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Differential regulation of protein phosphatase-1_I by neurabin

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Abstract

Neurabin is a brain-specific actin and protein phosphatase-1 (PP-1) binding protein that inhibits the purified catalytic subunit of protein phosphatase-1 (PP-1_C). However, endogenous PP-1 exists primarily as multimeric complexes of PP-1_C bound to various regulatory proteins that determine its activity, substrate specificity, subcellular localization and function. The major form of endogenous PP-1 in brain is protein phosphatase-1_I (PP-1_I), a Mg^{2+}/ATP-dependent form of PP-1 that consists of PP-1_C, the inhibitor-2 regulatory subunit, an activating protein kinase and other unidentified proteins. We have identified four PP-1_I holoenzyme fractions (PP-1_{IA}, PP-1_{IB}, PP-1_{IC}, and PP-1_{ID}) in freshly harvested pig brain separable by poly-L-lysine chromatography. Purified recombinant neurabin (amino acid residues 1–485) inhibited PP-1_{IB} ($IC_{50} = 1.1$ μM), PP-1_{IC} ($IC_{50} = 0.1$ μM), and PP-1_{ID} ($IC_{50} = 0.2$ μM), but activated PP-1_{IA} by up to threefold ($EC_{50} = 40$ nM). The PP-1_{IA} activation domain was localized to neurabin$^{1–210}$. Our results indicate a novel mechanism of PP-1 regulation by neurabin as both an inhibitor and an activator of distinct forms of PP-1_I in brain.

Keywords: Protein phosphatase-1; Neurabin; PP-1 inhibitor; Actin binding protein

Neurabin is a brain-specific actin and protein phosphatase-1 binding protein found predominantly in dendritic spines, but also in dendrites, axons, axon terminals, and glia [1–3]. Previous studies on the regulation of protein phosphatase-1 (PP-1) by neurabin showed that it is a potent and specific inhibitor of the catalytic subunit of PP-1 (PP-1_C) [1,4]. PP-1_C interacts with various proteins, such as targeting subunits, inhibitory subunits and substrates [5]. Most, but not all, of these interactions are mediated by the PP-1_C binding motif (R/K)X(V/I)XF [5]. Targeting subunits determine the subcellular localization and therefore the specialized functions of the various forms of PP-1 [5,6]. The predominant cytosolic form of PP-1 in pig brain is protein phosphatase-1_I (PP-1_I) which consists of the catalytic subunit PP-1_C, the inhibitor-2 regulatory subunit (I-2), and other regulatory subunits, including an unidentified activating protein kinase [7–10].

Neurabin interacts with both PP-1_C and I-2 [11]; it binds PP-$1_{C\gamma1}$ with higher affinity than PP-$1_{C\alpha}$ or PP-$1_{C\beta}$ [12,13]. It has been suggested that PP-1_C bridges the association of I-2 with neurabin, and that neurabin acts as a targeting protein that directs PP-1_C and I-2 to polymerized actin in the control of cell morphology [14]. However, the PP-1_C and I-2 complex (PP-1_I) is inactive in its basal state and requires phosphorylation by an activating kinase for activation. Whether neurabin regulates the activity of native brain PP-1_I is unknown since previous studies were performed with isolated PP-1_C or immunoprecipitates in the absence of kinase activation. We determined the effects of recombinant neurabin on the activity of endogenous PP-1_I complexes purified from fresh pig brain. These studies indicate that neurabin has differential effects on the activity of specific forms of native brain PP-1_I.

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Experimental procedures

Reagents. TP, benzamidine, DEAE–Sepharose, poly-L-lysine agarose, phenylmethylsulfonyl fluoride (PMSF), and bovine serum albumin (BSA) were from Sigma (St. Louis, MO). Superdex 200 and [γ-^{32}P]ATP were from GE Healthcare (Piscataway, NJ).

Protein preparation. ^{32}P-labeled phosphorylase a was prepared by phosphorylation of phosphorylase b by phosphorylase kinase as described [15]. The catalytic subunits of protein phosphatase-1 (PP-1$_C$) and protein phosphatase-2A (PP-2A$_C$) were purified to homogeneity from pig brain as described [15]. Human DARPP-32 was cloned from a human cDNA library, overexpressed and purified from transfected *Escherichia coli*. (Invitrogen, CA). Phospho-Thr^{34}-DARRP-32 was prepared by phosphorylation of DARPP-32 with PKA as described [16].

Preparation of neurabin. Full-length neurabin forms high molecular weight oliomers that cannot be purified (data not shown). Soluble C-terminals frament of rat neurabin (neurabin$^{1–485}$, neurabin$^{1–210}$, neurabin$^{1–102}$, neurabin$^{103–210}$, neurabin$^{50–150}$, and neurabin$^{50–210}$) were obtained by amplifying the rat cDNA [2] by PCR with the appropriate primers. The cDNA for each fragment with a polyhistidine (6 His) or glutathione-*S*-transferase (GST) tag was subcloned into the expression vector pTrcHis2-TOPO and used to transform BL21 *E. coli* as recommended by the manufacturer (Invitrogen, CA). Fragments were purified by chromatography on glutathione–agarose or Ni^{2+}–NTA–agarose (GE Healthcare, Piscataway, NJ). Neurabin$^{1–485}$ was further purified by chromatography on Superdex 200 and Mono Q FPLC.

Assay of PP-1$_I$. Protein phosphatase-1$_I$ was assayed by its ability to dephosphorylate ^{32}P-labeled phosphorylase a following preincubation with ATP and Mg^{2+}. Briefly, the assay consisted of 0.01 ml of PP-1$_I$ in 50 mM imidazole–Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/ml BSA (Enzyme Diluent Buffer); 0.01 ml of Reaction Buffer (50 mM imidazole–Cl pH 7.2, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mg/ml BSA, 0.375 mM ATP and 3.75 mM $MgCl_2$ (Enzyme Assay Buffer); and 0.01 ml of 3 mg/ml ^{32}P-labeled phosphorylase a in Enzyme Diluent Buffer plus 75 mM caffeine. Protein phosphatase-1$_I$ complex was preincubated with ATP and Mg^{2+} in Enzyme Assay Buffer for 5 min at 30 °C prior to initiation of the reaction with ^{32}P-labeled phosphorylase a. After 10 min at 30 °C, the reaction was terminated with 0.2 ml of 25% (w/v) trichloroacetic acid. The suspension was centrifuged at 10,000*g* for 5 min and 0.2 ml of the supernatant containing the released [^{32}P]phosphate was analyzed for Cerenkov radiation in a liquid scintillation counter. One unit of phosphatase activity catalyzes the dephosphorylation of 1 nmol of substrate per min at 30 °C.

Purification of PP-1$_I$. A 50 kg pig was anesthetized with an intramuscular injection of xylazine/ketamine in accordance with a protocol approved by the IACUC of Weill Medical College of Cornell University. Cardiac arrest was induced by intravenous injection of cold saturated KCl. The brain (~100 g) was removed within 5 min of cardiac arrest by extraction through the maxilla, chilled on ice, cut into 1 cm pieces, and homogenized in 5 volumes of 50 mM imidazole–Cl pH 7.3, 2 mM EDTA, 2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF and 5% (v/v) glycerol by 6 × 30 s pulses at low speed in a blender. All procedures were performed at 4 °C. The homogenate was centrifuged at 10,000*g* for 30 min. The supernatant (the soluble extract) was collected, diluted twofold in 25 mM imidazole–Cl pH 7.3, 0.2 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 1 mM benzamidine, 0.1 mM PMSF and 10% (v/v) glycerol (Buffer A), and loaded onto a DEAE–Sepharose column (2.5 × 20 cm) equilibrated in Buffer A. The column was washed with 300 ml of Buffer A plus 50 mM NaCl, and then eluted with Buffer A plus 300 mM NaCl. Active fractions of PP-1$_I$ were collected, diluted 10-fold with Buffer A, and loaded onto a poly-L-lysine column (1.5 × 10 cm) equilibrated in Buffer A. The column was washed with 150 ml of Buffer A, then eluted sequentially with Buffer A plus 50 mM NaCl, 100 mM NaCl, 250 mM, and 500 mM NaCl. Active fractions from each step were collected, concentrated by vacuum dialysis and separated on a Superdex 200 column (1.5 × 60 cm) equilibrated in Buffer A plus 200 mM NaCl at a flow rate of 0.75 ml/min. Active fractions were collected, concentrated by vacuum dialysis and stored at −20 °C in Buffer A plus 50% (v/v) glycerol.

Miscellaneous methods. Protein concentration was determined by the method of Bradford using BSA as standard [17]. Data were analyzed using GraphPad Prism 4.2 (San Diego, CA) and expressed as means ± SD. IC_{50} and EC_{50} values were determined using a sigmoidal dose-response curve fit program.

Results

Protein phosphatase-1$_I$ was the major form of protein phosphatase-1 in cytosolic extracts of pig brain obtained immediately after cardiac arrest (Fig. 1A). The

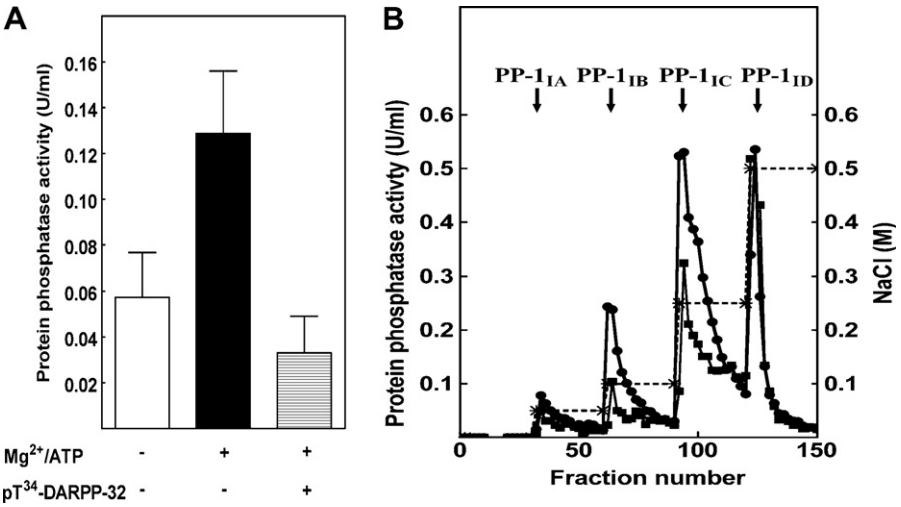

Fig. 1. Cytosolic protein phosphatase activity in pig brain and Identification of four forms of PP-1$_I$ by chromatography on poly-L-lysine agarose. (A) Pig brain cytosol extract was prepared and assayed for phosphorylase phosphatase activity in the absence or presence of 1.25 mM Mg^{2+} and 0.125 mM ATP. The contribution of PP-1 activity to the phosphorylase phosphatase activity was determined using 100 nM phospho-Thr^{34}-DARPP-32, a specific inhibitor of PP-1$_C$. (B) PP-1$_I$ eluted from DEAE–Sepharose was separated by chromatography on poly-L-lysine agarose column. The column was washed with Buffer A plus 0 NaCl and then eluted sequentially with Buffer A plus 50 mM NaCl, 100 mM, 250 mM NaCl or 500 mM NaCl, as indicated. The eluates were assayed for PP-1$_I$ activity in the presence (●) and absence of (■) of Mg^{2+} and ATP. Similar results were obtained in three independent experiments.

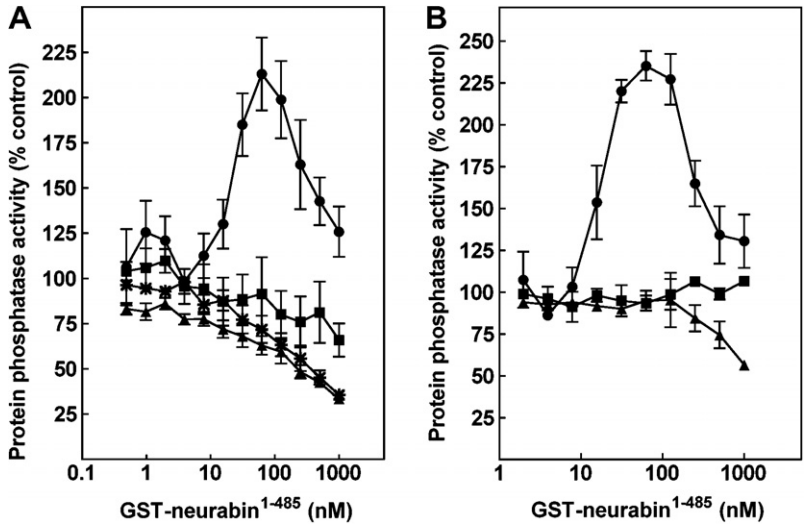

Fig. 2. Effect of neurabin$^{1–485}$ on the activities of PP-1_I, PP-1_C, and PP-$2A_C$. (A) PP-1_{IA} (●), PP-1_{IB} (■), PP-1_{IC} (▲) and PP-1_{ID} (*) were assayed in the presence of various concentrations of GST-neurabin$^{1–485}$ with 0.005 unit of PP-1_I. (B) PP-1_{IA} (●), PP-1_C, (▲) and PP-$2A_C$ (■) were assayed in the presence of various concentrations of neurabin$^{1–485}$ with 0.005 unit PP-1_{IA}, 0.01 unit PP-1_C or 0.01 unit PP-$2A_C$.

Mg^{2+}/ATP dependent phosphorylase phosphatase activity was inhibited by phospho-Thr^{34}-DARPP-32, a specific inhibitor of PP-1 [18]. PP-1_I activity was further purified by DEAE–Sepharose and poly-L-lysine agarose chromatography. Four forms of PP-1_I were identified by differential elution from poly-L-lysine agarose with NaCl, termed PP-1_{IA}, PP-1_{IB}, PP-1_{IC}, and PP-1_{ID} (Fig. 1B), and were further purified by gel filtration on Superdex 200. GST-neurabin$^{1–485}$ activated PP-1_{IA} with an EC_{50} of 40 nM, and inhibited PP-1_{IB}, PP-1_{IC}, and PP-1_{ID} with IC_{50} values of 1.1, 0.1, and 0.2 μM, respectively. The effect of neurabin$^{1–485}$ on PP-1_{IA} was biphasic; reduced activation occurred above ~90 nM (Fig. 2A). The effect of GST-neurabin$^{1–485}$ on PP-1_{IA} was specific for PP-1_{IA}; it did not activate purified PP-1_C or PP-$2A_C$ (Fig. 2B). Purified GST alone had no effect on these phosphatases (data not shown). PP-1_{IA} had an apparent molecular mass of 250 kDa by gel filtration chromatography (Fig. 3), while PP-1_{IB}, PP-1_{IC}, and PP-1_{ID} had apparent molecular masses of 250, 115, and 210 kDa, respectively, as determined by gel filtration (data not shown). Thus all four forms of native brain PP-1_I are multiprotein complexes consisting of PP-1_C, I-2 and additional unidentified proteins.

To identify the domain in neurabin responsible for activation of PP-1_{IA}, 6His-N-terminal fragments of neurabin were prepared and tested for their effects on PP-1_{IA} activity. Neurabin$^{1–210}$ activated PP-1_{IA} with an EC_{50} of ~30 nM and inhibited PP-1_{IB}, PP-1_{IC}, and PP-1_{ID} with IC_{50} values of ~1 μM, ~1 and ~30 nM, respectively (Fig. 4A). Neurabin fragments consisting of amino acid residues 1–102, 103–210, 50–150, and 50–210 had no effect on PP-1_{IA} activity (data not shown). The effect of neurabin$^{1–210}$ on the activity of PP-1_{IA} required preincubation with Mg^{2+} and ATP. Thus, the effect was specific for Mg^{2+}/ATP-dependent PP-1_I (Fig. 4B).

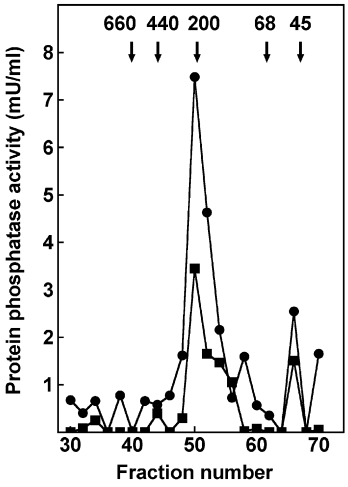

Fig. 3. Chromatography of PP-1_{IA} on Superdex 200. PP-1_{IA} obtained from the poly-L-lysine agarose column was separated on a Superdex 200 gel filtration column. Protein phosphatase activity was assayed in the absence (■) or presence of (●) 200 nM GST-neurabin$^{1–485}$ in Mg^{2+} and ATP. The arrows indicate the elution positions of the marker proteins thyroglobulin (660 kDa), apoferritin (440 kDa), catalase (200 kDa), bovine serum albumin (68 kDa), and ovalbumin (45 kDa). The calculated molecular mass of PP-1_{IA} is 250 kDa.

Discussion

Protein phosphatase-1 is a highly regulated enzyme with multiple functions determined by complex interactions with a number of regulatory proteins and substrates [5,8,19]. The composition of the endogenous PP-1 complexes present in brain and their specific functions are poorly understood. Only PP-1_I has been characterized in any detail in brain [7,9,20]. Another form of PP-1, termed PP-1_{PN}, has also been identified in brain [21]. PP-1_{PN} is present predominantly in the nucleus and may regulate

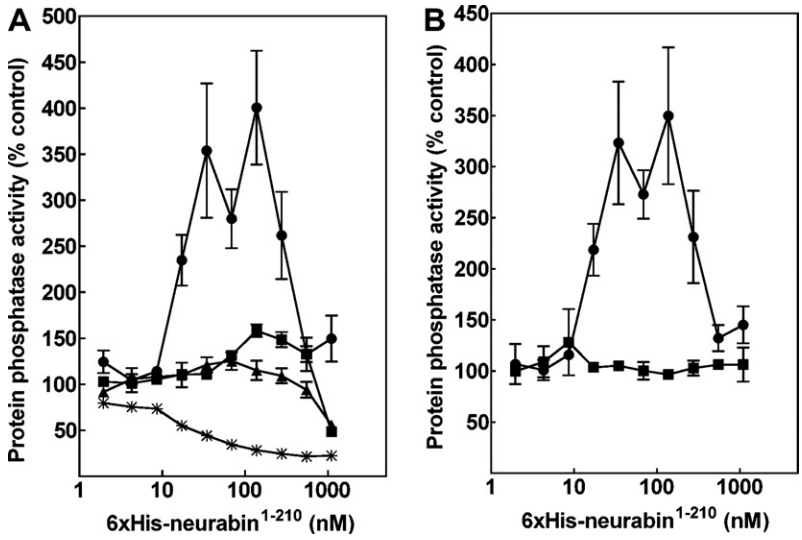

Fig. 4. Effect of neurabin$^{1–210}$ on the activity of PP-1_I. (A) PP-1_{IA} (●), PP-1_{IB} (■), PP-1_I (▲), and PP-1_{ID} (*) purified through the Superdex 200 step were assayed in the presence of various concentrations of 6× His-neurabin$^{1–210}$ with 0.005 unit of PP-I_{IA}. (B) PP-1_{IA} was assayed in the presence of various concentrations of 6× His-neurabin$^{1–210}$ in the presence (●) or absence (■) of Mg^{2+}/ATP. 100% activity is equivalent to 0.005 unit of phosphorylase phosphatase activity with (●) or without (■) preincubation with Mg^{2+}/ATP.

mRNA splicing [22]. Other forms of PP-1 have been identified in brain but they remain to be purified and characterized [23].

Previous work has shown that N-terminal fragments of neurabin inhibit PP-1_C with IC_{50} values of 0.2–10 nM [1,14]. Most cellular PP-1_C exists bound to regulatory proteins and substrates rather than as the free catalytic subunit [5,8]. A major form of PP-1 in brain is PP-1_I identified by its association with I-2 and requirement for Mg^{2+}/ATP [7,9]. In the present work, we identified four forms of PP-1_I, termed PP-1_{IA}, PP-1_{IB}, PP-1_{IC}, and PP-1_{ID}. Neurabin inhibited the major form of PP-1, PP-1_{IC}, which accounted for ~60% of total PP-1_I activity. Interestingly, neurabin had differential effects on the various forms of native PP-1_I. Recombinant neurabin$^{1–485}$ and neurabin$^{1–210}$ activated PP-1_{IA} but inhibited PP-1_{IB}, PP-1_{IC}, and PP-1_{ID} activity. These results indicate that neurabin has two domains of interaction with PP-1_I, an activation domain within amino acid residues 1–210 and an inhibitory domain within amino acid residues 210–485 that contains the canonical KIKF PP-1_C binding motif [1,14]. The molecular basis for the interaction between neurabin$^{1–210}$ and PP-1_{IA} that results in activation is currently under investigation. Activation by neurabin$^{1–210}$ is specific for Mg^{2+}/ATP-activated PP-1_{IA}, indicating specificity for a single form of PP-1_I. This indicates that activation requires modification of PP-1_C by other components of the PP-1_A holoenzyme.

The likely functions of PP-1_I can be deduced from its binding partners. The finding that neurabin interacts with two essential components of PP-1_I (PP-1_C and I-2) and that neurabin targets PP-1_C and I-2 to polymerized actin suggest that PP-1_I is involved in regulating cell morphology [14]. This role is supported by the observation that overexpression of neurabin induces filopodia and dendritic spines in cultured hippocampal neurons [14]. Protein phosphatase-1 has also been implicated in modulating the actions of the D1 dopamine receptor, AMPA- and NMDA-type glutamate receptors, $GABA_A$ receptors, and Ca^{2+}, K^+, and Na^+ channels [19,24]. Further studies are indicated to determine which of the PP-1_I enzymes identified here interact with these substrates and how neurabin might mediate these interactions to affect channel function.

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