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Chimeric phosphatase type 2A enzymes are substrates for nucleoredoxin

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ABSTRACT

This report deals with the effects of binding between two enzymatically active proteins: catalytic subunit of protein phosphatase type 2A (PP2Ac) and nucleoredoxin (NRX). We hypothesized that the PP2Ac constitutes a substrate for NRX, as we already detected their interaction in several independent experimental settings[6]. To distinguish the effect of binding of the NRX to its substrate from its action as an enzyme, we measured the phosphatase activity in the presence and absence of NRX and its artificial cofactor DTT. NRX inhibited activity of recombinant catalytic subunit by 35 to 40% in dose dependent manner, whereas the C terminus of the NRX (ΔN-NRX) slightly activated phosphatase activity. Upon addition of DTT the activity of recombinant catalytic subunit of protein phosphatase 2A was inhibited by 60% and in the presence of NRX the inhibitory effect was enhanced up to 80%. A chimera of HA-PP2Ac-PR65/A was rapidly activated (up to 3.2 nmol/minxml) upon exposure to H_2O_2, whereas HA-PP2A-PR65/A-myc-NRX activity was unaffected. In conclusion, we found that the activity of recombinant PP2Ac is lowered by DTT and NRX and that oxidation of PP2Ac upon purification procedure results in formation of internal disulfide bridge, which is a target of NRX in *in vitro* assays.

KEYWORDS

DTT;
Cell culture;
Inorganic phosphate release measurements;
Immunoprecipitation.

INTRODUCTION

Reversible phosphorylation is catalyzed by set of kinases and phosphatases that respond in cascades of intracellular signaling and lead to changes in genes expression and/or to apoptosis/necrosis death[1]. The main subjects of studies in this field are temporary modifiers of phospho-proteins known as protein kinases and protein phosphatases. Those enzymes modify approximately 30% of cellular proteins, and their genes occupy about 3% of human genome. One of the major known enzymes involved in those processes is protein phosphatase type 2A (PP2A)[2]. It belongs to serine/threonine phosphatases, PPP group of metaloenzymes, which catalyzes dephosphorylation of phosphoserines and phosphothreonines. Typical PP2A holoenzyme is made of two components: the constant one (AC) - a heterodimer of catalytic (PP2Ac/C) and regulatory (PR65/A) subunits and variable one[3-5]. The variable subunits fall into three, structurally unrelated, classes of B subunits. Apart from typical variable subunits, the core dimer can associate with multitude of so-called acces-

sory proteins, which number reached over 70 genetically unrelated polypeptides. In general, both types of proteins: variable subunits and accessory proteins are being thought to regulate PP2A localization and substrate specificity. However, the mechanisms underlying such regulatory functions are still not elucidated[4]. Previously, we had shown that nucleoredoxin bound to phosphatase dimer and interacted predominantly with PP2Ac, in *in vitro* experiments[6]. Over-expressed HA-tagged nucleoredoxin bound to approximately 2-5% of total PP2Ac in exponentially growing HEK 293 cells. NRX was initially discovered in mouse embryos, and it belongs to the family of oxidoreductases. Its cellular substrates have not been found by now[15], and in *in vitro* assays it is potent reducer of insulin disulfide bridges. We made a hypothesis that PP2Ac was a substrate for NRX, and we have designed a set of experiments to determine whether the presence of free radicals has any effect on the amount of PP2Ac bound to nucleoredoxin, as well as on phosphatase activity measured as velocity of the dephosphorylation of its substrate, a phosphorylase a.

MATERIALS AND METHODS

All constructs and antibodies used in this studies were described previously[6], apart from pCMV5-myc-NRX, which was cloned by PCR with primers: . All antibodies used in this study were gift from dr B.A Hemmings, FMI, Basel, Switzerland.

Cell culture, transfections

Human embryonic kidney 293 cells were maintained in Dulbecco's.

Modified Eagle's medium supplemented with 10% fetal calf serum. Cells were seeded at 0.5×10^7 per 65-mm dish and transfected by the DNA-calcium phosphate co-precipitation method after 20h. 1 microgram of plasmid DNA per ml medium was applied. 24 h post-transfection, cells were lyzed in buffer containing 1xTBS, 0.1 mM EDTA, 0.2% Triton X-100, 1 mM PMSF, 2 mM benzamidine, and 100 μg/ml N-tosyl-L-lysinechloromethyl ketone (TLCK). Cytoplasmic fractions used for immunoprecipitations, were incubated with protein A–agarose conjugated to 12CA5 monoclonal antibodies directed against the HA epitope. For immunoprecipitations, 50-250 micrograms of total protein was used and experiments were done as previously described[6-8]. Immunodetection was performed with antibodies against PP2Acα, PR65α/Aα and NRX. For H_2O_2 experiments cells were transfected and starved for 20 hours in DMEM serum free medium.

Antibodies and immunodetection

Polyclonal antibodies against peptides derived from PP2A subunits were described previously[6]. Anti-rabbit IgG coupled to alkaline phosphatase were purchased in Sigma. Westernblotting and immunodetection were performed as previously described[6,7].

Phosphatase activity measurements

Protein phosphatase 2A activity in the cell extracts was measured with ^{32}P-phosphorylase a as a substrate in the presence of protamine (30 microgram/ml) and ammonium sulfate (16 mM), as previously described[6,7]. The same conditions were used for the activity assays with PP2A in immunoprecipitates. Recombinant PP2Ac activity was measured in the buffer without protamine and ammonium sulfate and in the presence of 10 fold excess of GST-NRX, GST and GST-ΔN-NRX.

H_2O_2 treatment of cells

HEK 293 cells were maintained/transfected as described previously. 20 hours before experiment cells were placed in DMEM medium. Freshly prepared stock of H_2O_2 was used to the final concentration of 500 micromolar, and cells were stimulated for the period of time ranging from 2.5 to 60 minutes.

RESULTS

We have been interested to explain the function of the complex of NRX and PP2A, and since NRX is a cytoplasmic protein, then we focused on the activity changes of PP2A-NRX complex versus PP2A alone. Our first observation was that in the HEK 293 cells lyzates, the total phosphatase activity and 10 nanomolar okadaic acid sensitive activity are decreasing when HA-NRX is overexpressed[10]. This effect was dose-dependent, and with the low dose of expressed HA-NRX the okadaic acid sensitive activity was 70% of the control, and the higher amount of HA-NRX resulted in inhibiting the okadaic acid sensitive phos-

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phatase to 50%, and also influenced total phosphatase activity, reducing it to two-third of the control value. The data are shown on the figure 1, and it includes two additional controls as we wanted to see if the expression of other PP2Ac interacting protein, HA-eRF-1 has any effect on phosphatase activity, and whether the expression of constant parts of PP2A like HA-PP2Ac and HA-PR65/A affected the overall phosphatase activity. The overexpression of HA-PP2Ac resulted in similar inhibition of total phosphatase activity, yet the okadaic acid sensitive activity as inhibited only to 70% of the mock control. Expression of HA-eRF-1 did not significantly changed the values of phosphatase activities. As we noticed the HA-NRX caused dose dependent inhibition of PP2A like activity we decided to remove the most of the variables from the experiment and focus on in vitro studies, where the set of conditions is definable. We have used recombinant purified PP2Ac and first determined whether any of the most often used thiol reactive reagents affect phosphatase activity. As shown on figure 2A and 2B the addition of DTT to the incubation buffer caused significant inhibition of PP2Ac activity, down to 40% of the control value. 100 micromolar of H_2O_2 exerted similar effect on PP2Ac activity as DTT, and addition of recombinant GST-?N-NRX or GST-NRX had opposite effects. When the full length NRX was present in the reaction the inhibitory effect exerted by it was additive with inhibitory effects of H_2O_2, 2-ME and DTT (figure 2A), although when we used a C terminal part of NRX protein, which possesses the reductase activity but lacks the coiled coil motif and one of three direct repeats,

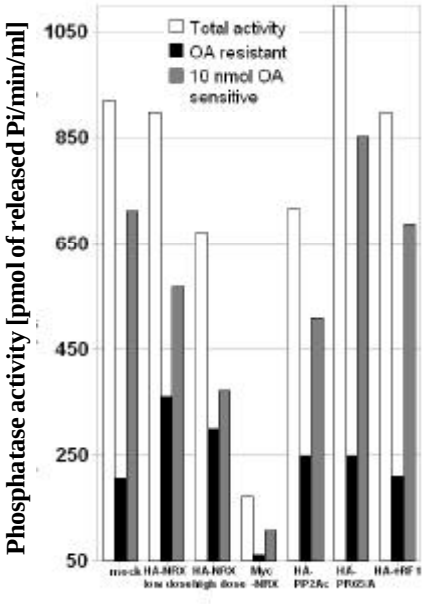

Figure 1: *In vitro* activity measurements of endogenous phosphatase activity in exponentially growing HEK 293 cells. Cells were transfected with indicated cDNAs and assayed for the expression of HA tagged proteins. 1 and 2 micrograms of total protein was assayed in standard phosphatase buffer plus 30 micrograms/ml of protamine and 16 micrograms/ml of ammonium sulfate. Samples were treated with okadaic acid to final concentration of 10 nmol/ml

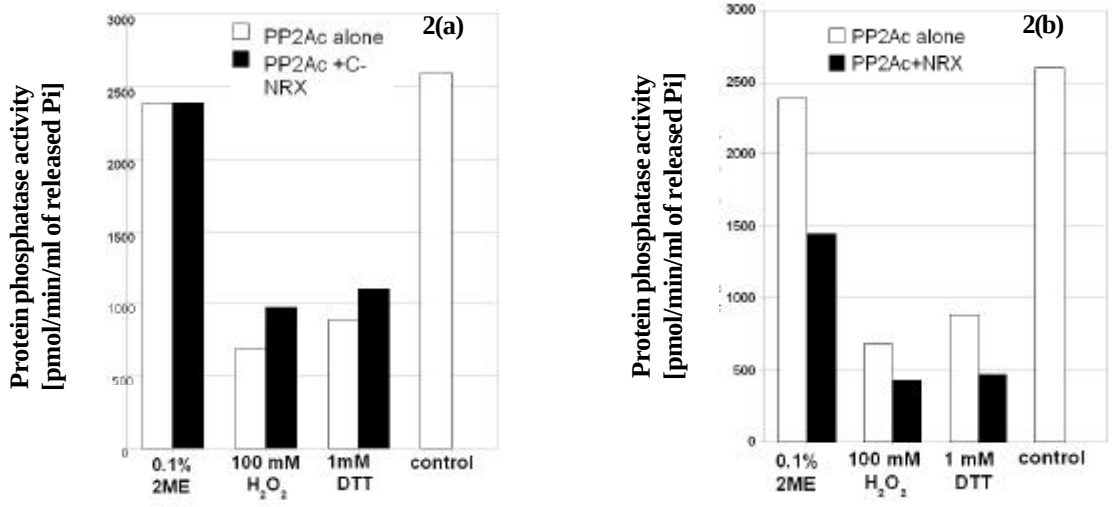

Figure 2 : *In vitro* measurements of recombinant PP2Ac activity upon addition of 1 mM DTT, 0.1% 2-ME and 100 mM H_2O_2. Assays were carried out in the presence of A:GST-ΔN-NRX and B:GST-NRX in the standard phosphatase assay buffer. Prior addition of recombinant proteins, phosphatase was preincubated for 5 minutes with thiol reactive reagents. Experiment represents one out of 5, samples are prepared in duplicated

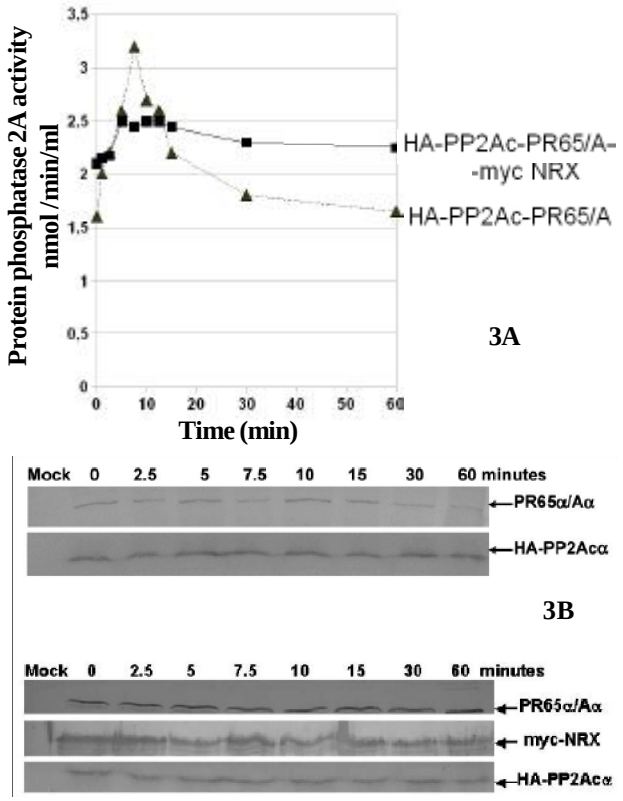

Figure 3: *In vitro* phosphatase activity measurements of chimeric HA-PP2Ac-PR65/A and HA-PP2Ac-PR65/A-myc-NRX after immunoprecipitation with anti HA antibodies from HEK 293 cells treated with 500 mM hydrogen peroxide. Points represent triplicate measurements, which originate from one pool of 12CA5- Protein A-Sepharose and were split before last wash, the fourth part of the immunoprecipitated material was separated on 10% SDS PAGE and blotted against PP2Ac, PR65/A and NRX

then we noticed slight activation of the enzyme,(up to 35% of the controls) or lack of effect when the 2ME was present in the buffer (figure 2B).

Such experiments suggested that NRX is a typical inhibitory protein, yet, we had to take into account that our recombinant PP2Ac displayed unique enzymatic properties in the sense that contrary to the yeast recombinant PP2Ac subunit it was inhibited and not activated by DTT or 2ME. When we take into account that both reagents are thought to prevent oxidation of SH groups in the proteins and are routinely added to the purified enzymes to increase their stability and protect them from oxidation, then we concluded that our protein was purified in an oxidized state with at least one disulfide bridge formed internally. As the reducing reagents caused the decrease of PP2A activity, then it seems that reversible and controlled formation of the disulfide bridge might be a way to regulate this enzyme in the cells. To prove that such fluctuations of PP2A activity are happening in the cells, we started the experiments designed to answer the question of what happens with PP2A activity when there is a change of the oxidizing potential of the cytoplasm. We used PP2Ac chimeric enzymes immuno-purified from HEK 293 cells to assay the activity changes upon exposure of cells to 500 micromolar of H_2O_2. We have reasoned that if we use hydrogen peroxide as a reagent to elicit the oxidation of the SH groups in the cytoplasmic proteins, then we can assay the activity changes of HA-PP2Ac containing enzymes in the presence or absence of any other cofactor. Figure 3 shows the experimental results, of A-immunodetection of core PP2A proteins in immunoprecipitates from HEK 293 cells which overexpress HA-PP2Ac; B- immunodetection of core PP2A proteins in immunoprecipitates from HEK 293 cells overexpressing HA-PP2Ac; and myc-NRX; C- the chimeric HA-PP2Ac-PR65/A and HA-PP2Ac-PR65/A-myc-NRX activity curves taken during exposure of HEK 293 cells onto the 500 micromolar H_2O_2.

At first we used quiescent HEK 293 cells and measured the changes in PP2A activity within 0-60 minutes of H_2O_2 treatment. The reason to use 500 micromolar concentration of H_2O_2 was that at this concentration we noticed maximal stimulation of PP2A activity in the cell lyzates, whereas higher concentrations were inhibitory and lower concentrations did not stimulate phosphatase activity. Figure 3 presents the amount of the chimeric PP2A dimer proteins, which were immunoprecipitated from H_2O_2 treated cells and the corresponding activity measurements. There is an rapid increase of phosphatase activity within first 10 minutes of the stimulation and the activity is almost doubled (1.69 versus 3.2 nmol/min/ml) whereas the amount of the catalytic subunit detected with anti HA antibody seems to be on the same level throughout the whole experiment. As the starting material and the amount of antibody was constant at each point of experiment the changes of activity must be attributed to the enzyme modifications, preferable oxidation of SH groups. When we add myc-NRX to such experiment and immunoprecipitate chimeric complex the phosphatase activity does not fluc-

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tuate (the difference between zero point to the highest measured activity is 0.35 nmol/min/ml what makes 23% of the zero point value) and NRX acts as a stabilizer.

DISCUSSION

The data presented in this study points out two major features of the NRX-PP2A complex. One is that *in vitro,* nucleoredoxin is an inhibitor of the phosphatase activity. This inhibition is enhanced when the artificial cofactor of NRX, like DTT fine tunes the reduction of the catalytic subunit of PP2A by NRX, as the catalytic cycle of NRX cannot be completed without redox equivalents which reduce its catalytic center. Contrary to the full length protein, the C terminus of NRX which is redox active but lacks the the protein-protein interaction motif, slightly activates the protein. In HEK 293 cells, which produce chimeric PP2A-NRX complexes, the inhibition of PP2A like activity depends on the amount of the NRX. Prolonged stimulation of the cells with H_2O_2, resulted in PP2A activation by the factor of 2 within10 minutes of stimulation. When we assayed the chimeric trimer of myc-NRX bound to HA-PP2A-PR65/A it had a higher basal activity (1.69 nmol/min/ml versus 2.1 nmol/min/ml) and there is no further activation. This suggests at least two possible explanations, first that the NRX binding site overlaps with H_2O_2 sensitive moieties, after the complex forms the SH group is protected and cannot be oxidized, or that NRX actually acts as redoxin in the real time of H_2O_2 treatment. The last possibility gives the explanation for both type of experiments, *in vitro* and *in vivo*. It suggests, that PP2Ac has a potential to form an internal disulfide bond which enhance its activity towards phosphorylase a, and which makes an opportunity for enzyme based regulation of PP2A activity in the modulative way.

Further question, which we would be keen to answer, is what is the binding site of NRX to PP2A and whether there is any additional component of such redox regulatory system. During the catalytic cycle an enzymatic center of redoxin has to be reduced by its reductase, otherwise the enzyme makes a single turn and it stops being locked in the inactive conformation. NRX support system is missing in *in vitro* studies and may not be functional in HEK 293 cells, therefore the DTT although it proved an efficient NRX cofactor has to be replaced by the other enzymatic activity to open the real biological pathway for the PP2A regulation via opening and closing a disulfide bond. Our data are only the introduction of the problem and they did not answer one of the most interesting question is what are the biologically relevant processes in which the elevated oxidative potential of the cytoplasm pushes PP2A activity higher and where does this oxidative potential originate from. In the protein tyrosine phosphatases (PTPs) field, for instance, there is a lot of data about their irreversible inactivation by reactive oxygen species, yet there are no enzymatic mechanisms which would counteract such inactivation[10-12]. Tyrosine phosphatases differ from serine/threonine ones in the catalytic mechanism, and in PTPs, the crucial catalytic residue is a single cystein, whereas PPPs are utilizing histidines, aspartic acid and water -coordinated metal ions to break the phosphate bond[13,14] in its substrates. What we found instead, is that the overall activity of PP2Ac depends on the intra molecular disulfide bonding and that the presence of such bond increases the activity by 60%. We had found an enzymatic activity, which binds and modulates PP2A activity *in vitro* and *in vivo,* and is potent reducer of disulfide bridges in polypeptides, fe. insulin. Other studies suggested that few out of 10 cystein residues are targets of chemical modifications, yet the method of detection of those changes was based on the mass difference, and the mass difference between two SH groups and one disulfide bond is too small to be detected in such approach[18]. Therefore we find our report interesting, as it shows that there is protein-based mechanism of correcting the alterations of PP2A redox status. The question is left where this disulfide bridge is formed and the answer is that it can be formed in the C-terminal groove of the PP2Ac between the 269 and 272 cysteins[17] as judged from computer model of PP2Ac subunit. We would be very interested to answer this question in the next set of experiments.

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