cAMP-dependent protein kinase and phosphoproteins in mammalian mitochondria. An extension of the cAMP-mediated intracellular signal transduction

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Abstract Evidence has been obtained for the occurrence of a cAMP-dependent serine protein kinase associated with the inner membrane/matrix of mammalian mitochondria. The catalytic site of this kinase is localized at the inner side of the inner membrane, where it phosphorylates a number of mitochondrial proteins. One of these has been identified as the AQDQ subunit of complex I. cAMP-dependent phosphorylation of this protein promotes the activity of complex I and mitochondrial respiration. A 5 bp duplication in the nuclear gene encoding this protein has been found in a human patient, which eliminates the phosphorylation site. PKA anchoring proteins have recently been identified in the outer membrane of mammalian mitochondria, which could direct phosphorylation of proteins at contact sites with other cell structures.

Key words: cAMP-dependent protein kinase; Mitochondrion; Protein phosphorylation; NADH-ubiquinone oxidoreductase

1. Introduction

In mammalian cells the cAMP-dependent protein kinase (PKA) [1] represents the central mediator of intracellular signaling by cAMP, the second messenger produced in response to hormones and neurotransmitters, which include catecholamines, glucacon, gonadotropins, some prostaglandins, serotonin [2]. The cAMP cascade regulates cellular processes like utilization of glycogen and lipid stores, steroidogenesis, neuronal activity, transcription of specific genes, cell proliferation and differentiation [2,3]. A variety of proteins like enzymes, membrane receptors, ion channels, transcription factors, are PKA substrates and their activities are reversibly modulated by phosphorylation and dephosphorylation [2–4].

Although different hormones all activate the same enzyme, PKA, they still trigger distinct physiological processes in the same cell. It follows that different hormones, each capable of raising intracellular cAMP, can result in the preferential phosphorylation and functional modulation of specific target proteins. A breakthrough in understanding this selectivity was provided by the recognition of the existence of intracellular gradients of cAMP [5] and the discovery of a new family of proteins (AKAP), which specifically anchor PKA to different cellular structures including endoplasmic reticulum, plasma membrane, mitochondria, peroxisomes, Golgi, microtubules [6,7]. These acquisitions indicate that the selectivity of the diverse effects elicited by the PKA pathway, once the system is activated in a cell by a given neuro-hormonal effector-receptor interaction, is determined by specific changes in the cAMP level in various cell compartments and by the subcellular sites where the PKA holoenzyme is localized or where the catalytic subunits, once released from the cytosolic holoenzyme, upon binding of cAMP to the R-subunits, migrate [8].

In the resting state PKA exists as an inactive tetrameric complex consisting of two regulatory subunits (R-subunits), which have a high affinity for cAMP, and two catalytic subunits (C-subunits). Four R-subunit isoforms (RIα, RIIα, RIβ, RIIβ) [9] and three C-subunit isoforms [10] have been identified. It is thought that the RI isoforms serve as a cytoplasmic reservoir sequestering the C-subunits in an inactive state [11], until the holoenzyme dissociates in response to cAMP, the cPKA subunits are released in an active state and can even migrate to other compartments, like the nucleus [8]. The RII isoforms can be associated to the anchoring proteins (AKAPs) in various cellular structures [6,7] and this complex can serve to localize the PKA holoenzyme in different subcellular compartments, thus determining the physiological substrates to be phosphorylated.

From time to time glucacon [12,13] and gonadotropins [14] have been reported to affect mitochondrial processes. Glucacon has also been found to promote phosphorylation of mitochondrial proteins in vivo [15,16] or in isolated preparations [17]. In yeast cAMP has been found to activate the expression of mitochondrial [18] and nuclear encoded subunits [19] of cytochrome c oxidase. cAMP binding proteins in yeast mitochondria have been thoroughly characterized but it is not clear if these are associated with PKA enzymes or act as direct modulators of cellular processes ([20] see also [21]). These various observations are, at any event, suggestive of the existence of a PKA associated with mitochondria.

2. The occurrence of PKA in mitochondria

Fragmentary and contradictory reports had appeared in the past on the occurrence of a cAMP-dependent protein kinase in mitochondria from rat liver ([22,23]; but see [24]) pig ovaries ([14]; but see [25]), and rat adrenal mitochondria ([26]; but see [11]). Somewhat more recently, Burgess and Yamada [27] showed a substantial activity of cAMP-dependent protein phosphorylation in bovine heart mitochondria and Schwoch et al. [28] produced immunological and enzymatic evidence showing the existence of PKA in the inner membrane/matrix

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of mitochondria from various rat tissues including heart and liver. Immunoblotting results of these authors suggested that in rat heart mitochondria a fraction of the C-subunit of PKA is bound to the R-subunit in the inner mitochondrial membrane from which it is released, upon cAMP binding, in the matrix.

Recent work from the authors' laboratories has provided definite evidence for the existence of a PKA associated with the inner membrane of bovine heart mitochondria [29–34]. It was found that cAMP promotes in intact bovine mitochondria phosphorylation by [γ-^{32}P]ATP of various mitochondrial proteins [30,31]. Mitoplasts, which consist of inner membrane vesicles and their matrix content, with removal from mitochondria of the outer membrane as well as components of the intermembrane space and cytosolic contaminants, exhibited the same pattern as intact mitochondria of cAMP-dependent phosphorylation of mitochondrial proteins (results to be reported elsewhere). In intact mitochondria the phosphorylation by added ATP of mitochondrial proteins in response to cAMP or dibutyryl-cAMP, was blocked by carboxyatractyloside [33], which inhibits the translocation of added ATP to the mitochondrial matrix [35]. This finding, in addition to providing unequivocal evidence for the existence of a mitochondrial PKA holoenzyme, shows that both the mitochondrial proteins phosphorylated in the presence of cAMP and the catalytic site of the C-subunit of mtPKA are localized at the matrix side of the inner mitochondrial membrane. The fact that protein phosphorylation by mtPKA was equally promoted by both, cAMP and its permeable derivate dibutyryl-cAMP, might suggest that the binding site for cAMP on the regulatory subunit is exposed at the cytosolic side of the inner mitochondrial membrane. This is a point which requires further investigation. If the cAMP binding site is exposed to the intermembrane space, then the PKA holoenzyme would respond to changes in the cytosolic level of cAMP and transfer the signal across the inner membrane, dissociating the catalytic subunit at the inner surface where it will phosphorylate proteins localized there. A localization of the cAMP binding site on the R subunit at the matrix side of the inner membrane would, on the other hand, raise the problem of how the concentration of the cAMP in the mitochondrial matrix is regulated.

mtPKA is loosely associated to the inner mitochondrial membrane from which it could be released by exposure of mitochondria to ultrasounds and high ionic strength media [30]. Immunoblotting with a specific antibody against phosphoserine showed that the cAMP-dependent ^{32}P-labelling of proteins in bovine heart mitochondria was due to esterification of protein serine residues [34], as expected for a canonical PKA which belongs to the class of serine (threonine) kinases [36] (Table 1).

Recently, Rubin et al. have identified two PKA anchoring proteins, S-AKAP84 [37,38] and AKAP121 [39], which tether PKAIIalpha to the cytoplasmic surface of the outer membrane of mammalian mitochondria. S-AKAP84 seems to be involved in mammalian spermiogenesis. It has been proposed that it serves to recruit PKA on the surface of mitochondria where it will phosphorylate proteins of the outer mitochondrial membrane and microtubule motor system involved in translocation of mitochondria to the site of cytoskeleton assembly [38]. The expression of AKAP121 is regulated by the thyroid stimulating hormone (TSH) and cAMP. This protein could enhance the sensitivity of mitochondria to cAMP [39]. It might be possible that AKAP proteins in the outer membrane are also involved in the import of PKA in mitochondria.

3. The mitochondrial proteins phosphorylated by the mtPKA

Reports have appeared in literature on cAMP-dependent and cAMP-independent phosphorylation of mitochondrial proteins in mammalian mitochondria in addition to the

Table 1
Consensus phosphorylation sites for substrates of PKA

	Motif	Frequency	Refs.
cAMP-dependent protein kinase	**R**X**S***	21/46	[36]
	RRX**S***	12/46	
	RXX**S***	11/46	
	KRXX**S***	2/46	
Complex I	Phosphorylation sites		
NADH-ubiquinone oxidoreductase			
18 kD (AQDQ) subunit beef heart	T_{-11}**RSLST**$_{-6}$...T_{128}**RVST**$_{132}$		SWISSPROT
18 kD (AQDQ) subunit human	L_{-8}**RTST**$_{-4}$...T_{128}**RVST**$_{132}$		[42]
18 kD (AQDQ) subunit human patient	L_{-8}**RTST**$_{-4}$...K_{128}EQEY$_{132}$		[42]
21 kD subunit Neurospora crassa	L_{-19}**RTSN**$_{-15}$...R_{-3}**RALSD**$_{3}$... A_{8}**RVPSV**$_{13}$...K_{33}**RNSP**$_{37}$		[43]
Complex IV			
Cytochrome c oxidase			
Subunit IV beef heart	T_{-19}**RVFSL**$_{-14}$...R_{-11}**RAIST**$_{-6}$		SWISSPROT
Subunit IV rat liver	T_{-19}**RALSL**$_{-14}$...K_{-11}**RAIST**$_{-6}$		SWISSPROT
Complex V			
Fo-F1 ATP synthase			
Subunit c (isoform P1) beef heart	I_{-40}**RVPSL**$_{-35}$...A_{37}**RNPSL**$_{42}$		SWISSPROT
Subunit c (isoform P2) beef heart	I_{-55}**RRTST**$_{-50}$...S_{-47}**RSLSA**$_{-42}$...L_{37}**RNPSL**$_{42}$		SWISSPROT

Canonical phosphorylation consensus sites for protein substrates of PKA, with their relative frequency are from Pearson and Kemp [36]. The asterisk denotes the residue which is phosphorylated.
Phosphorylation consensus sites for subunit AQDQ 18 kDa of complex I of beef heart and corresponding subunits in Neurospora crassa, and in humans, normal and patient, include sites in the leader (denoted by negative sequence numbers) and mature protein sequences.
Phosphorylation consensus sites in the leader sequence of subunit IV of cytochrome c oxidase are reported for the beef heart and rat liver enzymes.
Phosphorylation consensus sites for the two isoforms of subunit c of complex V in the leader and the mature sequence are reported for the beef heart enzyme. Where not otherwise specified sequences were obtained from the SWISSPROT database (Rel.36).

well-known case of subunits of pyruvate dehydrogenase and branched chain α-oxoacid dehydrogenase which are phosphorylated by their specific cAMP-independent protein kinases respectively. cAMP-independent phosphorylation of unidentified proteins has been ascribed to mitochondrial casein kinase [40].

Mitochondrial fractionation studies showed that cAMP-dependent phosphoproteins of 42 and 29 kDa are loosely associated, as the same PKA activity, with the inner membrane of bovine heart mitochondria, from which they are released by exposure to ultrasounds and high ionic strength. cAMP-dependent phosphoproteins of 18 and 6.5 kDa are more firmly associated to the inner membrane [30]. Progress in the identification of the inner membrane proteins phosphorylated by mtPKA was made by fractionation of membrane proteins by blue native electrophoresis followed by Tricine-SDS-PAGE [31]. This procedure, which results in the separation of the subunits of the five mitochondrial complexes of oxidative phosphorylation, showed a marked ^{32}P cAMP-dependent labelling of a 42 kDa protein band associated to complexes I, III, IV and V as well as labelling of the 18 kDa protein band associated with complex I. Incubation of the purified complex I with the isolated C subunit of PKA or with the inner membrane fraction (+cAMP) resulted in phosphorylation of a serine residue in its constituent 18 kDa subunit. The 42 kDa phosphoprotein which was apparently loosely associated with complexes I, III, IV and V of oxidative phosphorylation might represent subunits of PKA itself, an assembly factor for the complexes or a member of the PKA anchoring proteins. Both PKA subunits and AKAP proteins can be phosphorylated by PKA [7].

Sequence analysis of the 18 kDa phosphoprotein from the isolated bovine heart complex I identified it as the nuclear encoded 18 kDa AQDQ subunit of this complex of the respiratory chain (NADH-ubiquinone oxidoreductase (E.C.1.6.5.3.)) [41]. This protein is apparently located at the inner surface of the mitochondrial membrane at the junction between the peripheral mass protruding in the matrix and the membrane moiety of complex I [41]. The mature 18 kDa protein consists of 133 residues, at position 129–131 there is a phosphorylation consensus site RVS (Table 1). The protein has a leader sequence, removed after import in mitochondria [41], which also presents a phosphorylation consensus site at position −10 −7. The human AQDQ corresponding subunit has the same phosphorylation consensus sites in the leader and mature sequence (see Table 1) [42]. The corresponding subunit in *Neurospora crassa* complex I [43] has two phosphorylation consensus sites, at positions 9–12 and 34–36 respectively (Table 1). The *Neurospora crassa* 21 kDa protein has also a leader sequence, removed after import, with phosphorylation consensus sites at positions −18 −16 and −2 +2. The latter encompasses the C-terminus of the leader sequence and the first two N-terminal residues of the mature protein. The presence of phosphorylation consensus sites in both the leader sequences and the mature proteins can result in phosphorylation of the precursor proteins by cAMP-dependent protein kinase in both the cytosolic and mitochondrial compartments [29]. This dual phosphorylation might regulate import and/or maturation of the protein. The 18 kDa (IP) AQDQ subunit of bovine heart complex I and the homologous human and Neurospora crassa proteins do not show by the FASTA program any significant homology with other proteins in the SWISSPROT, NBRF/PIR databases neither with other cDNA sequences in EMBL databases. In this context is it worth noting that evidence has been presented showing that the 24 kDa subunit of the bovine heart complex I is a G protein [44].

Recently, the nuclear encoded subunit IV of cytochrome c oxidase has been shown to be phosphorylated in liver mitochondria in the absence of added cAMP [45]. The mature protein has various potential phosphorylation consensus sites for different kinases located within evolutionary conserved regions, in particular three sites for casein kinase II. There are two PKA consensus sites in the presequence which might be relevant for import of the protein in mitochondria and/or assembly of cytochrome c oxidase (Table 1). In the inner membrane of potato mitochondria, phosphorylation of the F1-δ and Fo-b subunits of the ATP synthase has also been shown to take place in the absence of added cAMP [46].

In mitochondria isolated from glucacon treated hepatocytes, a ^{32}P-labelled 20 kDa protein of the inner mitochondrial membrane was isolated and tentatively identified as cytokeratin by Gorlach et al. [16]. These authors have, however, revised this conclusion and proposed that this phosphoprotein is subunit IV of cytochrome c oxidase (S. Soboll, personal communication). Evidence for phosphorylation by PKA of the 18 kDa mitochondrial benzodiazepine receptor protein, involved in steroidogenesis, in adrenal mitochondria and in the steroidogenic MA-10 tumor cells was presented [47]. Very recently, 'in vitro' phosphorylation, by the purified catalytic subunit of PKA, of the mitochondrial telomere-binding protein (mTBP) of Candida parapsilosis has been reported [48]. The phosphorylated mTBP showed a reduced binding for the 5′ single-stranded overhang of the linear mitochondrial DNA in this yeast. This observation is of particular interest since PKA-mediated phosphorylation of the CREB protein modulates the transcription of genes regulated by CRE promoters [3].

Inspection in the SWISSPROT database (Rel.36) of the amino acid sequences of the subunits of the five complexes of oxidative phosphorylation shows potential consensus sites in 22 constituent subunits of complex I, 1 subunit of complex II, 6 subunits of complex III, 8 subunits of complex IV and 12 subunits of complex V. Of particular interest in the present context is subunit c of complex V. The mature protein has a M.W. of 8 kDa, and could correspond to the cAMP-dependent phosphoprotein of corresponding apparent M.W. identified in the inner membrane of bovine heart mitochondria [30]. There are two isoforms of the c subunit which are expressed in a tissue-specific manner. Both have canonical protein phosphorylation consensus sites in the leader and in the mature sequence (Table 1). It should be stressed that the presence of a phosphorylation consensus site in a protein and its phosphorylation in the purified state by C-PKA are necessary but not sufficient conditions to predict in vivo phosphorylation of that protein under physiological conditions. Other determinants for phosphorylation can reside in the sequence and folding of the protein. The situation that emerges from what has been reviewed here suggests that more surprises might be expected as far as the number of mitochondrial proteins phosphorylated in vivo by PKA and the physiological role of this phosphorylation is concerned.

4. The role of cAMP-dependent protein phosphorylation in mitochondria

Of all the mitochondrial proteins only 13 subunits of the five complexes of oxidative phosphorylation (subunits ND1-ND6 and ND4L of complex I, cytochrome b of complex III, subunits 1, 2 and 3 of complex IV and subunits ATPase 6 and A6L of complex V) are encoded by the mitochondrial genome and synthesized in mitochondria, all the other proteins are encoded by nuclear genes, synthesized on cytoplasmic ribosomes directly as mature proteins or as precursors with leader sequences, imported in mitochondria, processed and assembled in the enzyme complexes [49]. Whilst the mitochondrially encoded proteins can only be phosphorylated by the mtPKA, the nuclear encoded proteins can be first phosphorylated in the cytosol where the major pool of PKA is localized and then imported in mitochondria where they can also be phosphorylated. Thus, a complex pattern of phosphorylation-dephosphorylation in the two cell compartments could take place whose physiological significance remains to be understood. Clues for the elucidation of these aspects are emerging from studies on the occurrence and role of cAMP-dependent phosphorylation of mitochondrial proteins in cell cultures. Studies along these lines, carried out in our laboratory in collaboration with R. Scarpulla at the Northwestern University in Chicago, have shown that an intracellular increase in the level of cAMP induced by the addition of cholera toxin to 3T3 Balb/c mouse fibroblasts, in the serum starved resting state, promoted phosphorylation of the 18 kDa subunit of complex I associated to the inner mitochondrial membrane [50]. This phosphorylation was accompanied by marked enhancement of the activity of complex I as well as stimulation of respiration supported by NAD-linked substrates. A short term stimulatory effect on complex I activity was also produced by incubation of mitoplasts from starved fibroblasts with dibutyryl-cAMP, the effect being potentiated when mitoplasts were supplemented with the cytosolic fraction (results to be reported elsewhere). Thus, phosphorylation of the 18 kDa subunit by PKA regulates the activity of complex I. Through this effect, a rise in the cellular level of cAMP can result in a rapid activation of mitochondrial respiration and ATP synthesis. In fibroblasts in the exponential growth phase, increase in the cellular level of cAMP resulted in phosphorylation of serine in subunit 4 of complex IV (cytochrome c oxidase), likely at the phosphorylation consensus site present in the leader sequence (see Table 1) accompanied by enhanced activity of cytochrome c oxidase (results to be reported elsewhere). This is apparently a long term effect which might involve activation of the biogenesis of this respiratory complex. It can be recalled that in yeast mitochondria cAMP was reported to activate the expression of the three mitochondrially encoded subunits of complex IV [18], as well as of nuclear encoded subunits [19].

Evidence for a critical role of PKA-mediated phosphorylation of the AQDQ subunit of complex I in the regulation of the activity of this complex seems to be provided by the recent finding in a child affected by a severe deficiency of complex I, who died at the age of 16 months, of a 5 bp duplication in the nuclear gene encoding this subunit [42]. This mutation, which was transmitted with autosomal recessive inheritance, caused a shift in the translational reading frame resulting in an elongation of the mature protein by 14 amino acids and destruction of the phosphorylation consensus site (see Table 1).

5. Conclusions

The occurrence of a mitochondrial PKA and phosphorylation of mitochondrial proteins reveals an extension of the cAMP-mediated intracellular signal transduction to these organelles where the biogenesis, membrane assembly and activity of mitochondrial enzymes and transporters can be modulated. The finding of PKA anchoring proteins in the outer membrane suggests also a role of cAMP-dependent protein phosphorylation in the interaction of the mitochondrial surface with other cellular structures. The present paper concerns the first aspects of an emerging topic in the field of cellular signal transduction. Clearly, much more work is expected in order to understand the role that cAMP signalling in mitochondria plays. Specific aspects which deserve further study are the characterization of the compartimentalization of PKA in mitochondria and the membrane location of the cAMP binding sites on the R subunits as well as identification of the regulatory and catalytic isoforms of PKA associated to mitochondria. Another aspect to be addressed is represented by the regulation of the phosphorylation-dephosphorylation process of mitochondrial proteins. Further investigations should be directed to isolate and sequence the various mitochondrial proteins that are phosphorylated in vivo by mitochondrial PKA and to elucidate the role that phosphorylation of the specific proteins may play in the control of mitochondrial biogenesis and functions.

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