HomoloGene:39776), an essential gene called Suppressor of Glycerol Defect 1 (Sgd1p).

In addition to in vitro binding activity, we also found that overexpression of NOM1 leads to accumulation of GFP-tagged PP1α in nucleoli, a region of the cell from which PP1α is normally excluded. As noted, this targeting activity requires both the NOL1 NoLS and the NOM1 RVXF motif at 307. The fact that the 247–860 NOM1 deletion mutant that includes all five candidate RVXF motifs but that lacks the NOM1 NoLS does not lead to nucleolar accumulation of PP1 indicates that the NOM1 targeting activity is mediated directly by NOM1 localization to the nucleolus, and it is an indirect effect of overexpressing NOM1. It is also important that PP1α accumulated in nucleoli within only about 4 h after expression of NOM1, again indicating a direct effect of NOM1 expression.

All three PP1 isoforms are included in the nucleolar proteome, and PP1γ and to a lesser extent PP1β are enriched in this subcellular location, suggesting that PP1 plays an important role in this region of the cell. It is therefore important to define cellular components that regulate nucleolar localization of PP1. One report identified a single amino-terminal residue that differed between PP1α (Gln-20) versus PP1β and PP1γ (Arg-19 for PP1β and Arg-20 for PP1γ) that was critical for their different localization patterns. In particular, it was found that a Q20R substitution in PP1α led to its accumulation in nucleoli and that an R19Q substitution in PP1β led to its exclusion from nucleoli. Interestingly, it was also reported that substitution of the Gln-20 in PP1α and the Arg-19/20 in PP1β and PP1γ with alanine led to nucleolar accumulation of all three isoforms. One interpretation of these data is that a protein required for PP1 nucleolar localization interacts with the amino terminus of PP1 and that the presence of a Gln at position 19/20 interferes with binding. Given the ability of NOM1 to localize PP1 to nucleoli, we are investigating whether NOM1 might form additional contacts with PP1 and whether residues at the amino terminus of PP1 contribute to NOM1 affinity.

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REFERENCES

1. Bollen, M. (2001) Trends Biochem. Sci. 26, 426–431
2. Ceulemans, H., and Bollen, M. (2004) Physiol. Rev. 84, 1–39
3. Cohen, P. T. (2002) J. Cell Sci. 115, 241–256
4. Andreassen, P. R., Lacroix, F. B., Villa-Moruzzi, E., and Margolis, R. L. (1998) J. Cell Biol. 141, 1207–1215
5. Kotani, H., Ito, M., Hamaguchi, T., Ichikawa, K., Nakano, T., Shimah, H., Nagao, M., Ohta, N., Furuichi, Y., Takahashi, T., and Umekawa, H. (1998) Biochem. Biophys. Res. Commun. 249, 292–296
6. Trinkle-Mulcahy, L., Andrews, P. D., Wickramasinghe, S., Sleeman, I., Prescott, A., Lam, Y. W., Lyon, C., Swedlow, J. R., and Lamond, A. I. (2003) Mol. Biol. Cell 14, 107–117
7. Trinkle-Mulcahy, L., Chusainow, J., Lam, Y. W., Swift, S., and Lamond, A. (2006) Methods Mol. Biol. 365, 133–154
8. Trinkle-Mulcahy, L., Sleeman, J. E., and Lamond, A. I. (2001) J. Cell Sci. 114, 4219–4228
9. Lesage, B., Beullens, M., Nuytten, M., Van Eynde, A., Keppens, S., Himpen, B., and Bollen, M. (2004) J. Biol. Chem. 279, 55978–55984
10. Trinkle-Mulcahy, L., Andersen, I., Lam, Y. W., Moorhead, G., Mann, M., and Lamond, A. I. (2006) J. Cell Biol. 172, 679–692
11. Vagnarelli, P., Hudson, D. F., Ribeiro, S. A., Trinkle-Mulcahy, L., Spence, J. M., Lai, F., Farr, C. J., Lamond, A. I., and Earnshaw, W. C. (2006) Nat. Cell Biol. 8, 1133–1142
12. Ito, M., Nakano, T., Erdodi, F., and Hartshorne, D. J. (2004) Mol. Cell. Biochem. 259, 197–209
13. Parra, M., Mahmoudi, T., and Verdin, E. (2007) Genes Dev. 21, 638–643
14. Allen, P. B., Kwon, Y. G., Nairn, A. C., and Greengard, P. (1998) J. Biol. Chem. 273, 4089–4095
15. Egloff, M. P., Johnson, D. F., Moorhead, G., Cohen, P. T., Cohen, P., and Barford, D. (1997) EMBO J. 16, 1876–1887
16. Meiselbach, B., Sticht, H., and Enz, R. (2006) Chem. Biol. 13, 49–59
17. Wakula, P., Beullens, M., Ceulemans, H., Stalmans, W., and Bollen, M. (2003) J. Biol. Chem. 278, 18817–18823
18. Huang, H. S., Pozarowski, P., Gao, Y., Darzynkiewicz, Z., and Lee, E. Y. (2005) Arch. Biochem. Biophys. 443, 33–44
19. Boisvert, F. M., van Koningbruggen, S., Navascues, J., and Lamond, A. I. (2007) Nat. Rev. Mol. Cell Biol. 8, 574–585
20. Angus, S. P., Solomon, D. A., Kuschel, L., Hennigan, R. F., and Knudsen, E. S. (2003) Mol. Cell. Biol. 23, 8172–8188
21. Iao, W., Datta, J., Lin, H. M., Durdin, M., and Rane, S. G. (2006) J. Biol. Chem. 281, 38098–38108
22. Takemura, M., Ohoka, F., Perpelescu, M., Ogawa, M., Matsushita, H., Takaba, T., Akiyama, T., Umekawa, H., Furuichi, Y., Cook, P. R., and Yoshida, S. (2002) Exp. Cell Res. 276, 233–241
23. Negi, S. S., and Olson, M. O. (2006) J. Cell Sci. 119, 3676–3685
24. Albers, A. S., Thorburn, A. M., Shenolikar, S., Mumbey, M. C., and Fersht, A. R. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 388–392
25. Berndt, N., Dohadwala, M., and Liu, C. W. (2007) EMBO J. 26, 375–386
26. Durfee, T., Becherer, K., Chen, P. L., Yeh, S. H., Yang, Y., Kilburn, A. E., Lee, W. H., and Elledge, S. J. (1997) Genes Dev. 7, 555–569
27. Nelson, D. A., Krucher, N. A., and Ludlow, J. W. (1997) J. Biol. Chem. 272, 4528–4535
28. Rubin, E., Mitochondria, S., Villa-Moruzzi, E., and Ludlow, J. W. (2001) Oncogene 20, 3776–3785
29. Vietri, M., Bianchi, M., Ludlow, J. W., Mittnacht, S., and Villa-Moruzzi, E. (2006) Cancer Cell Int. 6, 3
30. Simmons, H. M., Oseth, L., Nguyen, P., O’Leary, M., Conklin, K. F., and Hirsch, B. (2002) Leukemia (Baltimore) 16, 2408–2416
31. Simmons, H. M., Ruis, B. L., Kapoor, M., Hudacek, A. W., and Conklin, K. F. (2005) Gene (Amst.) 347, 137–145
32. Miyoshi, H., Blomer, U., Takahashi, M., Gage, F. H., and Verma, I. M. (1998) J. Virol. 72, 8150–8157
33. Kim, D. W., Uetsuki, T., Kaziro, Y., Yamaguchi, N., and Sugano, S. (1990) Gene (Amst.) 91, 217–223
34. Shiner, N. C., Campbell, R. E., Steinbach, P. A., Giepmans, B. N., Palmer, A. E., and Tsien, R. Y. (2004) Nat. Biotechnol. 22, 1567–1572
35. Wu, X., and Tatchell, K. (2001) Biochemistry 40, 7410–7420
36. Okada, T., Fujii, T., Tanuma, N., Mitsuhashi, S., Urano, T., Araki, Y., Shima, H., and Kikuchi, K. (2004) Int. J. Oncol. 25, 1383–1388