Neurabins Recruit Protein Phosphatase-1 and Inhibitor-2 to the Actin Cytoskeleton*

Inhibitor-2 (I-2) bound protein phosphatase-1 (PP1) and several PP1-binding proteins from rat brain extracts, including the actin-binding proteins, neurabin I and neurabin II. Neurabins from rat brain lysates were sedimented by I-2 and its structural homologue, I-4. The central domain of both neurabins bound PP1 and I-2, and mutation of a conserved PP1-binding motif abolished neurabin binding to both proteins. Microcystin-LR, a PP1 inhibitor, also attenuated I-2 binding to neurabins. Immunoprecipitation of neurabin I established its association with PP1 and I-2 in HEK293T cells and suggested that PP1 mediated I-2 binding to neurabins. The C terminus of I-2, although not required for PP1 binding, facilitated PP1 recruitment by neurabins, which also targeted I-2 to polymeric F-actin. Mutations that attenuated PP1 binding to I-2 and neurabin I suggested distinct and overlapping sites for these two proteins on the PP1 catalytic subunit. Immunocytochemistry in epithelial cells and cultured hippocampal neurons showed that endogenous neurabin I and I-2 colocalized at actin-rich structures, consistent with the ability of neurabins to target the PP1-I-2 complex to actin cytoskeleton and regulate cell morphology.

The type 1 protein serine/threonine phosphatase or protein phosphatase-1 (PP1) is highly conserved in all eukaryotes and regulates a variety of functions including protein synthesis, gene transcription, glycolysis, lipid metabolism, muscle contractility, and learning and memory (1, 2). These diverse functions are directed by the unique localization and substrate recognition by PP1 conferred via its association with regulatory or targeting subunits. Hormonal control of cellular PP1 activity regulates a variety of functions including protein synthesis, gene transcription, glycogen and lipid metabolism, muscle contractility, and learning and memory (1, 2). These diverse functions are directed by the unique localization and substrate recognition by PP1 conferred via its association with regulatory or targeting subunits. Hormonal control of cellular PP1 activity.

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MATERIALS AND METHODS

Antibodies (Dilutions Used for Western Blotting Are Shown in Parentheses)—A monoclonal antibody against neurabin I (1:250) and an anti-PP1 antibody (1:2000) were obtained from BD Biosciences. The rabbit polyclonal antibody was made against rat neurabin II/spinophilin (1:500 as described previously (14). The anti-NF-L monoclonal antibody (1:1000) was obtained from Sigma. The rabbit polyclonal anti-GADD34 antibody S20 (1:500) was purchased from Santa Cruz Biotechnology. The monoclonal anti-PDSD-95 antibody (1:1000) was provided by Morgan Sheng, Massachusetts Institute of Technology. The monoclonal anti-HA (hemagglutinin) antibody (1:1000) was from Covance, and the monoclonal anti-GFP antibody (1:2000) was from Clontech. The sheep anti-I-2 polyclonal antibody (1:1000) was described previously (17).

For Western immunoblotting, proteins were subjected to electro-
Phosphorylation on 10% polyacrylamide gels in the presence of SDS-PAGE. The gels transferred electrophoretically to polyvinylidene difluoride membranes, which were blocked in TBS (10 mM Tris-HCl, pH 7.5, 150 mM NaCl) plus 0.05% Tween and 4% (w/v) milk. The membranes were then incubated with primary antibody at 4°C overnight, washed with TBS plus 0.05% Tween, and incubated with appropriate secondary antibodies for 1 h at room temperature. The immunoreactive proteins were visualized with chemiluminescence (PerkinElmer Life Sciences).

Expression of Recombinant Proteins—The plasmid DNA encoding human 1-2 (1-205) fused to GST was kindly provided by Heman Patel, McGill University (18). GST-hI-2 (1-205) was expressed in *E. coli* BL21 (Strategene) and purified with glutathione beads (Amersham Biosciences) as per the manufacturer's instructions. To generate library I-2–(197), encoding hI-2 (1-2-118) clones were cloned into the KpnI and XhoI restriction sites of pET30b (Novagen). The hI-2 (1-149) cDNA was cloned into BamHI and EcoRI sites of pET30a. Hexahistidine-tagged proteins were expressed in *E. coli* BL21(DE3)pLysS (Strategene) and purified on Ni2+-NTA-agarose (Qiagen) as described previously (24). Briefly, GST-GM-240 phosphorylated in vitro with PKA was also used in pulldowns as described previously (25).

For codestimation of purified proteins, GST-NrbII (354-494) (5 μg of total protein) was incubated with glutathione-Sepharose for 1 h at 4°C. The beads were washed twice with TBS, and increasing concentrations of purified skeletal muscle PPP (26) dialyzed into 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 15 mM β-mercaptoethanol, and bovine serum albumin (1 mg/ml) or the buffer alone were added and mixtures incubated for 1 h. Following this, purified rabbit skeletal muscle I-2 (1 μg of total protein) was added and the incubation continued for 1 h. The beads were then washed 3 times with TBS, and the proteins were eluted with 25 μl of SDS sample buffer and analyzed by SDS-PAGE.

For quantitative assays of β-galactosidase expression, dilution plasters were grown in selective liquid media to A 0.6. An aliquot (1.5 ml) of the cultures was subjected to centrifugation, and yeasts were resuspended in 200 μl of Z buffer (100 mM H3PO4, 1 M KCl, 1 mM MgSO4, pH 7.0). The cells were permeabilized in 50 μl of 0.1% (w/v) SDS, and 50 μl of chloroform by vortexing three times for 30 s each, and the lysates were assayed for β-galactosidase using O-nitrophenyl β-D-galactoside as described (31).

**Immunoprecipitation**—HER283T cells, grown to near-confluence in 6-well plates, were transfected with 1.5 μg of DNA encoding HA-hI-2 (1–197), 1.5 μg of GFP-NrbII (1–1095) DNA, and 6 μl of LipofectAMINE (Invitrogen), according to the manufacturer's instructions. After 24 h, cells were washed with PBS and lysed in 500 μl of RIPA buffer (10 mM sodium phosphate pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% SDS) for 15 min on ice. Cells were scraped and the lysates cleared by centrifugation at 20,000 × g for 3 min. 1 μl of Living Colors polyclonal anti-GFP antibody (Clontech) and protein G-Sepharose (Sigma) were added, and the mixture incubated for 1 h (14). A 1:1 slurry of protein A-agarse (Bio-Rad) and protein G-Sepharose (Sigma) were added for 1 h, and beads were washed 4 times with NETN-250 prior to solubilizing the immunoprecipitates in SDS sample buffer and analysis on SDS-PAGE.

**Actin Sedimentation**—The Actin Binding Protein Biochem Kit (Cytoskeleton) (14). Briefly, 40 μl of rabbit (or 40 μl of polymerase) were incubated with combinations of 7.5 μg of hexahistidine-NrbI (1–516), 5 μg of PPP, and 2 μg of I-2, both purified from rabbit skeletal muscle, were added in a total volume of 60 μl, and the mixture was incubated for 30 min at room temperature. The polymerized actin was sedimented by centrifugation at 150,000 × g for 1.5 h at 24°C, and the pellet containing F-actin and the supernatant proteins or the soluble fraction was analyzed by SDS-PAGE. To establish actin-mediated sedimentation, we included a depolymerization step that solubilized the F-actin-bound proteins in 200 μl of 1 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, and 0.1% β-mercaptoethanol, followed by dialysis against 1,000-fold excess volume of the same buffer for 3 h at room temperature and centrifugation at
150,000 x g for 1.5 h. This procedure solubilized >90% of F-actin and the actin-associated proteins, which were analyzed by SDS-PAGE.

**Immunocytochemistry**—Madin-Darby canine kidney (MDCKII) cells were grown to confluence on fibronectin-coated coverslips in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cells were washed with PBS containing 1 mM CaCl$_2$ and 0.5 mM MgCl$_2$, permeabilized with a 1:1 mixture of methanol/aceton for 10 min at −20 °C, rinsed twice with PBS containing 0.05% Tween 20 (PBS-T), and incubated in a 10% fetal bovine serum/PBS-T blocking solution for 1 h at room temperature. Sheep polyclonal antibody against human I-2 (17) and rabbit polyclonal anti-rat spinophilin/NrbII (Upstate Biotechnology, Inc.) were diluted in PBS-T at 1:500 and 1:400 respectively, and applied to the coverslips for 1 h at room temperature, and quenched in 50 mM ammonium chloride for 10 min at room temperature prior to permeabilization in PBS containing 0.2% Triton X-100 for 2 min. The coverslips were rinsed with PBS, blocked with 3% bovine serum albumin for 1 h at room temperature, and stained as described above using an Alexa Fluor 488-labeled donkey anti-sheep antibody (Molecular Probes).

Primary rat E19 embryonic hippocampal neurons were plated at a density of 2 x 10^4 cells/60-mm dish on polylisin-treated coverslips and cocultured with rat glial cells for 14 days (32) in minimum essential medium (Invitrogen) supplemented with 10% horse serum. The cultures were rinsed with PBS, fixed in 4% paraformaldehyde for 15 min at room temperature, and quenched in 50 mM ammonium chloride for 10 min at room temperature prior to permeabilization in PBS containing 0.2% Triton X-100 for 2 min. The coverslips were rinsed with PBS, blocked with 3% bovine serum albumin for 1 h at room temperature, and stained as described above using an Alexa Fluor 488-labeled donkey anti-sheep antibody (Molecular Probes).

Digital images were captured using a Nikon Microphot-SA epifluorescence microscope with Nikon Plan Apo 60x/1.4 oil immersion objective and appropriate filters for fluorescein isothiocyanate, rhodamine, and DAPI and a Hamamatsu Orca C4742-95 digital camera. The image files created using Openlab software (Improvision) were contrast-enhanced, pseudocolored, and merged using Photoshop 6.0 software.

**RESULTS**

**Identification of Neurabin I and Neurabin II as I-2-binding Proteins**—To analyze neuronal signaling complexes assembled by I-2, we undertook affinity chromatography of rat brain deoxycholate (DOX)-extracted lysates (enriched in plasma membrane and cytoskeletal proteins) on immobilized GST-hI-2-(1–205). This full-length human I-2 bound several PP1-binding proteins that were visualized by far Westerns using digoxigenin-conjugated PP1 as probe (Fig. 1A). GST alone did not bind these proteins. In contrast, PP1-binding proteins from rat brain cytosol were not concentrated by GST-hI-2-(1–205) (Fig. 1A). By utilizing antibodies against known neuronal PP1-binding proteins, we identified two of these I-2-binding proteins as neurabin I (NrbI) and neurabin II/spinophilin (NrbII) (Fig. 1B). GST-hI-2-(1–205) failed to bind other PP1-binding proteins, such as GADD34 (10) and NF-L (34), or PSD-95, a major neuronal protein adapter (35).

**Structural Determinants in I-2 Required for Neurabin Binding**—I-2 contains numerous domains that mediate PP1 binding and inhibition (20, 30). To identify the key determinants required for neurabin binding, we undertook pulldowns using recombinant hexahistidine (His)-tagged I-2 proteins. His-hI-2-(1–197) bound and sedimented PP1, NrbI, and NrbII from rat brain DOX lysates (Fig. 2A). His-hI-2-(1–197), lacking a domain critical for potent PP1 inhibition (30), showed decreased binding to PP1 and neurabin I (Fig. 2A). His-hI-2-(1–118), which sedimented PP1 effectively, bound no detectable NrbI and low levels of NrbII similar to Ni$^{2+}$/NTA beads alone. This suggested that the C terminus of I-2, although not essential for PP1 binding, was required for effective neurabin recruitment. I-2 shares ~50% sequence identity with I-4, another PP1 inhibitor (19). Full-length GST-hI-4-(1–202) sedimented PP1, NrbI, and NrbII from brain DOX lysates (Fig. 2B), suggesting that sequences common to I-2 and I-4 mediated binding to PP1 and neurabins. As seen with His-hI-2-(1–118), GST-hI-4-(1–123), lacking C-terminal sequences, bound PP1 effectively but failed to sediment NrbI or NrbII from brain lysates (Fig. 2B). This emphasized that the C-terminal sequences shared by I-2 and I-4 facilitated their binding to neurabins. However, the C-terminal domain of the I-2, His-hI-2-(197–198), alone failed to bind detectable PP1, and GST-hI-4-(124–202) bound less than 10% of PP1 compared with WT I-4; particularly significant for our studies, neither polypeptide sedimented neurabins from tissue lysates (data not shown). This suggested that the C termini of I-2 and I-4 were necessary but not sufficient to bind the neurabin-PPI complex.

**Structural Determinants in Neurabin Required for I-2 Binding**—To define domains in rat NrbI and NrbII that dictated their association with I-2, we conducted pulldowns from rat brain DOX lysates using several recombinant neurabin domains. The central domains represented by GST-NrbI-(374–516) and GST-NrbII-(354–494), previously shown to bind PP1 (21), bound I-2 (Fig. 3A). Smaller peptides, GST-NrbI-(436–479) and GST-NrbII-(427–470), retained the tetrapeptide PP1-binding motif and bound PP1 (21) and I-2 weakly. A point mutation in the PP1-binding motif eliminated both PP1 and I-2 binding to GST-NrbI-(374–516, F460A). This suggested that PP1 was a required intermediate for recruitment of I-2 by neurabin. Two other PP1-binding peptides, GST-GM-(1–240) derived from human skeletal muscle glycogen-targeting subunit (3),...
and GST-GADD34-(513–674), a fragment of human GADD34 (10, 36), bound PP1 from rat brain DOX lysates. However, only GST-G3M-(1–240) also recruited I-2 (Fig. 3A). This demonstrated that a subset of PP1-targeting subunits in mammalian tissues associate with both PP1 and I-2. PKA phosphorylation of G3M at serine 67 within the PP1-binding motif abrogated PP1 binding both in vivo and in vitro (37), and in pulldowns from NIH3T3 cell extracts using GST-G3M-(1–240) phosphorylated in vitro by PKA, neither PP1 nor I-2 binding was observed (Fig. 3B). This suggested that, as noted with neurabins, I-2 associates with G3M via PP1, and phosphorylation within the PP1-docking motif regulates the association of a PP1-I-2 complex with G3M.

Further evidence for PP1 functioning as a bridge to link I-2 to neurabins was obtained using the purified proteins. GST-NrbII-(354–494) preloaded with increasing concentrations of rabbit skeletal muscle PP1 sedimented rabbit skeletal muscle I-2 in a dose-dependent manner (Fig. 3C). No association of I-2 with GST-NrbII-(354–494) was detected in the absence of PP1. GST-NrbII-(354–394) also sedimented a preformed complex of PP1 bound to full-length hI-2-(1–205). Under similar conditions, GST-NrbII-(354–394) bound PP1 but did not recruit hI-2-(1–114) (data not shown). These data showed that PP1 bridged full-length hI-2-(1–205) to neurabins, but the N-terminal domain, I-2-(1–114), lacked critical determinants required for the formation of a stable NrbII-PP1-I-2 complex.

Microcystin-LR, a PP1 Inhibitor, Attenuates I-2 Binding to Neurabin (PP1—MC, a hepatotoxin, binds the PP1 catalytic site and inhibits enzyme activity. The high affinity of MC for PP1 and other serine/threonine phosphatases has prompted the use of MC-Sepharose for affinity isolation of numerous cellular phosphatase complexes (38). MC-Sepharose concentrated PP1 associated with NrbI and NrbII from rat brain lysates, but these complexes contained no detectable I-2 (Fig. 4A). This was consistent with earlier observations suggesting a competition between MC and I-2 for PP1 binding (20). Thus, MC-Sepharose excluded PP1 complexes in which the PP1 catalytic site was occupied by I-2.

To investigate this further, we also undertook pulldowns with GST-neurabins from DOX lysates in the presence and absence of 10 μM MC (Fig. 4B). The addition of MC prevented binding of both PP1 and neurabin to GST-hI-2-(1–205). In the converse experiment, GST-NrbI-(374–516) and GST-NrbII-(354–494) bound both PP1 and I-2 from DOX lysates. Although addition of 10 μM MC abolished I-2 binding, both GST-neurabins showed an increased association with PP1 under these conditions (Fig. 4B). These in vitro studies not only suggested that I-2 regulated levels of PP1 recruited by neurabins but also supported the notion that I-2 bound the active site of the PP1-neurabin complex and generated an inactive phosphatase complex.
Interaction of PP1 Catalytic Subunit with Neurabin I and I-2—A single gene (GLC7) encodes the yeast PP1 catalytic subunit, which shares 87% overall sequence identity to human PP1. Mutational analyses of GLC7 have provided insights into the interactions of PP1 with several mammalian regulators (29, 30, 39). By using a yeast two-hybrid assay, we analyzed the association of rat NrbI and human I-2 with 39 yeast PP1 mutants (27, 28). Surprisingly, NrbI proteins containing the N-terminal actin-binding domain failed to bind PP1, and a deletion that yielded NrbI-(103–1095) was required to promote PP1 binding. Whereas a majority of PP1 mutants bound both regulators effectively, a subset of the mutants showed deficits in their association to hI-2-(1–205) or NrbI-(103–1095) (Fig. 5A). PP1(K259A,R260A) and PP1(D229A,R233A) bound hI-2-(1–205) similar to WT PP1, but PP1(K110A,K112A) showed a diminished association with hI-2-(1–205). This mutation, however, had little or no effect on PP1 binding to NrbI-(103–1095). Enzyme assays using partially purified WT and mutant PP1 catalytic subunits correlated the loss of two-hybrid interaction with a reduced sensitivity of PP1 to inhibition by I-2 (data not shown). The amino acids that selectively attenuated the association of PP1 with I-2 and/or NrbI were mapped on the three-dimensional structure of the PP1 catalytic subunit (Fig. 5B), and the data suggested some differences in PP1 docking by the two regulators.

Cellular PP1 Complexes Contain Neurabin I and I-2—To establish the presence of I-2 in a cellular neurabin-PP1 complex, we coexpressed GFP-NrbI proteins with HA-hI-2-(1–197)
To investigate I-2 targeting by neurabins, we undertook and target neurabin-bound proteins to the actin cytoskeleton F-actin through their N-terminal actin-binding domain (15, 40) that even in cells, NrbI required PP1 binding to recruit I-2. The core PP1-binding sequence, KIKF, abrogated the association were present in both immunoprecipitates. A point mutation in the protein (IP-GFP) were also immunoblotted (IB) with antibodies against GFP, HA, and PP1. Cell lysates were also subjected to immunoblotting with anti-GFP and anti-HA antibodies to establish the expression levels of GFP-NrbI and HA-hI-2.

in HEK293T cells. Anti-GFP immunoprecipitates contained equivalent levels of GFP-NrbI-(1–1095) (the full-length protein) and GFP-NrbI-(1–552) (Fig. 6). HA-hI-2-(1–197) and PP1 were present in both immunoprecipitates. A point mutation in the core PP1-binding sequence, KIKF, abrogated the association of GFP-NrbI-(F460A) with both PP1 and I-2. This established that even in cells, NrbI required PP1 binding to recruit I-2.

**Neurabins Recruit PP1 and I-2 to F-actin**—Neurabins bind F-actin through their N-terminal actin-binding domain (15, 40) and target neurabin-bound proteins to the actin cytoskeleton (14). To investigate I-2 targeting by neurabins, we undertook *in vitro* actin sedimentation assays with hexahistidine-tagged NrbI-(1–516) that contained an actin-binding domain and the central region that binds both PP1 and I-2 (Fig. 3). F-actin sedimented more than 90% of the His-NrbI-(1–516) (data not shown). In the absence of His-NrbI-(1–516), rabbit I-2-(1–205) bound F-actin weakly, and no PP1 binding was observed (Fig. 7A). His-NrbI-(1–516) increased the sedimentation of both I-2 and PP1 with F-actin. Interestingly, PP1 association with the F-actin-bound His-NrbI-(1–516) was further enhanced when PP1 and I-2 were presented together. These data suggested that actin-bound NrbI interacted independently with PP1 and I-2, but the synergism between the NrbI-bound I-2 and PP1 further enhanced PP1 targeting to polymerized actin.

In contrast to WT hI-2-(1–205), the N-terminal fragment, hI-2-(1–114), showed no detectable binding to F-actin and required His-NrbI-(1–516) to sediment with the polymerized actin (Fig. 7B). PP1 severely attenuated the NrbI-mediated recruitment of hI-2-(1–114) to F-actin. Unlike WT hI-2-(1–205), hI-2-(1–114) did not enhance PP1 binding to His-NrbI-(1–516) associated with F-actin. Instead, hI-2-(1–114) diminished the levels of NrbI-bound PP1 (Fig. 7B). Increasing PP1 levels eliminated hI-2-(1–114) binding to the F-actin-bound His-NrbI-(1–516), and elevating hI-2-(1–114) concentrations abolished NrbI-mediated sedimentation of PP1 with the polymerized actin (data not shown). These data suggested that PP1 competed with the N terminus of I-2 for neurabin binding, and the additional interactions between PP1 and I-2 generated a complex that showed enhanced association with the actin-bound neurabin (Fig. 7C).

**Co-localization of Neurabin II and I-2 in Cells**—Prior immunocytochemical studies showed that NrbII was concentrated at adherens junctions in Madin-Darby canine kidney (MDCK) polarized epithelial cells (41). Immunocytochemistry using an anti-NrbII antibody confirmed that in MDCK II cells, NrbII was concentrated at the cell periphery consistent with adherens junctions (Fig. 8A). Double staining with anti-I-2 antibody showed a punctate distribution of I-2 throughout the cytoplasm, but a significant amount of I-2 was concentrated at the periphery of adherent cells (Fig. 8A) in regions that also stained for F-actin (data not shown). Merging of the images

![Image](http://www.jbc.org/)

**Fig. 6. Assembly of neurabin:PP1-I-2 complex in HEK293T cells.** HEK293T cells were transfected with plasmids encoding HA-hI-2-(1–197) and GFP-NrbI proteins (1.5 μg of DNA) with antibodies against GFP, HA, and PP1. Cell lysates were also subjected to immunoblotting with anti-GFP and anti-HA antibodies to establish the expression levels of GFP-NrbI and HA-hI-2.

**Fig. 7. Neurabin I recruits PP1 and I-2 to F-actin.** A, polymerized F-actin (40 μg of protein) was incubated in the presence (+) or absence (−) of 7.5 μg of His-NrbI-(1–516), 5 μg of PP1, and/or 2 μg of I-2. Following actin sedimentation and extraction as described under “Materials and Methods,” the F-actin-bound proteins were analyzed by SDS-PAGE. Actin and NrbI-(1–516) were visualized by protein staining with Coomassie Blue, and the other proteins were immunoblotted with antibodies against PP1 and I-2. B shows identical F-actin sedimentation assays using 2 μg of I-2-(1–114). C incorporates the data into a model that shows independent and mutually exclusive association of PP1 and I-2 with neurabins at the actin cytoskeleton. A conformation change in PP1 and/or I-2 converts this complex (indicated by arrow) into one where the association of one or both proteins is promoted or stabilized with the actin-bound neurabins.
established that NrbII and I-2 colocalized at the actin-rich adherens junctions in polarized epithelial cells.

NrbI and NrbII are highly expressed in neurons and concentrated in the post-synaptic density (34). Immunocytochemistry of cultured rat hippocampal neurons showed that I-2 was distributed throughout the cytoplasm and concentrated in puncta representing spines (Fig. 8B). I-2 staining overlapped with that for NrbII, which was even more concentrated in spines (16). Higher magnification (Fig. 8C) emphasized the localization of I-2 and NrbII in foci representing spines. I-2 and NrbII also colocalized at growth cones in younger neurons (data not shown). The data suggested that neurabins targeted I-2 to actin-rich structures in both neurons and non-neuronal cells.

DISCUSSION

Two classes of PP1 regulators, termed targeting subunits and inhibitors, control dephosphorylation events that regulate eukaryotic cell physiology (4, 5). Previous data (4, 6) suggested a mutually exclusive interaction of these regulators with the PP1 catalytic subunit, based on the presence of a consensus motif, (K/R)(I/V)X, required for PP1 binding and/or regulation. PP1 regulators also contain other sequences, such as ankyrin (42) and leucine-rich (43) repeats, which can contribute to PP1 binding. This suggests that by utilizing a subset of interactions with PP1, targeting subunits and inhibitors may not always compete but instead collaborate in PP1 regulation. CPI-17 (protein kinase C-potentiated inhibitor protein of 17 kDa) and the structurally related PHII (phosphatase holoenzyme inhibitor) lack recognizable KVXF motifs and inhibit myosin phosphatases composed of PP1 bound to a myosin-binding subunit (11, 12). Several protein kinases regulate the smooth muscle myosin phosphatase activity by phosphorylating either CPI-17 (44) or myosin-binding subunit (45), providing direct evidence that these two PP1 regulators cooperate in the hormonal control of smooth muscle contraction.

Another example of coordinated control of PP1 activity by more than one regulator comes from the discovery that the growth arrest and DNA damage-inducible protein, GADD34, binds both PP1 and I-1, a PKA-activated PP1 inhibitor (10). Whereas GADD34 and I-1 both contain KIXF motifs essential for PP1 binding, independent interactions between the three proteins override potential competition for this key PP1-binding site and allow effective regulation of the GADD34-bound PP1 by the PKA-phosphorylated I-1 (10). In this study, we identified another heterotrimeric PP1 complex containing I-2 and neurabins. Unlike the GADD34-PP1-I-1 complex, PP1 is a necessary bridge in the assembly of the Nrb-II-PP1-I-2 complex. Several lines of evidence support this conclusion. First, GST-NrbI-(436–479) consisting of 43 amino acids representing less than 5% of the full-length NrbI bound both PP1 and I-2, and mutating a single amino acid abolished NrbI binding to both proteins. Furthermore, no other proteins appeared to be required as the heterotrimeric complex could be reconstituted using purified PP1, I-2, and recombinant Nrb polypeptides.

Interestingly, deletion of the N-terminal actin-binding domain was required for NrbI-PP1 association in a yeast two-hybrid assay. As the GAL4-NrbI was targeted to the yeast
neurabin II (21, 49) binding. These data suggested that a consensus recognition site for NrbI and I-2 on the PP1 catalytic subunits and identified additional residues that impaired I-2 but not NrbI binding. Yet other residues (blue in Fig. 5B) abrogated the association of PP1 with NrbI (103–1095) but not I-2. I-2 and NrbI also shared common docking sites on PP1 (green in Fig. 5B). These include the hydrophobic pocket that bound the tetrapeptide motif (6), KIKF in NrbI (21, 46) and KLHY in I-2 (20, 47), and the β12–β13 loop, which promoted I-2 (48) and NrbII (21, 49) binding. These data suggested that a subset of these unique and overlapping sites are utilized for PP1 binding to neurabin I and II within a heterotrimeric complex. MC, a cyclic heptapeptide inhibitor, docks in the PP1 catalytic site (6, 48, 50) and competes with I-2 for PP1 binding (20). MC abolished I-2 binding to an NrbI-PP1 complex formed by incubating GST-Nrb with rat brain DOX lysates and enhanced PP1 recruitment by GST-Nrb (Fig. 4). The ability of MC to displace I-2 from PP1 bound to the neurabin central domains argued that in this complex I-2 occupied the PP1 catalytic site and produced an inactive protein phosphatase. Yet, NrbII complexes immunoprecipitated from cultured mammalian cells were shown to be active protein phosphatases (14). This may indicate that the heterotrimeric complexes formed by full-length NrbI are different from those assembled by the truncated Nrb central domains or suggest that cellular mechanisms activate an NrbII-PP1-1 complex. One such mechanism may involve the phosphorylation of I-2 on threonine 72. In vitro studies showed that GSK-3 (51), mitogen-activated protein kinases (53), and CDK5, a neuronal protein kinase (18), phosphorylated I-2 within a dimeric PP1-1 complex and transiently activated this “ATP-Mg-dependent protein phosphatase” (51, 52). Alternatively, NrbI can be phosphorylated by PKA in vitro (55) and in vivo (14). This phosphorylation occurs at serine 461, immediately adjacent to the PP1-binding motif, and attenuates PP1 binding to NrbI (55). Thus, as shown in this study with the prototypic PP1-targeting subunit, Gαs, NrbI phosphorylation by PKA may also displace I-2. Finally, neurabins shuttled on and off the actin cytoskeleton in response to the Rac-1 GTPase by growth factors (54) providing yet another mechanism for regulating the NrbII-PP1-2 complex.

Immunocytochemistry established that I-2 and NrbII colocalized at actin-rich adherens junctions that mediate cell-cell contact in MDCK-polarized epithelial cells and in dendritic spines and growth cones of cultured rat hippocampal neurons. Whereas the function of PP1 at the adherens junctions remains unknown, extensive studies suggest that PP1 dephosphorylates receptors, ion channels, and other signaling molecules in dendritic spines (14, 16, 56) to regulate synaptic transmission (57). Thus, the disruption of the mouse NrbI gene resulted in altered regulation of two prominent PP1 substrates, the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate and N-methyl-D-aspartate receptors, and impaired synaptic plasticity (33), a cellular form of learning. This may suggest that the neurabin-PP1-1 complex controls the function and morphology of neurons and non-neuronal cells.

In conclusion, we identified a novel PP1 complex containing the two neurabin isoforms (NrbI or NrbII) and I-2. By using purified proteins, cultured cells, and tissue extracts from several species including human, mouse, rat, dog, and rabbit, we established that neurabins target the PP1-1-2 complex to F-actin. The widespread expression of PP1, I-2, and NrbII suggests that this complex may regulate cytoskeletal functions and cell morphology in many tissues. Our data suggest that PP1 and I-2 are also recruited by other targetting substrates, including Gαs, the skeletal muscle glycogen targeting subunit. Thus, analysis of the NrbII-PP1-1 complex may provide important insights in the collaborations between I-2 and other PP1 regulators that dictate PP1 functions in mammalian tissues.

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