Serine (threonine) phosphatase(s) acting on cAMP-dependent phosphoproteins in mammalian mitochondria

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Abstract Immunochemical and functional evidence showing the existence in the inner membrane and matrix fraction of mammalian mitochondria of serine/threonine phosphatases acting on cAMP-dependent phosphoproteins is presented. Mg^{2+}-dependent Ca^{2+}-inhibitable PP2C phosphatase, associated to the inner membrane, dephosphorylates the 18 kDa (NDUFS4 gene) of complex I. © 2002 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Mitochondrial phosphatase; cAMP-dependent protein phosphorylation; Mitochondrion; Complex I

1. Introduction

Reversible protein phosphorylation catalyzed by protein kinases (PKs) and phosphatases (PPs) plays a crucial role in cellular signal transduction [1]. Protein PPs have been classified in three families: the PTP PPs specific for tyrosine residues, the PPP and PPM PPs specific for serine/threonine [2,5]. Various PPP and PPM have been described, which include: PP1, PP2A, PP2B (calcineurin), and PP2C (α,β,γ), pyruvate dehydrogenase PP [6] and others [2–5]. These PPs differ in structure, substrate specificity, response to divalent cations, and sensitivity to inhibitors [2–6].

PKs and PPs are targeted to subcellular compartments through association with specific membrane anchoring proteins [2,7]. A family of these anchoring proteins (AKAP) bind both cAMP-dependent PK (PKA) and PPs targeting them to specific cellular membranes [7–9].

The pyruvate dehydrogenase PP, which dephosphorylates the E1α subunit of pyruvate dehydrogenase (PDH) and activates this enzyme, is associated together with PDH to the inner mitochondrial membrane [10]. In mitochondria a PP2A phosphate dephosphorylates and activates the anti-apoptotic Bcl2 protein [11]. Recent work showed a PP activity in the matrix (M) of plant mitochondria which dephosphorylates inner membrane (IM) phosphoproteins [12].

Our group has obtained evidence showing the occurrence of a cAMP-dependent PK associated with the IM/M of mammalian mitochondria where it phosphorylates various mitochondrial proteins [13–16]. A cAMP-dependent phosphoprotein of 18 kDa was identified as the AQDQ subunit of complex I [14,15] encoded by the nuclear gene NDUFS4 [17]. In mouse [16,18] and human cell cultures [19,20] production of intracellular cAMP promoted phosphorylation of the 18-kDa subunit and functional activity of complex I. Human pathological NDUFS4 mutations abolished these effects of cAMP on complex I [19–22].

This paper reports the detection and characterization of serine/threonine PPs in the IM and M of bovine heart mitochondria. A IM Mg^{2+}-dependent Ca^{2+}-inhibitable PP (type PP2C-γ) is found to specifically dephosphorylate the 18-kDa subunit of complex I.

2. Materials and methods

2.1. Materials

C2C12 mouse myoblasts from ATCC. Dulbecco's modified Eagle's medium (DMEM), phosphate-buffered saline (PBS), Trypsin (0.05%)–EDTA (0.02%), penicillin, streptomycin, calf serum and fetal bovine serum from EuroClone; catalytic subunit of cAMP-dependent PK, PK inhibitor (PKI), cyclosporin A, hexokinase, protease inhibitor cocktail and phosphatidic acid from Sigma; calyculin A from Alexis; okadaic acid, horseradish peroxidase conjugate goat IgG antibody and digitonin from Calbiochem; sheep monoclonal antibody against PP2Cα and sheep peroxidase conjugate goat IgG antibody from Upstate Biotechnology; polyclonal antibodies against 20-residue phosphorylated C-terminus and 20-residue N-terminus peptides of the 18-kDa protein of complex I were produced on our request by Neosystem; [γ-^{32}P]ATP 3000 Ci/nmol from NEN Life Science; Hyperfilm-MP, Hybond™ ECL™ nitrocellulose membrane from Amersham International.

2.2. Sample preparation

Cytosol and heavy mitochondria were isolated from beef heart as in [23]. Fractionation of mitochondria, to obtain the IM and supernatant M fractions was carried out as in [13]. The presence of cytosol in the mitochondrial fraction was estimated as in [16]. Cytosolic contamination of the IM and M mitochondrial fractions amounted to 0.4% and 5% respectively. Complex I was purified from bovine heart mitochondria as in [24].

2.3. Cell culture and mitoplast preparation

C2C12 mouse myoblasts were cultivated with DMEM with 10% calf serum at 37°C [16]. Mitoplasts and their fractionation were carried out as in [16,25,26].

2.4. Substrates phosphorylation

Phosphorylation of purified complex I (25 μg protein) was carried out by incubation, at 30°C for 10 min under stirring, in 25 μl of 10 mM Tris–HCl pH 7.5, 8 mM $MgCl_2$, 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 4 U C-cPKA and 70 μM [γ-^{32}P]ATP (1000 cpm/pmol). Phosphorylation of IM protein was carried out as in [16]. The reaction was stopped by the addition of 0.5 μM PKI, 1.4 U Hexokinase and 10 mM glucose to remove ATP excess.

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Abbreviations: PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecylsulfate

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2.5. Protein PP assay

The PP activity was assayed by incubating at 30°C ^{32}P-labelled IM or ^{32}P-labelled complex I, in the presence, where indicated, of cold cytosol (C), IM or M fraction from bovine heart in a medium containing 10 mM Tris–HCl pH 7.5, 4 mM $MgCl_2$, 0.25 mM PMSF, 1 μl/100 μg protein of protease inhibitor cocktail. Where present $CaCl_2$, $MgCl_2$ EGTA and a PP inhibitor cocktail (100 nM okadaic acid, 200 nM phosphatidic acid, 200 nM calyculin A, 300 nM cyclosporin A, 20 mM NaF) were added as specified in the legends to figures. The reaction was stopped by the addition of 25 μl of 50 mM Tris–HCl pH 6.8, 10% v/v glycerol, 15% w/v sodium dodecylsulfate (SDS), 25% v/v β-mercaptoethanol, and boiled for 3 min.

2.6. Gel electrophoresis, autoradiography and immunodetection

Polyacrylamide gel electrophoresis (SDS–PAGE) of ^{32}P-labelled complex I and IM fraction was carried as in [13]. For molecular weight (M.W.) assignment Multimark Multicolored Invitrogen standards were used. Radioactive bands were visualized and radioactivity was measured as in [13]. The amount of ^{32}P incorporated in the protein was calculated from the specific activity of added [γ-^{32}P]ATP (1000–2000 cpm/pmol). The PP activity was expressed as pmol of ^{32}P removed from the phosphorylated substrates per mg protein of IM or M fraction.

For immunodetection of PP2C, phosphorylated C-terminus and N-terminus of the 18-kDa subunit of complex I, proteins, resolved by SDS–PAGE, were electrotransferred, to nitrocellulose membranes [16]. The membrane was washed in 20 mM Tris–HCl pH 7.5, 0.5 M NaCl, 0,05% Tween 20 (TTBS) and 5% fatty acid-free dry milk. The nitrocellulose membranes were then incubated with the antibodies against PP2Cα (diluted 1:100) and phosphorylated C-terminus (diluted 1:100) of the 18-kDa subunit of complex I for 12 h, antibody against N-terminus (diluted 1:200) of the 18-kDa subunit of complex I for 2 h. Immunodetection was performed as in [16].

3. Results

3.1. Dephosphorylation of cAMP-dependent phosphoprotein subunits of mitochondrial enzymes

The 18-kDa (IP) subunit of complex I is the only polypeptide to be labelled by ^{32}P when the isolated complex is incubated with [γ-^{32}P]ATP and the catalytic subunit of PKA [15]. PP activity was detected by incubating the ^{32}P-labelled complex I with the IM or M from bovine heart mitochondria. The autoradiograms in Fig. 1A show that the ^{32}P-labelled 18 kDa subunit of complex I was dephosphorylated by the two mitochondrial fractions. Both PP activities were promoted by Mg^{2+}, but only the IM activity was inhibited by Ca^{2+}.

Immunoblot with an antibody against a C-terminal segment, phosphorylated at Ser-131 of the 18 kDa subunit of complex I, confirmed serine dephosphorylation of the subunit by the Mg^{2+}-dependent PP in the IM (Fig. 1B). Immunoblot of the 18-kDa subunit with an antibody against an N-terminal segment of the 18-kDa protein showed that the incubation of the complex I with the IM did not cause decrease of the content of this protein. This control excluded proteolytic degradation of the 18-kDa subunit during the incubation of complex I with the IM fraction.

Fig. 2 illustrates Ca^{2+}-sensitivity of dephosphorylation by submitochondrial fractions of the 18-kDa phosphoprotein of complex I and other phosphoproteins of the IM. Dephosphorylation by the IM of the 18-kDa phosphoprotein of complex I was inhibited by Ca^{2+} in the same concentration range at which dephosphorylation of the 42-kDa phosphoprotein of the IM (subunit E1α of the pyruvate dehydrogenase) by its specific PP [6] was stimulated by this cation. Dephosphorylation by the IM of a 6.5-kDa phosphoprotein of this fraction was, on the other hand, unaffected by Ca^{2+}. The M fraction exhibited a Ca^{2+}-insensitive PP acting on the 18-kDa phosphoprotein of complex I and the 42- and 6.5-kDa phosphoproteins of the IM.

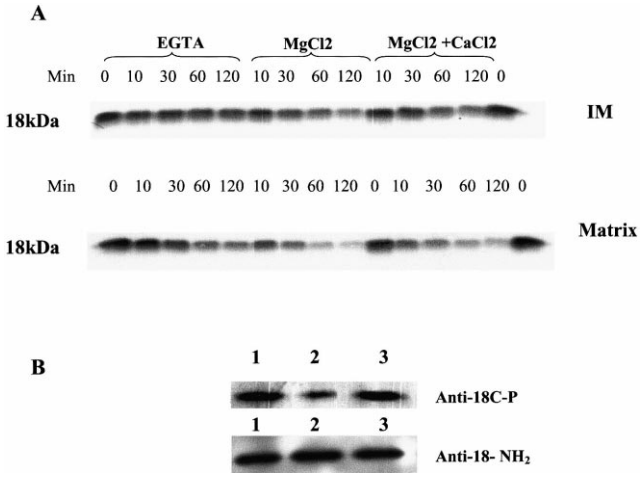

Fig. 1. Dephosphorylation of the 18-kDa subunit of complex I by mitochondrial phosphatases. Dephosphorylation of the 18-kDa subunit was analyzed by incubating ^{32}P-labelled complex I (25 μg protein), prepared as described under Section 2, with IM or M from bovine heart mitochondria (100 μg protein for each fraction) in the reaction mixture described under Section 2, final volume 50 ml. A: The reaction was stopped at the times shown in and ^{32}P-labelling of the 18-kDa subunit of complex I was detected by autoradiography of SDS–PAGE slabs. B: Cold complex I was incubated 30 min with IM as in A in the presence of 5 mM EDTA plus 5 mM EGTA (lane 1), 4 mM $MgCl_2$ (lane 2) or 4 mM $MgCl_2$+PP (lane 3) inhibitors. Complex I, separated by blue native PAGE, was resolved in its subunits by SDS–PAGE. Proteins were electrotransferred to nitrocellulose sheets for immunodetection of the phosphorylated C-terminus (Anti 18C-P) and N-terminus (Anti 18-NH) of the 18-kDa subunit with the specific antibodies. For other details see Section 2.

The data summarized in Fig. 3 show that the specific activity of the Mg^{2+}-dependent Ca^{2+}-inhibitable PP of the IM acting on the 18 kDa subunit of complex I was practically equivalent to a cytosolic Mg^{2+}-dependent, Ca^{2+}-insensitive PP acting on the same protein. The matrix PP acting on the 18-kDa subunit of complex I was Mg^{2+}-dependent but only partially inhibited by Ca^{2+}.

3.2. Immunochemical detection of mitochondrial PPs

Available data show that member(s) of the PP2C type of mammalian PP are Mg^{2+}-dependent and Ca^{2+}-inhibitable [27–28]. Western blot analysis using an antibody against the PP2C PP shows that this enzyme, relatively abundant in the cytosol of bovine heart and mouse myoblasts, was also present in mitochondria (Fig. 4). Bovine heart mitochondria exhibited a double PP2C immunoreactive band of ≈75 kDa, which was enriched in the M fraction, although some of it was also present in the IM. In the last fraction a PP2C immunoreactive band of a M.W. higher than 75 kDa was also detectable. Myoblast mitoplasts showed two PP2C immunoreactive bands. One of 75 kDa was enriched in the M fraction, the other of a higher M.W. was detected in the IM (Fig. 4).

4. Discussion

The data presented show the presence in mammalian mitochondria of serine (threonine) PPs acting on mitochondrial proteins phosphorylated by cAMP-dependent PK. One of the proteins phosphorylated by PKA is the 18-kDa (NDUFS4

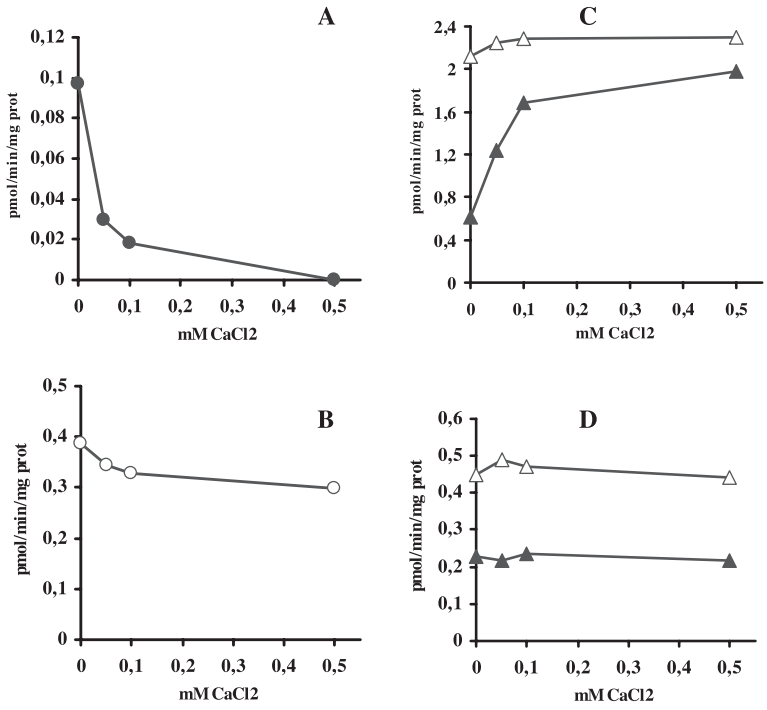

Fig. 2. Effect of Ca^{2+} on dephosphorylation of mitochondrial cAMP-dependent phosphoproteins. Dephosphorylation of the 18-kDa subunit of complex I was assayed by incubating for 30 min ^{32}P-labelled purified complex I (25 μg protein) with cold IM (●), or M (○) (100 μg protein of each fraction). Dephosphorylation of the 42-kDa and 6.5-kDa phosphoproteins of the IM was assayed by 30 min incubation of ^{32}P-labelled IM (100 μg protein) in the absence (▲) or presence (△) of cold M fraction (50 μg). The reaction mixture was that described under Section 2. $CaCl_2$ was added at the concentrations indicated. After stopping the reaction ^{32}P radioactivity was measured on the protein bands of the reported M.W. separated by SDS–PAGE.

gene) of complex I [15]. Evidence has been obtained indicating that a 6.5-kDa cAMP-dependent serine phosphoprotein is subunit c of the F_oF_1 ATP synthase [Gaballo, A., Sardanelli, A.M., Signorile, A., Scacco, S., and Papa, S., manuscript in preparation].

A PP acting on the 18-kDa subunit of complex I has been characterized here, associated with the IM of bovine heart mitochondria, which is Mg^{2+}-dependent and Ca^{2+}-inhibitable. The 18-kDa phosphoprotein of complex I is also dephosphorylated by the cytosol and the mitochondrial matrix of bovine heart. The PP activity of the cytosol is Ca^{2+}-insensitive. The partial inhibition by $Ca^{2+}$of the matrix PP could result from partial release, during mitochondria fractionation, of the membrane PP with decreased sensitivity to $Ca^{2+}$and/or presence of a Ca^{2+}-insensitive PP in this fraction. The Ca^{2+}-inhibition of the PP acting on the 18-kDa subunit of complex I (phosphorylation of this subunit activates the complex [18–20]) occurs in the 10^{-5}–10^{-4} concentration range at which this cation stimulates the pyruvate dehydrogenase PP (see Fig. 2) and activates the dehydrogenase [6]. In this way Ca^{2+}can exert two convergent stimulatory effects on NAD-linked respiration in mitochondria. It can be pointed out that the intramitochondrial Ca^{2+} concentration has been shown to reach under certain conditions the concentration of 10^{-5}–10^{-4} M [29]. The PP acting on the cAMP-dependent phosphoprotein of 6.5 kDa, is Mg^{2+}-dependent, but different from that acting on the other two phosphoproteins, is insensitive to Ca^{2+} and is distributed between the IM and M. The present observations thus indicate that in addition to PDH PP there exist in mitochondria other PPM PPs of the type PP2C. The presence in mitochondria of these PPs was confirmed by direct immunodetection. The PP detected in M was more abundant and exhibited a M.W. of 75 kDa characteristic of the PP2Cγ isoform [27]. The PP detected in the IM gave a weaker immuno-

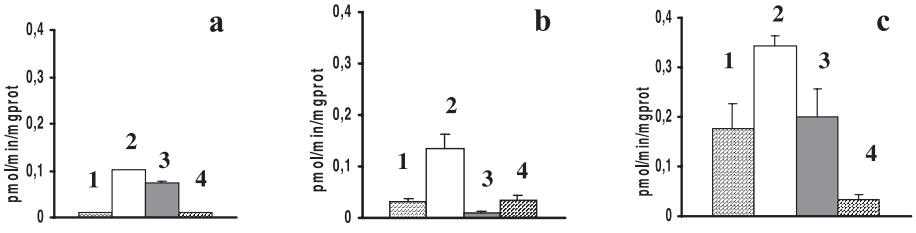

Fig. 3. Dephosphorylation of the 18-kDa subunit of complex I by bovine heart subcellular fractions. Dephosphorylation of the 18-kDa subunit was assayed by incubating ^{32}P-labelled complex I (25 μg proteins) for 10 min with cytosol (a), IM (b) and M (c) fraction (100 μg proteins for each fraction) in the reaction mixture described under Section 2 in the presence of: 5 mM EGTA (column 1), 4 mM $MgCl_2$ (column 2), 4 mM $MgCl_2$ and 0.1 mM $CaCl_2$ (column 3), 4 mM $MgCl_2$ and PPs inhibitors (column 4). ^{32}P radioactivity of the 18-kDa subunit of complex I was measured as described in the legend to Fig. 2. The data of b are the mean and standard error from three experiments

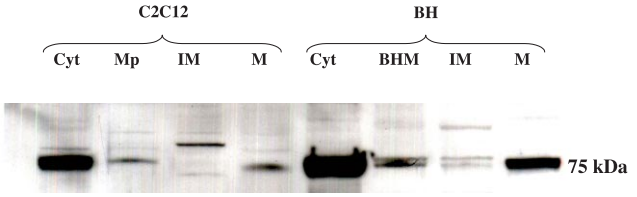

Fig. 4. Immunodetection of PP2C PP in subcellular fractions from bovine heart and mouse fibroblasts. The subcellular fractions of bovine heart (BH) and C2C12 mouse myoblast cultures were prepared as described in Section 2. Proteins of the bovine heart (50 μg) and mouse myoblasts fractions (20 μg) were separated by SDS–PAGE, transferred to nitrocellulose membrane and immunodecorated with an antibody against PP2C PP. Cyt: Cytosol, Mp: mitoplast, BHM: bovine heart mitochondria.

logical response to the PP2C antibody and exhibited a M.W. higher than 75 kDa. This, together with the Mg^{2+}-dependence and Ca^{2+}inhibition of this PP activity, suggests that the IM PP is an isoform of the PP2Cγ type. This PP acting on the cAMP-dependent phosphoprotein of complex I could reverse the stimulatory effect of the cAMP cascade on mitochondrial respiration [30].

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