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Interaction of nucleoredoxin with protein phosphatase 2A

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Abstract **A trimeric protein phosphatase 2A ($PP2A_{T55}$) composed of the catalytic (PP2Ac), structural (PR65/A), and regulatory (PR55/B) subunits was isolated from rabbit skeletal muscle by thiophosphorylase affinity chromatography, and contained two additional proteins of 54 and 55 kDa, respectively. The 54 kDa protein was identified as eukaryotic translation termination factor 1 (eRF1) and as a PP2A interacting protein [Andjelkovic et al. (1996) EMBO J. 15, 101–112]. The 55 kDa protein is now identified as nucleoredoxin (NRX). The formation of a complex between GST–NRX, $PP2A_C$ and $PP2A_D$ was demonstrated by pull-down experiments with purified forms of PP2A, and by immunoprecipitation of HA-tagged NRX expressed in HEK293 cells complexed endogenous PP2A subunits. Analysis of PP2A activity in the presence of GST–NRX showed that NRX competed with polycations for both stimulatory and inhibitory effects on different forms of PP2A.**

Keywords: Reversible phosphorylation; Protein–protein interaction; Protein phosphatase type 2A; Redox regulation

1. Introduction

Reversible protein phosphorylation catalyzed by protein kinases and phosphoprotein phosphatases is a major regulatory mechanism in mammalian cells utilized in response to external signals [1,2]. Protein phosphatase 2A (PP2A) is a large, diverse family of enzymes specific for phosphoserine and phosphothreonine residues and is widely expressed in mammalian cells [3]. PP2A is implicated in the regulation of metabolism, transcription, RNA splicing, translation, differentiation, DNA replication, oncogenic transformation and signal transduction [3]. To perform these diverse functions PP2A trimeric holoenzymes consisting of the catalytic subunit (PP2Ac) and the structural subunit (PR65/A) containing one of the regulatory subunit (PR55/B, PR61/B′, PR72/B″, striatin/SG2NA) dephosphorylates specific substrates [3–5]. The regulatory subunits determine enzymatic activity, substrate selectivity and subcellular localization of PP2A holoenzymes. Proteins such as PHAPI (I_1^{PP2A}), PHAPII (I_2^{PP2A}, SET and TAF-1) and PTPA regulate PP2A activity [3]. In addition, PP2Ac undergoes phosphorylation [6] and methylation [7,8]. Methylation of the C-terminal leucine of PP2Ac is implicated in dimer–trimer inter-conversion [9–11]. Several proteins of cellular and viral origin form complexes with PP2A and affect phosphatase activity [4,12]. PP2A not only dephosphorylates many kinases [14] but forms 'signaling cassettes' with some of them, wherein they reciprocally influence each others activity [13,14].

To search for new PP2A regulatory subunits and interacting proteins, we modified existing purification protocols and employed FPLC ion exchange chromatography as a final purification step. This led to the dissociation of an apparently homogenous PP2A holoenzyme preparation into trimeric and dimeric PP2As, as well as free PR55/B subunit and two novel proteins of 54 and 55 kDa, respectively [15]. Previously we reported the identification of the 54 kDa polypeptide as eukaryotic release factor 1 (eRF1) and showed that it interacts with dimeric PP2A and target it to polysomes in COS-7 cells.

In this paper we identify the 55 kDa protein as a rabbit homologue of nucleoredoxin (NRX), a protein that was previously discovered as the gene product of the murine *red-1* gene, discovered during extensive transcript mapping of mouse chromosome 11 in the vicinity of the *nude* locus [16]. Overexpression studies revealed that NRX resides in the nucleus. Bacterially expressed NRX possess intrinsic oxidoreductase activity against insulin disulfide bonds, but its physiological targets are unknown. In this study we elucidate the relationship between NRX and PP2A subunits as well as the potential function of NRX–PP2A complexes. We hypothesized that NRX represents a novel PP2A interacting protein and therefore we assayed the complex formation and its activity towards known PP2A substrates. We further assessed whether NRX is a PP2A specific binding protein or it interacts with closely related catalytic subunit of protein phosphatase 1 (PP1c). Our goal was to understand the role which NRX plays upon binding to PP2A and the effects such binding exerts on phosphatase activity.

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† This manuscript is a tribute to our colleague Dr. Stanislaw Zolnierowicz who passed away on the 13th of February 2001 when the experiments presented in this study had been finished.

Abbreviations: eRF1, translation termination factor 1; GSTf, glutathione *S*-transferase; GST–NRX, fusion between GST and NRX; HA, hemagglutinin; NRX, nucleoredoxin; OA, okadaic acid; PP1c, catalytic subunit of protein phosphatase 1; PP2A, protein phosphatase type 2A; PP2Ac, catalytic subunit of PP2A; $PP2A_D$, dimeric form of PP2A composed of PR65/A and $PP2A_C$; PR65/A, the structural subunit of PP2A; PR55/B, PR72/B″ and PR61/B′, regulatory subunits of PP2A; $PP2A_{T55}$, trimeric form of PP2A composed of $PP2A_D$ and a third regulatory subunit indicated as suffix

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2. Experimental procedures

2.1. Purification of PP2A holoenzymes and determination of amino acid sequences

PP2A holoenzymes were purified from rabbit skeletal muscle as described previously [15]. After the final purification step, proteins were subjected to SDS–PAGE followed by western blotting and immunodetection with a panel of antibodies specific for PP2A subunits (see below). Trypsinization of the 55-kDa protein, separation of tryptic peptides, and microsequencing were carried out as described previously [15].

2.2. Molecular cloning of constructs encoding nucleoredoxin, PP2A, and eRF1

PCR was used to amplify full-length NRX cDNA. pTV/GN25 plasmid [16] served as the template, with a sense oligonucleotide containing *Bam*HI restriction site 5′-CGGGATCCATGTCGGGCTTCCTGGAGG-3′ and an antisense 5′-ACCGTCGACCTAGATGGGCTCAGGCTTGAG-3′ primer (*Sal*I site underlined). In order to obtain an N-terminal deletion mutant of NRX, termed ΔN(1–161)NRX, PCR was run with a sense oligonucleotide 5′-GACGAATTCCCCTTGGGGACCTAAACCCTTC-3′ (*Eco*RI site underlined). PCR amplifications were performed with *Pfu* DNA polymerase (Roche) in the presence of 5% dimethylsulfoxide. For expression in mammalian cells, NRX cDNA was designed to encode a hemagglutinin (HA)-tag at the N terminus and subcloned into the pCMV-5 plasmid. PCR was run as described above using the sense primer 5′-CGAGATCTACCATGTATCCATATGATGTTCCAGATTATGCTTCGGGCTTCCTGGAGGAGC-3′ (*Bgl*II restriction site underlined) and an antisense *Xba*I restriction site containing primer 5′-ACTCTAGACTAGACT AGATGGGCTCAGGCTTGAG-3′. All plasmids were sequenced using an ABI PRISM (Perkin–Elmer) model 377 automated sequencer.

2.3. Bacterial expression and purification of nucleoredoxin

Escherichia coli DH5α transformants containing pGEX4T-3/NRX and pGEX4T-3/ΔN(1–161)NRX were grown to OD_{600} 0.8 in Luria–Bertani medium containing ampicillin at 100 μg/ml and induced with 100 μM isopropyl β-D-thiogalactopyranoside for 3 h at 22 °C. DH5α cells were isolated by centrifugation (500 × *g* for 10 min) and disrupted in ice-cold 1 × PBS supplemented with 1 mM PMSF and 2 mM benzamidine by passing twice through a French press (500 psi). GST–NRX and its deletion mutant GST–ΔN(1–161)NRX was purified over a glutathione–agarose column according to the manufacturer's recommendations.

2.4. Oxidoreductase activity of purified nucleoredoxin

The oxidoreductase activity of purified recombinant proteins was assayed as described [16], with GST as the negative control.

2.5. Cell culture, transfection, and immunoprecipitation

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Cells were seeded at 5×10^6 per 65-mm dish and transfected by the DNA–calcium phosphate co-precipitation method after 20 h. 1 μg plasmid DNA per ml medium was applied. 36 h post-transfection, cells were lyzed in buffer containing 1 × TBS, 0.1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, 2 mM benzamidine, and 100 μg/ml N^{α}-tosyl-L-lysine chloromethyl ketone (TLCK). Cytoplasmic fractions were used for immunoprecipitations, with protein A–agarose conjugated 12CA5 monoclonal antibodies directed against the HA epitope. For immunoprecipitations, 50–250 μg total protein was used and experiments were done as previously described [15]. Immunodetection was done with antibodies against PP2Acα, PR65α/Aα, PR55α/Bα, eRF1, and NRX.

2.6. Antibodies and immunodetection

Polyclonal antibodies were raised in rabbits against peptides conjugated to *Limulus polyphemus* hemocyanin (Sigma). Standard intra-dermal injections of conjugated peptides emulsified with complete (the first injection) or incomplete (all subsequent injections) Freund's adjuvant were carried out. Antibodies against GST fusion proteins were raised in rabbits by injecting them with recombinant GST–NRX. Rabbit polyclonal anti-PP1cα antibodies were purchased from Upstate Biotechnology. Anti-rabbit IgG raised in goat and coupled to alkaline phosphatase was used as a secondary antibody (Santa Cruz Biotechnology Inc.) and the color reaction developed in the presence of nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.7. GST pull-down Assay

Purified PP2A preparations (PP2Ac, PR65/A-PP2Ac dimer and trimers containing either PR55/B or PR72/B′) containing about 1–2 μg catalytic subunit (approximately 1.5 U) were mixed with 1–2 μg of GST, GST–NRX and GST–CDCL5 and incubated for 1 h at 4 °C. Next, glutathione–agarose was added and incubation continued for 2 h at 4 °C. Agarose beads were washed three times with 1 × TBS containing 0.2% Triton X-100, 1 mM PMSF and 2 mM benzamidine, and once with the same buffer containing 0.5 M NaCl. Elution was carried out with 10 mM glutathione followed by boiling the beads in SDS–PAGE buffer. Alternatively, cell-free extracts were prepared from exponentially growing HEK293 cells. Cell-free extracts (1 mg protein) were mixed with 3 μg GST–NRX, 5 μg deletion mutant GST–ΔN(1–161)NRX, or 10 μg GST coupled to glutathione–agarose beads, and treated, as described above.

After SDS–PAGE, samples were transferred onto nitrocellulose membranes, blocked with 5% skimmed powdered milk in 1 × TBS supplemented with 0.1% Triton X-100 and 0.05% Tween 20, and incubated with polyclonal antibodies (diluted 1:100 in Blotto) against PP2Acα, PR65α/Aα, PR55α/Bα, and eRF1 for 2 h at room temperature. Immunodetection was performed as described above.

2.8. Purification of protein phosphatase 2A from rabbit skeletal muscle

PP2A holoenzymes, PR65/A-PP2Ac dimer complexed with either the PR55/B-, and PR72/B″ subunits, were purified from rabbit skeletal muscle according to Waelkens et al. [17]. PP2Ac and PP1c were purified from bovine heart as described for rabbit skeletal muscle by Ramachandran et al. [18].

2.9. Protein phosphatase 2A activity assay

Protein phosphatase 2A activity was measured with ^{32}P-phosphorylase *a* as a substrate in the presence of protamine (30 μg/ml) and ammonium sulfate (16 mM) as previously described [3,17]. For activity assays of PP2A present in immunoprecipitates, 5 nM okadaic acid (OA) was included in the assay buffer. To measure in vitro effects of GST–NRX, GST–ΔN(1–161)NRX and GST alone on PP2A activity, assays were performed with or without protamine or polylysine and 16 mM ammonium sulfate. One unit of activity corresponds to 1 nmol of ^{32}P-phosphate released from ^{32}P-phosphorylase *a* (10 μM) per min at 30 °C in a 30-μl assay.

3. Results

3.1. Identification of the 55-kDa $PP2A_{T55}$-associated protein as a nucleoredoxin

To identify the 55-kDa protein that copurified with $PP2A_{T55}$ [15], amino acid sequences of seven tryptic peptides were obtained by automated Edman degradation (Table 1). All peptides from the rabbit protein matched the murine NRX sequence [16] found in the GenBank/EMBL databases (Accession No. X92750). Peptides 15 + 16 differed from the murine NRX sequence by insertion of 2 Pro residues and substitution of Ala to Glu, and in peptides 40 + 41 a Glu was changed to a Gly. These alterations may result from species differences. Finally, peptide 21 corresponded in part to the C-terminus of murine NRX and contained additional amino acids (EDDXXD), thus reflecting a divergence of the C-terminus. Alternatively, peptide 21 could be a mixture of two peptides, one from the C-terminus of rabbit NRX and another corresponding to the murine sequence ^{375}EDDMTD^{380}.

Fig. 1 shows that NRX is built from repetitive sequence elements. The repetition in the sequence preceding and

Table 1
Peptides derived from the 55-kDa protein purified from rabbit skeletal muscle together with protein phosphatase $2A_{T55}$ and their counterparts identified in murine nucleoredoxin

Number of peptide	Sequences of peptides derived from the rabbit 55-kDa protein	Sequences of corresponding peptides present in murine nucleoredoxin
p. 15 + 16	^{3}AAAGPGAGPGPGAGAAAEPEPR	^{61}RGD**AAAGPGAG**A**GAGAAAEPEPR**
p. 21	^{1}LKPEEDDXXD	^{429}K**LKPE**PI
p. 23 + 24	^{1}QLIQPIAEK	^{345}K**QLIQPIAEK**
p. 27 + 28	^{1}LVTGGGEEVDVHSLGA	^{12}K**LVTGGGEEVDVHSLGA**
p. 32	^{2}FPWHP	^{304}RE**FPWHP**
p. 40 + 41	^{1}EVIAEPLLR	^{171}R**EVIA**G**PLLR**
p. 54 + 55	^{3}PEGL	^{155}RDD**PEGL**

The number of each peptide derives from the fraction(s) collected from microbore HPLC during separation of peptides obtained after in situ trypsinization of the 55-kDa protein on polyvinylidene difluoride (PVDF) membrane. Superscript numbers preceding rabbit protein-derived peptides refer to the cycle number of an automated Edman degradation which gave interpretable phenylhydantoin-derivatives of amino acids. Sequences of murine nucleoredoxin-derived peptides start from ariginine (R) or lysine (K) representing possible trypsin digestion sites. Superscript numbers preceding nucleoredoxin-derived peptides indicate the position of given amino acid within the nucleoredoxin. Murine nucleoredoxin peptide sequences corresponding to sequences of peptides derived from the rabbit 55-kDa protein are indicated in bold.

Fig. 1. Repeated sequences present in the murine nucleoredoxin. (A) Three direct repeats present in the murine nucleoredoxin. Each repeat contains 2 copies of motif [h(4)–x–s–x(12,21)–a–h–P–W–x–s–x–P–x(2)–a] where capital letters mean conserved amino acids and lowercase letters stand for amino acids with acidic (a), hydrophobic (h) and small (s) side chains. (B) Five repeated motifs [h(4)–x–s–x(12,21)–a–h–P–W–x–s–x–P–x(2)–a] present in the murine nucleoredoxin. (C) Sequence of murine NRX with indication of the position of the direct (italic) and internal (bold) repeats. The catalytic centre sequence is underlined.

following the catalytic center (WCPPC) has been described before [16]. Within this repeat, we found an internally repeated motif, described as [h(4)–x–s–x(12,21)–a–h–P–W–x–s–x–P–x(2)–a] with conserved amino acids written in capital letters and amino acids with acidic (a), hydrophobic (h), and small (s) side chains, which occurs twice in each repeat (Fig. 1B). Furthermore, the 80 amino acids at the C terminal part of NRX show relevant sequence similarity with each of the two long repeats (18% identity, 34% similarity when compared with repeat 1; 14% identity, 40% similarity when compared with repeat 2). Alignment of this C-terminal sequence against these two repeats is shown in Fig. 1A and the position of the repeats in NRX is shown in Fig. 1C. Nucleoredoxin homologues are found in a variety of species of both higher and lower eukaryotes, such as mouse NRX (X92750), human NRX (Al121672), *D. rerio* (AW184272) *X. laevis* (AW200621), *C. elegans* (U235511.1) *A. thaliana* (AAC24068), *Z. mays* (U90944), but is clearly absent in yeast. A structural model of mouse NRX based on existing structures of oxidoreductases in the public data bases, and three different algorithms, shows a nearly 100% match between nucleoredoxin [Ile174 to Leu315] and tryparedoxin (Accession No. O7740). Despite these two proteins sharing only an overall identity of 27.6%, it is clear that part of the

sequence, harboring the catalytic centre, forms the structure of an oxidoreductase fold. It was indeed previously demonstrated that murine NRX functions as an oxidoreductase [16], and this has now been confirmed for the rabbit homologue (results not shown). In summary, NRX is built of five similar structural elements, of which two occur in duplication before and after the catalytic center, followed by a less-conserved C-terminal element.

3.2. Association between GST–NRX and PP2A

To confirm the formation of a complex between NRX and PP2A, we carried out GST pull-down experiments. NRX was able to bind purified PP2Ac, and $PP2A_D$ (Fig. 2B, upper and middle panel). When purified $PP2A_{T55}$ was loaded on GST–NRX beads, only $PP2A_D$ bound to GST–NRX, and the PR55/B subunit appeared in the flow-through fractions (Fig. 2B, lower panel). GST alone was used as the negative control (Fig. 2A). We also checked if PR65/A alone was sufficient to bind GST–NRX but purified recombinant PR65/A subunit did not bind to GST–NRX (data not shown). All these results point towards the C subunit as the binding partner for NRX, also in $PP2A_D$.

Fig. 3 presents results of similar experiments in which $PP2A_D$, present in cell-free extracts from HEK 293 cells, was captured by GST–NRX but not by GST alone. (GST–CDC5L was used as negative control.) Similar results were obtained when a truncated form of NRX (GST–Δ1–161 NRX) was used (data not shown). Taken together, these results clearly demonstrate that NRX–PP2A complex formation can be reproduced in vitro from recombinant NRX and native PP2As. Moreover, the interaction domain with PP2A is not located in the N terminal part of NRX, since the first 161 amino acids are dispensable for this function.

3.3. HA-tagged NRX coprecipitates $PP2A_D$ when overexpressed in HEK293 cells

Since we confirmed that recombinant GST–NRX fusion protein interacts with PP2A in vitro, we were interested in determining whether the same complex forms in vivo. Therefore, we performed co-immunoprecipitations from HEK293 cells transfected with an HA–NRX expressing construct. In HA–NRX immunoprecipitates we detected both PP2Ac and PR65/A; these were missing in the control reactions (Fig. 4A). None of the PP2A regulatory subunits, neither eRF1 nor PP1c, were present in HA–NRX immunoprecipitates (results not shown).

Our next question was whether NRX immunoprecipitates contain phosphatase activity with PP2A characteristics. The results in Fig. 4B show phosphorylase phosphatase activity in immunoprecipitates prepared from HA–PP2Ac, HA–PR65/A, HA–eRF1, and HA–NRX-overexpressing HEK293 cells. Levels of OA-sensitive phosphatase activity brought down with HA–eRF1 and HA–NRX were similar, implying that both proteins form equally stable complexes with PP2A under the conditions we used. Because HA-tagged versions of PP2A subunits can replace these in their respective complexes (dimers and trimers), the OA sensitive phosphorylase phosphatase activity in the immunoprecipitates containing HA–PP2Ac and HA–PR65/A subunits most probably represent trimeric PP2A forms, and dimeric enzyme forms in the

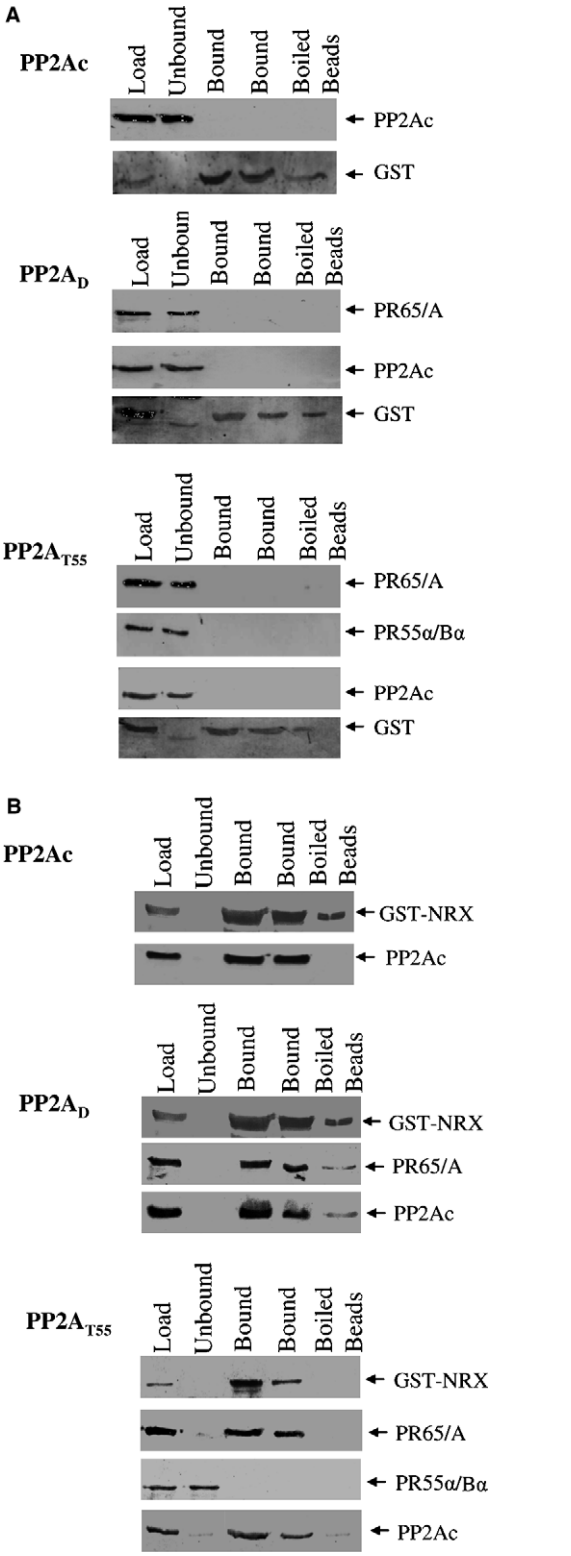

Fig. 2. GST–NRX associates with purified PP2Ac, PR65/A-PP2Ac dimer ($PP2A_D$), and dissociates the PR55/B-subunit from $PP2A_{T55}$. GST (panel A), and GST–NRX (panel B) were mixed with PP2Ac, $PP2A_D$ or $PP2A_{T555}$. Lane 1, load (10%); lane 2, unbound material; lanes 3–4, elution with 10 mM glutathione; lane 5, protein recovered after boiling agarose beads in SDS–PAGE buffer. Proteins were resolved on SDS–PAGE and immunodetection was performed.

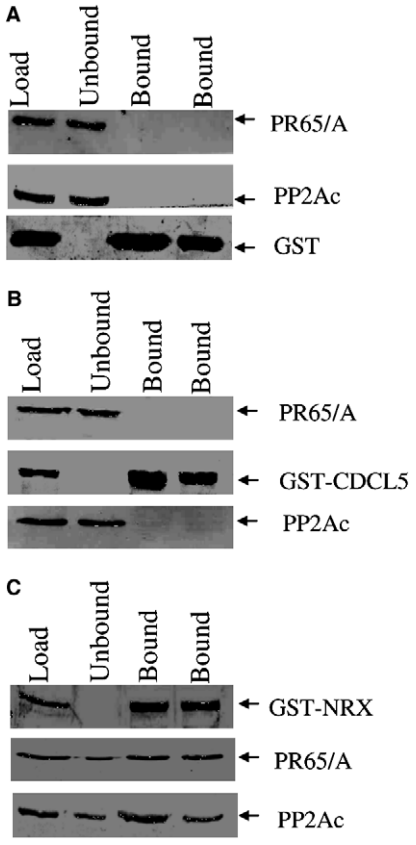

Fig. 3. GST–NRX associates with PR65/A-PP2Ac dimer present in HEK293 cells extracts. GST (A), GST–CDC5L (B), and GST–NRX (C) immobilized on glutathione–agarose were mixed with 1 mg protein present in extracts of HEK293 cells. Lane 1, load (10%); lane 2, unbound material; lanes 3–4, elution with 10 mM glutathione. Immunodetection was performed using antibodies specific for PP2A subunits and GST (indicated to the right).

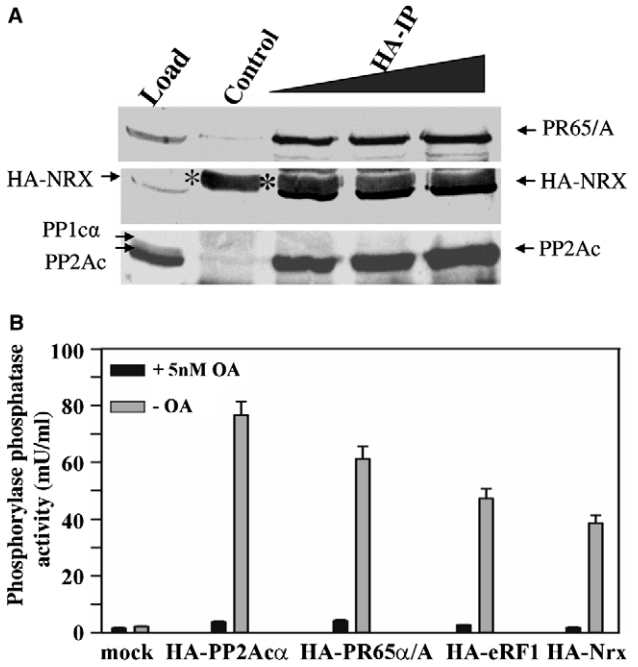

Fig. 4. (A) Co-immunoprecipitation of the PR65/A-PP2Ac dimer with HA–NRX overexpressed in HEK293 cells. HA–NRX cell-free extracts were immunoprecipitated with 12CA5 monoclonal antibodies bound to protein A–agarose and subjected to immunoblotting analysis using polyclonal antibodies directed against PP1 catalytic subunit, PP2A subunits and anti-HA antibodies. Lane1, HA–NRX transfected cell extract; lane 2, immunoprecipitates from cells transfected with the empty vector, lanes 3–5, immunoprecipitates from 100, 150, 200 μg of HA–NRX protein lysate. An asterisk indicates the position of IgG heavy chain that runs just above HA–NRX. (B) Phosphorylase phosphatase activity measured in immunoprecipitates from HEK293 cells transfected with HA–PP2Ac, HA–PR65/A, HA–eRF1, or HA–NRX overexpressing constructs. Phosphatase activity was measured in absence and presence of 5 nM OA. Reactions were done in triplicate. Standard deviations are indicated in both cases but are sometimes too small on this scale to be observed.

HA–ERF/1 and HA–NRX complexes, as formally proven for the HA–NRX immunoprecipitates (Fig. 4A).

In summary, our data indicate that NRX forms stable complexes with the PP2A dimer, corroborating our initial observation of an interaction between PP2A and NRX.

3.4. Nucleoredoxin specifically affects phosphatase activity of PP2A

Next, we measured the effects of NRX on PP2A activity. Phosphatase activity of PP2Ac and $PP2A_D$, but not PP1c, was stimulated about twofold when NRX was present in the

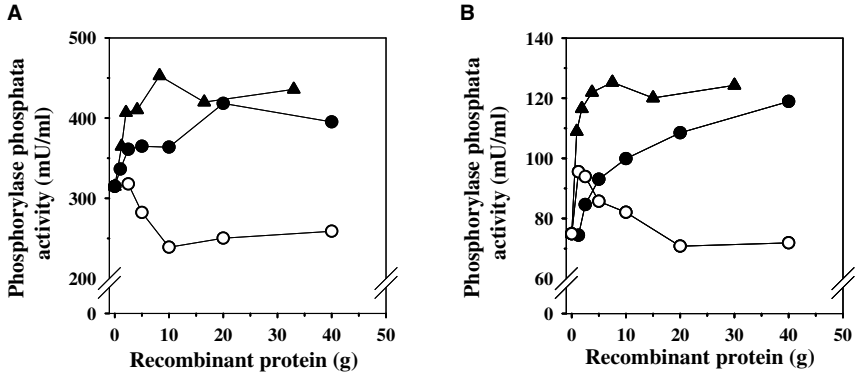

Fig. 5. Effect of GST–NRX, GST–ΔN(1–161)NRX and GST on phosphorylase phosphatase activity of $PP2A_C$ and $PP2A_D$. The indicated amount of $PP2A_C$ (A) or $PP2A_D$ (B) was mixed with the indicated concentrations of GST–NRX (▲), GST–ΔN(1–161)NRX (●) or the GST control (○) and ^{32}P-phosphorylase *a*. After 10 min incubation at 30 °C, the released ^{32}P-phosphate was measured as described in Section 2.

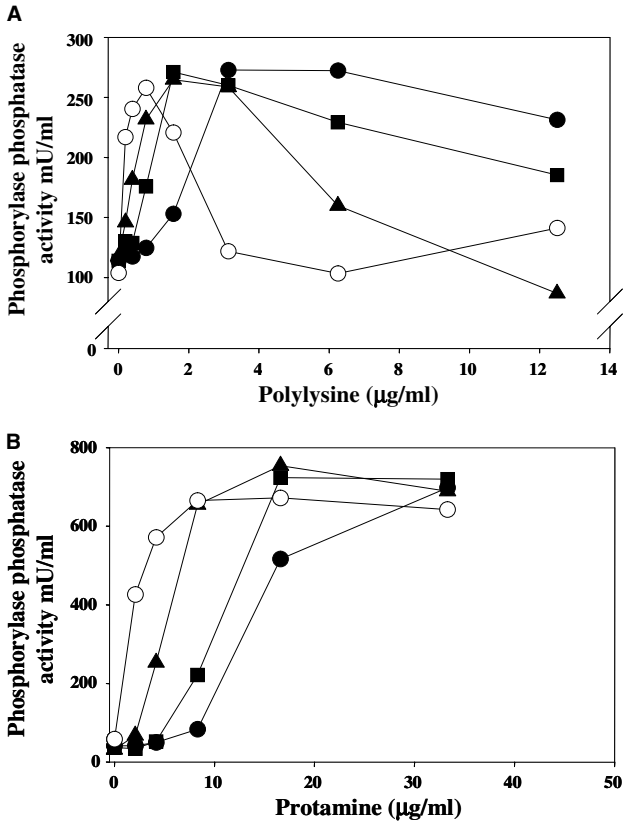

Fig. 6. Effect of GST–ΔN(1–161)NRX on the optimal polycation concentration for stimulation of trimeric $PP2A_{T72}$ and $PP2A_{T55}$. (A) The indicated amount of $PP2A_{T72}$ was incubated in the absence (○) or presence of 40 μg/ml (●), 20 μg/ml (■) or 10 μg/ml (▲) GST–Δ(1–161)NRX and the indicated concentrations of polylysine and ^{32}P-phosphorylase *a* as a substrate. (B) The indicated amount of $PP2A_{T55}$ trimer was incubated in the absence (○) or presence of 80 μg/ml (●), 40 μg/ml (■) or 20 μg/ml (▲) GST–ΔN(1–161)NRX and the indicated concentrations of protamine, plus 16 mM $(NH_4)_2SO_4$ and phosphatase activity was measured as in A.

reaction mixture (maximum effect at about 10 μg/ml) NRXΔ1–161, stimulated PP2A up to the same level, but about 4 times more protein was necessary to obtain the same effects (Fig. 5 and data not shown). Since PP2A is also stimulated by polycations, we investigated whether NRX was competitive or additive with polycations. Stimulatory effects of polycations on PP2A phosphorylase phosphatase activity are bi-phasic, with an optimal concentration typical for each holoenzyme [17]. Super-optimal concentrations of polycations are less stimulatory, and this effect is neutralized by $(NH_4)_2SO_4$ [17,19,20]. Therefore, NRX and/or NRXΔ1–161 were tested for effects on the optimal concentration of polycations required for maximal stimulation of $PP2A_{T55}$ and $PP2A_{T72}$. We preferred NRXΔ1–161 for this kind of experiments because full length NRX easily forms aggregates and the difference among the full length and N terminal deletion mutant seems to be rather quantitative than qualitative. We selected $PP2A_{T72}$ because it represents the most sensitive PP2A for polycations. Fig. 6A demonstrates the effect of NRX on the polylysine stimulation of $PP2A_{T72}$. In the absence of $(NH_4)_2SO_4$, NRX affected the optimal stimulatory polylysine concentration and prevented apparent inhibition by polylysine at super-optimal concentrations indicating that NRXΔ(1–161) indeed competed for both the stimulatory and apparent inhibitory effects. In the presence of protamine and $(NH_4)_2SO_4$, only inhibition of the phosphatase stimulation of $PP2A_{T55}$ was observed (Fig. 6B).

4. Discussion

In this paper, we show that NRX interacts with PP2A and affects its activity. Our data imply that the major target of NRX in PP2A complexes is the catalytic subunit both free and present in the PP2Ac-PR65/A dimer. The identity of interaction sites remains to be established, but it seems that the N-terminal region of NRX is dispensable for PP2A-NRX binding. A similar conclusion that NRX binds to the PP2Ac subunit may also be drawn from the effects of NRX on PP2A activity. Since NRX stimulates the phosphorylase phosphatase activity of the free catalytic subunit, a direct interaction with PP2Ac is implicated. Although we observed that, in vitro, catalytic subunit alone is sufficient to form a stable complex with NRX our in vivo studies clearly indicate that the dimer interacts with HA–NRX. Therefore we decided to check whether the trimeric complex $PP2A_D$–NRX retains the biochemical properties of dimeric phosphatase. It was shown earlier that the polycation effect on PP2A is dependent on the PR65/A subunit [19,20]. This effect is bi-phasic in nature, and shows concentration dependent stimulation or inhibition of phosphatase activity. It is believed that PR65/A possesses both high-affinity (stimulatory) sites as well as low affinity (inhibitory) sites for protamine binding. Therefore, the observed shift in optimal protamine concentration needed for maximal stimulation of the $PP2A_D$–NRX complex could be explained by interaction of NRX with both types of protamine binding sites on PR65/A.

Since $(NH_4)_2SO_4$ neutralizes the inhibitory polycation effect, only inhibitory effects of NRX were observed in the presence of $(NH_4)_2SO_4$ (Fig. 6B). To exclude simple electrostatic interactions between polycations and negatively charged GST–NRX we determined that binding of GST–NRX to protamine–agarose was negligible. Therefore the simple explanation that NRX depletes polycations from the reaction mixture cannot be applied to explain all observed effects and we conclude that NRX binds to $PP2A_D$ via interactions with both subunits.

Relatively little is known about NRX [16,21]. It belongs to a family of oxidoreductases that participates in the reversible regulation of disulfide bond formation and/or breakdown. Oxidoreductases have been implied in the regulation of transcription factors [22]. Several other examples of interactions between phosphatases and redox-active enzymes have been described. For example, calcineurin/PP2B has been reported to co-purify with superoxide dismutase, protecting PP2B from inactivation by reactive oxygen species [23]. This finding corroborates other observations suggesting that protein phosphatases are subjected to redox control mechanisms [23,24]. Also, kinases are subject to regulation by oxidative stress, e.g. apoptosis signal-regulating kinase 1 associates with thioredoxin [25]. In addition to its enzymatic oxidoreductase activity, NRX seem to displace the different 'third regulatory subunits' in the holoenzyme complexes, indicating that the physical binding might be another function of this protein.

In summary, data obtained using different techniques demonstrated that NRX interacts with both PP2Ac and $PP2A_D$. Since free PP2Ac does not seem to exist in cells in vivo, our data indicate that $PP2A_D$, which constitutes 30% of PP2A in murine 10T1/2 fibroblasts [26], is the most likely molecular target of NRX. In contrast, the question remains whether PP2Ac contains disulfide bridges which can be oxidized or reduced by NRX. Our hypothesis would be that two of ten cysteine residues present in PP2Ac, namely cysteine 269 and cysteine 272 could represent a target for formation of disulfide bonds catalyzed by NRX. Significantly, a recent paper reported that brain PP2A is susceptible to reversible inhibitory modification by thiol–disulfide exchange [27].

Future studies should aim to elucidate the molecular mechanisms of the interactions between NRX and PP2A, and to clarify the function of NRX in phosphatase regulation. We speculate that such interaction would be important to protect PP2A from the effects of oxidative stress and accumulation of free radicals.

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