Mitochondrial AKAP121 links cAMP and src signalling to oxidative metabolism

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Running title: AKAP121 regulates oxidative metabolism

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Summary

AKAP121 focuses distinct signalling events from membrane to mitochondria by binding and targeting cAMP-dependent protein kinase (PKA), protein tyrosine phosphatase (PTPD1) and mRNA. We find that AKAP121 also targets src tyrosine kinase to mitochondria via PTPD1. AKAP121 increased src-dependent phosphorylation of mitochondrial substrates and enhanced the activity of cytochrome c oxidase, a component of the mitochondrial respiratory chain. Mitochondrial membrane potential and ATP oxidative synthesis were enhanced by AKAP121 in an src- and PKA-dependent manner. Finally, siRNA-mediated silencing of endogenous AKAP121 drastically impaired synthesis and accumulation of mitochondrial ATP. These findings indicate that AKAP121, through its role in enhancing cAMP and tyrosine kinase signalling to distal organelles, is an important regulator in mitochondrial metabolism.
**Introduction**

Protein kinase A (PKA) is an essential mediator in most cAMP-dependent signalling pathways. A family of proteins named A-Kinase Anchor proteins (AKAPs) has been identified that enhance cAMP-dependent PKA signalling pathways (Rubin, 1994; Gray *et al.*, 1998; McKnight *et al.*, 1998; Feliciello *et al.*, 2001; Houslay and Adams, 2003; Tasken and Aandahl, 2004; Taylor *et al.*, 2004; Wong and Scott, 2004). AKAP121 (also called D-AKAP1), AKAP149 and AKAP84 arise from a single gene by alternative pre-mRNA splicing (Lin *et al.*, 1995; Trendelenburg *et al.*, 1996; Chen *et al.*, 1997; Huang *et al.*, 1997; Huang *et al.*, 1999; Furusawa *et al.*, 2002). AKAP121 and AKAP84 tether PKA to the mitochondrial outer surface. This localization is mediated by the interaction of AKAP121 and AKAP84 with β tubulin, an integral component of mitochondrial outer membrane (Cardone *et al.*, 2002). AKAP121 is widely expressed in several tissues and its accumulation is regulated at the transcriptional level by the cAMP/PKA pathway (Feliciello *et al.*, 1998). Anchoring of PKA to mitochondria supports cAMP signalling and suppresses apoptosis (Harada *et al.*, 1999; Affaitati *et al.*, 2003). AKAP121, via a KH domain at its COOH-terminus, binds at least two mRNAs that encode mitochondrial proteins (Ginsberg *et al.*, 2003; Ranganathan *et al.*, 2005). This multi-component system, reminiscent of other AKAP complexes at cell membranes, ensures efficient translation and import of nuclear-encoded mitochondrial proteins. It is suggested that PKA may phosphorylate some of these proteins co-translationally, as well as acting on AKAP121 itself to regulate the stability of the RNA-AKAP121 complex (Ginsberg *et al.*, 2003; Feliciello *et al.*, 2005).

In addition, AKAP121 and AKAP84 bind the central core of PTPD1, a classical non-receptor protein tyrosine phosphatase (Moller *et al.*, 1994). PTPD1 binds to and activates src, enhancing EGF-dependent mitogenic signalling (Cardone *et al.*, 2004). By translocating PTPD1 to the outer membrane of mitochondria, AKAP121 inhibits PTPD1-dependent EGF signalling to the nucleus. These data suggest a model whereby AKAP121, by targeting PTPD1/src complex to mitochondria, may shift the focus of tyrosine kinase signalling from membrane to specific distal organelles, such as mitochondria (Feliciello *et al.*, 2005).

We tested this hypothesis and found that AKAP121, indeed, targets src tyrosine kinase to mitochondria. By manipulating the localization and expression of AKAP121 we were able to modulate cAMP- and src-dependent signalling to mitochondria, affecting phosphorylation of mitochondrial substrates, activity of components of the respiratory chain, mitochondrial membrane potential and oxidative synthesis of ATP.
Experimental Procedures

Cell lines. The human embryonic kidney cell line HEK293 was cultured in Dulbecco modified Eagle’s medium containing 10% fetal calf serum in an atmosphere of 5% CO₂. Where indicated, HEK293 cells were stably transfected with a CMV–G418 vector expressing AKAP121 and selected for four weeks in medium containing G418 (800 µg/ml). Resistant clones were isolated, screened for expression of the transgene and pooled (ten and five independent positive clones for PTPD1 and CMV, respectively). Pools were expanded and grown in medium supplemented with 400 µg/ml G418. GC2 cells were derived from primary mouse pre-leptotene spermatocytes by stable co-transfection with transgenes encoding SV40 large T-antigen and a temperature-sensitive variant of the p53 transcriptional regulator protein (Wolkolwicz et al., 1996). GC2 cells were grown at 37 °C in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum (FCS). Primary cultures of human fibroblasts were obtained from cutaneous biopsy of healthy subjects. Primary human fibroblasts and NIH3T3 cells were propagated in DMEM medium supplemented with 10% calf serum.

Antibodies and chemicals. Polyclonal anti-AKAP121 antibody was purchased from Santa Cruz (C-20). We also used an anti-AKAP121 polyclonal antibody that has been previously described (9). Anti-PTPD1 polyclonal antibody was prepared as previously described (Moller et al., 1994). Mouse monoclonal anti-src antibody was purchased from Oncogene Research Products; anti-COXII from Molecular Probes; anti-hemoagglutinin epitope (HA.11) from Covance; anti-tubulin from Sigma; anti-SOD monoclonal antibody from Walter Occhiena; anti-AKT from Santa Cruz. CPT-cAMP from Sigma; H89 and PP2 from Calbiochem.

Plasmids and transfection. Mouse pCEP4-AKAP121 cDNA was a gift of Dr C. Rubin (Albert Einstein College of Medicine, NYC). An AKAP84 mutant lacking the first 30-aminoacid was generated by PCR using specific oligonucleotide. The PCR-product was sub-cloned in CMV-vector. cDNA coding the kinase-inactive form of Src (Lys259 changed to methionine) was cloned into pSG5 (Barone and Courtneidge, 1996) and kindly provided by Prof. A. Migliaccio (Second University of Naples, Italy). The vector encoding for human PTPD1 was previously described (Moller et al., 1994). A small DNA insert (about 70 bp) encoding for short hairpin RNA targeting mouse AKAP121 (nucleotide 301-321, ATG +1) was subcloned in pRNA-H1/neo vector (GenScript Corporation). Blast search confirmed that this sequence specifically recognizes mouse. A scrambled sequence subcloned in the same vector was used as experimental control. siRNA vectors were transiently transfected using lipofectamin protocol. All plasmids were purified using QIAGEN tip columns (Qiagen, Chatsworth, CA) and sequenced using the CEQ2000 DNA Analysis System and a Beckman automated sequencer.
**Immunoprecipitation and immunoblot analysis.** Cells were homogenized in lysis buffer (20 mM Tris-HCl pH 7.4, 0.15 M NaCl, 10 mM EDTA, 0.25 % Triton X-100, 0.05% Tween-20, 0.02% sodium azide) containing aprotinin (5 µg/ml), leupeptin (10 µg/ml), pepstatin (2 µg/ml) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). The lysates were cleared by centrifugation at 15,000 x g for 15 min. Cell lysates (2mg) were immunoprecipitated with the indicated antibodies. An aliquote of cell lysate (100µg) or immunoprecipitates were resolved by SDS-PAGE gel and transferred to Immobilon P membrane. The immunoblot analysis was performed as previously described (Cardone et al., 2004). Chemio-luminescent (ECL) signals were quantified by scanning densitometry (Molecular Dynamics). Highly purified mitochondria and supernatant fraction were isolated as described (Hovius et al., 1990).

**Immunofluorescence analysis.** Cells were rinsed with PBS and fixed in 3.7% formaldehyde for 20 min. After permeabilization with 0.5% Triton X-100 in PBS/5 min, the cells were incubated with PBS 1X/0.1 mg/ml bovine serum albumin for 1 hr at room temperature. Double immunofluorescence was carried out with the following antibodies: anti-superoxide dismutase monoclonal (1/200), anti-AKAP121 goat polyclonal (1/200) (Santa Cruz, sc-6439), anti-PTPD1 rabbit polyclonal (1/200), anti-AKAP121/84 rabbit polyclonal (1/100). Fluorescein- or rhodamine-tagged anti-rabbit and anti-mouse IgG (Technogenetics) secondary antibodies were used. Cover slips were analyzed by confocal microscopy.

**Assays for cytochrome c oxidase activity and mitochondrial ATP synthesis.** The activity of cytochrome c oxidase on purified mitochondria was determined by spectrophotometric measurement of the rate of reduced cytochrome c oxidation at 550 nm (Couperstein and Lazarov, 1951). Cytochrome c (type VI, Sigma) was reduced by dithiotriol (DTT) at a final concentration of 0.5 mM. Cytochrome c reduction was assessed measuring A550/A565 ratio. In our conditions, this ratio was between 15 and 20. The mitochondrial fraction was suspended in 60mM of phosphate buffer (pH=7.4) containing 0,6% lauryl maltoside and centrifuged for 10 min at 10.000 g. The protein concentration was determined according to Lowry et al. (1951). The assay was performed in a total volume of 1 ml of phosphate buffer (60mM) containing 0.1 mg of mitochondrial proteins, 30 µM final concentration of reduced cytochrome c. The decrease in absorbance at 550nm was measured for 1 min with 15” integration time (Stieglerova et al., 2000).

**Assay for oxidative ATP synthesis.** Cells were harvested by trypsinization 48 h after transfection, washed twice in phosphate buffer saline (PBS) and counted in a hemo-cytometer. A replicate for each sample was prepared that had been treated for 1hr with 4 µg/ml rotenone (Sigma). The emission recorded from samples treated with rotenone was defined as baseline luminescence corresponding to a non-mitochondrial source of ATP. Assays were performed using the ATP
luminescence assay kit HS II (Roche) according to manufacturer’s instructions, using 3000 cells per sample. Light emission was recorded in a single measure of 2 sec using a Lumat LB 9507 luminometer (Berthold technologies).

**Quantitative analysis of mitochondrial DNA.** Mitochondrial DNA content relative to nuclear genome from control (CMV) and AKAP121 expressing cells was evaluated by PCR using oligonucleotide primers specific for nuclear (β globin) and mitochondrial genes (NADH dehydrogenase and cytochrome b). The primer sequences used were: cytochrome b: FW-5’ CTTAGGCGACCCAGACAAATTAT; rev 5’-TCATTGGGCTTGGATGGTG; NADH: FW 5’- CAGCCATTCTCATCCAAAACC; rev5’-ATTATGATGCGACTGT GAGTGC; β globin: FW 5’- AGCCTGACCAACATGGTGAAA C; rev 5’-AGC CACCTGAATAGCTGGGACT. PCR reactions were carried out on the Applied Biosystem 7000 Real time PCR System using Syber Green method. All reactions were performed in a 30μl mixture containing 1X SYBR reaction buffer, 10μM primers and 20 ng of whole cellular DNA. PCR products range between 70-150 bp. Southern blot analysis was performed as described (Maniatis, 1989). Briefly, 20 μg of total genomic DNA were digested with EcoRI restriction enzyme, separated on 1% agarose gel, transferred to N-Hybond membrane and sequentially hybridized with the mitochondrial and nuclear (β globin) cDNA probes. The mitochondrial probe spanning the nucleotides 7392-8625 of human mitochondrial genome was obtained with the following oligonucleotide primers: FW-5’ GGATGCACCCACCCCTAC; rev-5’-GGAGGTGGGGATCAATAGAGG.

**Imaging mitochondrial membrane potential.** Mitochondrial membrane potential was assessed using the fluorescent dye tetra-methyl rhodamine ethyl ester (TMRE) in the “redistribution mode”. Cells transfected with CMV and AKAP121 were loaded with TMRE 20 nM for 30 min in a medium containing 156 mM NaCl, 3 mM KCl, 2 mM MgSO4, 1.25 mM KH2PO4, 2 mM CaCl2, 10 mM glucose, and 10 mM Hepes. The pH was adjusted to 7.35 with NaOH (Abramov et al., 2004). At the end of the incubation, cells were washed in the same medium containing 20 nM dye and allowed to equilibrate. A decline of mitochondria-localized intensity of fluorescence was indicative of mitochondrial membrane depolarization. Confocal images were obtained using a Zeiss inverted 510 confocal laser scanning microscopy and a 63X oil immersion objective. The illumination intensity of 543 Xenon laser, used to excite TMRE fluorescence, was kept to a minimum of 0.5% of laser output to avoid photo-toxicity.
Results

**AKAP121 assembles a tyrosine kinase/phosphatase signalling complex on mitochondria.**

We previously showed that AKAP121 binds and targets PTPD1 to sub-cellular organelles. Since PTPD1 associates with src, we hypothesized that AKAP121 may form a scaffold complex that includes not only PTPD1 and PKA, but src as well. In this context, PTPD1 may act as molecular bridge between the anchor protein and src. To test this notion, we performed co-immunoprecipitation experiments using total lysates prepared from HEK293 cells. This cell line expresses endogenous PTPD1 and low levels of AKAP121 (Figure 1A). Cells were transiently transfected with AKAP121 vector with or without PTPD1 vector. Forty-eight hr following transfection, total lysates were prepared and subjected to immunoprecipitation. Figure 1A shows that AKAP121 was precipitated by anti-src antibody only in AKAP121-transfected cells, and the amount of precipitated AKAP121 significantly increased when it was co-expressed with hemoagglutinin (HA)-tagged PTPD1. Conversely, anti-AKAP121 antibody precipitated significant amounts of PTPD1 and src (Figure 1B).

To determine if AKAP121 targets src tyrosine kinase on mitochondria in intact cells, we performed immunostaining analysis using specific antibodies directed against src, PTPD1 and AKAP121. Primary mouse pre-leptotene spermatocytes (GC2) express high levels of endogenous AKAP121 and PTPD1. Both proteins partly co-localize on mitochondria (Cardone et al., 2004). We analyzed src localization in GC2 cells by double immunostaining with anti-AKAP121 and anti-src antibody. The signals were collected and analyzed by confocal microscopy. As expected, AKAP121 selectively localized on mitochondria, as shown by double labeling with anti-AKAP121 and anti-superoxide dismutase, a protein specifically localized to the mitochondrial matrix (Figure 2, panels a-f). Some src immunostaining overlapped that of AKAP121, suggesting that both proteins co-localize on mitochondria in vivo. However, src staining was also evident at the cell periphery (membrane) as well as at the perinuclear and nuclear regions, where no mitochondria or AKAP121 were detectable. We confirmed this finding using different anti-AKAP121 or anti-src antibodies (data not shown; see Materials and Methods). We performed similar experiments examining src localization in human fibroblast primary cultures that express endogenous AKAP121. Growing fibroblasts were labeled in vivo with Texas red–conjugated mitotracker, which selectively accumulates into mitochondria, fixed in formaldehyde and immunostained with anti-src antibody. Src staining partly overlapped with that of mitotracker (Figure 2, panels g-i). Additionally, we asked whether exogenous AKAP121 expression in the HEK293 cell line, which costitutively expresses low AKAP121 levels, would target src to mitochondria. Indeed, HEK293 cells stably
transfected with AKAP121 showed significant amounts of src concentrated on mitochondria (Figure 2, panels j-l).

The ability of AKAP121 to direct src to mitochondria was also demonstrated in HEK293 cells by co-fractionation experiments. HEK293 were transiently transfected with expression vectors encoding PTPD1 and AKAP121. Forty-eight hr following transfection, mitochondrial and supernatant fractions were isolated, size-fractionated on SDS-PAGE and immunoblotted with the indicated antibodies. As shown in Figure 3, AKAP121 co-purified with the mitochondria-enriched fraction, as did the mitochondrial voltage-dependent anion channel (VDAC), whereas tubulin and MAPK were found exclusively in supernatant fractions. In un-transfected control cells most of endogenous c-src protein was found in the supernatant. Expression of AKAP121, PTPD1 or both, significantly increased the amount of c-src recovered in the mitochondrial fraction. Note that co-expression of AKAP121 and PTPD1 translocates an amount of src to mitochondria roughly equivalent to AKAP121 or PTPD1 alone. PTPD1 and src are not only localized to mitochondria. Significant amounts of these enzymes have been found associated with other organelles and cell structures. This suggests that interaction with specific targeting sites is critically dependent on the absolute levels and binding affinity of AKAP121, src and PTPD1.

Src activity was not required for mitochondrial localization promoted by AKAP121. Thus, co-transfection of HEK293 with AKAP121 and an src kinase dead (src K-) increased src levels in the mitochondrial fraction. A similar increase was seen when AKAP121 transfected cells were treated with the src inhibitor, PP2. We conclude that AKAP121 recruits c-src to mitochondria independently on its kinase activity.

**AKAP121 enhances src-dependent phosphorylation and activation of mitochondrial substrates.** Some src is normally found in the mitochondrial matrix, where it phosphorylates and stimulates the activity of cytochrome c oxidase (COX), a component of the mitochondrial respiratory chain (Miyazaki *et al.*, 2003). We wished to determine if src localization by AKAP121/PTPD1 on mitochondria correlated with enhanced phosphorylation of mitochondrial src substrates. HEK293 cells were transiently transfected with AKAP121 and PTPD1 vectors for 24 hr, and subsequently harvested and lysed. Mitochondrial fractions were prepared and subjected to immunoblot analysis with anti-phosphotyrosine antibody. As shown in Figure 4A, expression of AKAP121 or PTPD1, to a lesser degree, markedly enhanced tyrosine phosphorylation of mitochondrial proteins. In contrast to src localization, stimulation of tyrosine phosphorylation by AKAP121 required src activity. Thus, treatment with PP2 or expression of src K- reduced phosphorylation to control levels. The phosphorylation of some mitochondrial substrates in cells
transfected with AKAP121 and PTPD1 was reduced, compared to cells expressing AKAP121 alone. We have evidence that PTPD1 is not only localized to mitochondria, but in the absence of AKAP121, a significant fraction is linked to the actin cytoskeleton. In this compartment, PTPD1 regulates EGF-dependent src-FAK signalling (Carlucci et al., manuscript in preparation). This suggests that interaction with specific targeting sites and phosphorylation of distinct substrates are critically dependent on the absolute levels of AKAP121, src and PTPD1.

We then monitored the activity of cytochrome c oxidase (COX). HEK293 cells were transiently transfected with the indicated expression vectors. Forty-eight hr from transfection, cells were harvested, lysed and mitochondrial COX activity assayed. Figure 4B shows that AKAP121 increased COX activity by ca. 30%. HEK293 cells transiently expressing AKAP84, the smaller splice variant of AKAP121, also showed increased COX activity compared to their control HEK293 cells (data not shown). To prove that AKAP-mediated targeting of src to mitochondria was required for COX activity, we tested an AKAP84 mutant carrying a deletion of the mitochondrial targeting (MT) domain (Δ1-30). This mutant retained the ability to bind PTPD1/src and PKA, but failed to target these proteins to mitochondria (Cardone et al., 2004). AKAP84Δ1-30 was transiently transfected in HEK293 cells and COX activity assayed. The expression of mutant protein was comparable to that of wild-type AKAP121 (data not shown). Figure 4B shows that AKAP84Δ1-30 acted as dominant negative, reducing COX activity by ca. 40% relative to controls. Stimulation of COX activity by AKAP121 depended on active src kinase. Treatment of the transfected cells with PP2 reduced COX activity below control levels. The amount of COX protein, as shown by Western blot, was unaffected by transfection and treatment with PP2 (Figure 4B, inset). This indicates that the number of mitochondria per cell remained constant during these treatments.

Elevated oxidative respiratory chain activity is associated with increased mitochondrial DNA content (Deveaud et al., 2004). We therefore asked whether AKAP121 promoted mitochondrial DNA accumulation. Using semi-quantitative PCR, we monitored the accumulation of two mitochondrial genes, NADH de-hydrogenase and cytochrome B. The nuclear β globin gene was used as an internal control. Figure 4C shows that AKAP121 increased the levels of NADH de-hydrogenase and cytochrome B by ca. 50%, compared to control cells transfected with the CMV vector (CMV). To confirm this finding, we performed Southern blot analysis on total cellular DNA using as probes mitochondrial and β globin cDNAs. Control cells and cells expressing AKAP121 were serum-deprived overnight, transferred to 10% FCS and harvested at the indicated times. As shown in Figure 4D, AKAP121 increased both basal and serum-stimulated mitochondrial DNA levels by about 2-3 fold.
AKAP121 regulates mitochondrial membrane potential and oxidative ATP synthesis. Electron flux through the respiratory chain is used to reduce free oxygen at the level of complex IV. Hydrogen then moves from the mitochondrial matrix to the inter-membrane space. This generates an electrochemical gradient, expressed as mitochondrial membrane potential (ΔΨm) (Saris and Carafoli, 2005). The ΔΨm is fundamental for the efficient production of ATP and requires the coordinated activity of several enzyme complexes, including cytochrome c oxidase. Since AKAP121-src stimulated COX activity, we asked if this anchor protein enhanced the ΔΨm under basal or stress conditions using a fluorescence assay (Abramov et al., 2004). Indeed, mitochondria in HEK293 transiently transfected with AKAP121 were hyperpolarized in comparison to control cells (Figure 5A, a, d). Hyper-polarization was inhibited by 30 min exposure to H89 (10µM), an inhibitor of PKA and other kinases (Figure 5A, e). Similarly, treatment with PP2 (10µM) for 30 min reduced polarization (Figure 5A, f). Treatment with H89 or PP2 also decreased the intensity of mitochondria localized fluorescence in control cells (Figure 5A & Figure 5B). Similar results were obtained in HEK293 cells stably transfected with AKAP121 (figure 5C).

We then compared the response of cells transiently transfected with CMV or AKAP121 and exposed to serum deprivation (SD) or to chemical hypoxia. Cells were serum deprived for 18 hr or subjected to chemical hypoxia by exposure to oligomycin (5µg/ml) and 2 de-oxycyglucose (2DG, 2mM) for 45 min, followed by 15 hr of re-oxygenation. Either treatment dramatically reduced ΔΨm in control cells (Figure 6A, b, c & figure 6B). In contrast, these treatments only slightly reduced the intensity of mitochondria localized fluorescence in AKAP121-transfected cells (Figure 6A, e, f, & figure 6B). The localized fluorescence intensity of cells expressing AKAP121 was significantly higher than in control cells.

AKAP121, by facilitating the electrochemical gradient along the inner mitochondrial membrane, should enhance ATP synthesis. Figure 7A shows that cells transfected with AKAP121 had higher ATP levels than controls. H89 abrogated this increase. Treatment with CPT-cAMP, a cAMP analog, increased the ATP levels in control cells and, to a lesser extent, in AKAP121 expressing cells, compared to un-treated cells. This suggests that AKAP121 increased mitochondrial PKA signaling close to maximum levels. In fact, in serum-deprived AKAP121 cells, the effects of cAMP on mitochondrial PKA signaling were more evident (Affaitati et al. 2003; Ginsberg et al., 2003). Taken together, these data imply that PKA up-regulates ATP synthesis and this effect is enhanced by AKAP121. ATP accumulation was also dependent on src activity. Thus, ATP concentrations were lowered by PP2 in both control and, more dramatically, in AKAP121-transfected cells. Western blot analysis showed that neither PP2 nor H89 reduced expression of AKAP121 (data not shown).
We then examined the effects of serum deprivation on ATP accumulation. Twenty-four hr following transfection, cells were serum-deprived for eighteen hr and their mitochondrial ATP concentration determined. Serum-deprivation significantly reduced ATP levels in control cells and in cells expressing AKAP121. These findings, along with the data reported in figure 6B, suggest that growth factors stimulate oxidative phosphorylation and that AKAP121 increases mitochondrial robustness and resistance to oxidative stresses.

To confirm that AKAP121 regulates the mitochondrial respiratory chain, we silenced endogenous AKAP121 by DNA vector-based siRNA and measured ATP levels. As control, we used the same vector carrying a scrambled RNA sequence. Mouse fibroblasts (NIH3T3), which express AKAP121, were transiently transfected and the efficiency of silencing was evaluated by immunoblot analysis (Figure 7B). Expression of siRNA_{AKAP121} decreased endogenous AKAP121 levels ca. 2.5-fold. The control vector which expresses siRNA_{scrambled}, did not reduce AKAP121 concentrations. Consistent with our hypothesis, the synthesis of mitochondrial ATP was suppressed by siRNA_{AKAP121} but not siRNA_{scrambled}. The extent of inhibition by siRNA was comparable to that provoked by src inhibition with PP2 (Figure 7C).
Discussion

Our data shows that mitochondrial AKAP121 forms a signalling complex that includes, in addition to PKA, PTPD1 and src. AKAP121 increases cAMP and src signalling to mitochondria. Thus, tyrosine phosphorylation of some mitochondrial substrates, activity of cytochrome c oxidase, mitochondrial membrane potential ($\Delta\Psi_m$) and ATP synthesis were enhanced by AKAP121 in an src- and PKA-dependent manner.

AKAPs, by co-localizing signalling enzymes and their substrate(s), are proposed to ensure efficient propagation of transduction events generated at distal sites to specific intracellular compartments (Rubin, 1994; Feliciello et al., 1997; Gray et al., 1998; McKnight et al., 1998; Feliciello et al., 2001; Houslay and Adams, 2003; Tasken and Aandahl, 2004; Taylor et al., 2004; Wong and Scott, 2004). AKAP121 clearly plays this role in mitochondria. By localizing PKA at the outer membrane of mitochondria, AKAP121 increases PKA-dependent phosphorylation/inactivation of pro-apoptotic protein BAD and enhances cell survival (Harada et al., 1999; Affaitati et al., 2003). AKAP121 also facilitates PKA-dependent phosphorylation and activation of StAR, a mitochondrial steroidogenic factor that localizes in mitochondria of adrenal and testicular Leydig cells. In doing so, AKAP121 increases the biosynthesis of steroid hormones in a PKA-dependent manner (D. Stocco, M. Dyson, J. Jones & M.E.G., unpublished).

Signalling enzymes other than PKA are also bound and targeted by AKAP121. The aminoterminal of AKAP121 interacts with the central core of PTPD1, localizing the phosphatase on mitochondria (Cardone et al., 2004). PTPD1 is an effector for EGF signal transduction from the membrane to the nucleus. AKAP121 binding diverts PTPD1 to mitochondria and downregulates this transduction pathway. We report here that AKAP121, via PTPD1, targets src to mitochondria. AKAP121 enhances src-dependent tyrosine phosphorylation of some mitochondrial substrates, facilitating the mitochondrial respiratory chain and increasing ATP synthesis. This conclusion is based both on AKAP121 overproduction and AKAP121 knockdown by siRNA.

The importance of tyrosine kinase signalling in mitochondrial function is supported by several lines of evidence (Abram and Courtneidge, 1999; Ko et al., 2002; Boerner et al., 2004; Augereau et al., 2005; Salvi et al., 2005). Tyrosine-phosphorylation of mitochondrial proteins is stimulated in vitro by ATP and H$_2$O$_2$ (Augereau et al., 2005). ATP production at state 3 likewise enhances phosphorylation; this stimulation is ablated by PP2. Phosphorylation and activation of COX by mitochondrial src is postulated to play an important role in osteoclast function and bone remodeling (Miyazaki et al., 2003). The 39-kDa subunit of complex I is tyrosine-phosphorylated, and subunits of complexes II, III and IV may also be tyrosine kinase substrates (Augereau et al., 2005). PDGF signalling is linked to tyrosine phosphorylation of the c and $\delta$ subunits of the mitochondrial ATP
synthase complex. This accounts for the enhanced activity of ATP synthase seen in a variety of PDGF-treated cells, including cortical neurons, mouse fibroblasts and kidney cells (Evtodienko et al., 2000; Ko et al., 2002; Boerner et al., 2004). Moreover, serum-deprivation has been linked to loss of mitochondrial respiratory control (Gottlieb et al., 2002).

Components of the respiratory chain can be also phosphorylated and regulated by PKA (Yang et al., 1998; Ludwig et al., 2001, Papa et al., 2002). A functional interplay between cAMP, tyrosine kinase and mitochondrial COX has been recently described (Lee et al., 2005). These authors found that high cAMP levels induced phosphorylation of COX subunit I at tyrosine304 and inhibited COX activity. The responsible tyrosine kinase has not been identified.

We propose that AKAP121 is a nodal point where PKA and src signalling integrate, increasing the rate and magnitude of signalling to mitochondria. The mechanism by which src bound to AKAP121-PTPD1 phosphorylates substrates located within the mitochondrial matrix is still unknown. One possibility is that src may translocate inside mitochondria through the outer/inner mitochondrial transport system (Endo, et al., 2003). AKAP121 increases the absolute levels of src anchored at the outer membrane of mitochondria. This facilitates transport of src, as well as PKA, inside the organelle, where both kinases normally reside (Miyazaki, et al., 2003; Yang et al., 1998; Papa et al., 2003). This regulation is critical for mitochondrial physiology, and explains the essential role of AKAP121 in cell survival, steroidogenesis and oxidative phosphorylation. Identification of the critical mitochondrial substrates of PKA and src, and the functional relationship between these two signaling enzymes on mitochondria will, of course, require further study.

In this manuscript we also present evidence that mitochondrial DNA content is increased by AKAP121. In this regard, our preliminary data suggest that cAMP and PKA are functionally linked to this process. In S. cerevisiae, mitochondrial activity (citric acid cycle and oxidative respiration) and mtDNA content are co-regulated. Mitochondrial activities and mtDNA are down-regulated by growth in glucose, whereas growth in a non-fermentable carbon source stimulates oxidative phosphorylation and increases mtDNA content. The cAMP/PKA signal transduction pathway positively regulates both reactions (Robertson et al., 2000; Cho et al., 2001; Griffioen and Thevelein, 2002). We have shown that AKAP121 concomitantly regulates mtDNA content and oxidative ATP synthesis. We cannot, however, ascertain which of the two events is the primary regulator of mammalian mitochondrial metabolism.

In summary, we demonstrate for the first time that AKAP121 regulates src events on mitochondria and highlight a unique role of this protein in the regulation of oxidative metabolism (Figure 7D). This mechanism increases the complexity of the symbiotic relationship developed million years ago between primordial eukaryotic cells and the aerobic bacteria that are thought to be mitochondrial
ancestors. In view of the ubiquitous expression of AKAP121, our findings reveal an efficient mechanism that may be used in most or all mammalian cells to adapt physiologically to rapid changes in carbon source availability.
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Legends to the figures

Figure 1. PTPD1-src complex binds to AKAP121. A. HEK293 cells were transiently transfected with expression vector encoding for AKAP121 in the presence or absence of HA-PTPD1 vector. Cell lysates were immunoprecipitated with anti-src antibody. Immunoprecipitates and aliquots (80 µg) of lysates were resolved on SDS-PAGE and immunoblotted with anti-HA, anti-PTPD1, anti-AKAP121 and anti-src specific antibodies. B. Lysates from control or AKAP121/PTPD1 transfected cells were immunoprecipitated with anti-AKAP121 antibody. Precipitates were resolved on SDS-PAGE and sequentially immunoblotted with anti-AKAP121, anti-HA and anti-src antibodies.

Figure 2. AKAP121 targets src to mitochondria. a-c, double immunostaining of GC2 cells with anti-AKAP121 (red) and anti-src antibodies (green); d-f, double immunostaining of GC2 cells with anti-SOD (red) and anti-src (green) antibodies; g-i, primary culture of human fibroblasts labeled in vivo with mitotracker (red), fixed and immunostained with anti-src (green) antibody; j-l, HEK293 cells stably expressing transfected with AKAP121 vector were labeled in vivo with mitotracker (red), fixed and immunostained with anti-src (green) antibody.

Figure 3. AKAP121 and src co-purify with mitochondria. HEK293 cells were transiently transfected with vectors encoding AKAP121, PTPD1 and, where indicated, with a kinase-dead mutant of c-src (src K-). Treatment with src inhibitor PP2 was performed 30 min before harvesting. Forty-eight hr following transfection, cells were harvested and lysed. Mitochondrial and cytosolic fractions were prepared as described in the “Experimental Procedures”, size-fractionated on SDS-PAGE and immunoblotted with the indicated antibodies. The lower panel shows a quantitative analysis of src content of mitochondrial and supernatant fractions. VDAC and MAPK were used as loading controls. The data are expressed as arbitrary units and represent the mean of two independent experiments that yielded similar results.

Figure 4. AKAP121 increases src-dependent phosphorylation and activity of components of the mitochondrial respiratory chain. A. HEK293 cells were transiently transfected with vectors encoding AKAP121, PTPD1 and kinase-inactive form of Src (src K-). Where indicated, cells were treated with the src inhibitor PP2, 30 min before harvesting. Mitochondrial fractions were immunoblotted with anti-phosphotyrosine antibody. A representative set of auto-radiograms is shown. B. HEK293 cells were transiently transfected with control (CMV), AKAP121 or AKAP84Δ1-30 vectors and harvested forty-eight hr after transfection. Where indicated, PP2 was added to the
medium 30 min before harvesting. Cytochrome c oxidase activity was assayed with purified mitochondria. Inset, immunoblot analysis of cell lysates with anti-cytochrome c oxidase II subunit (1, CMV; 2, AKAP121; 3, AKAP84_∆1-30; AKAP121 +PP2). The data represent the mean ± S.E.M. of five independent experiments. C. Semi-quantitative PCR of total genomic DNA extracted from HEK293 cells stably or transiently transfected with the AKAP121 expression vector. Mitochondrial (Cytochrome B, Nadhd) and nuclear (β globin) genomic DNAs were amplified as described in Materials and Methods. The data are shown as fold increase of mitochondrial versus nuclear genomic DNA. D. Southern blot analysis of total genomic DNA extracted from stably transfected HEK293 cells. Mitochondrial (mito) and nuclear (β globin) cDNAs were used as probes. Indicated are the fold increases of mitochondrial versus β globin DNA. Values from control, CMV cells (0-time point) were set as 1.

**Figure 5.** AKAP121 increases mitochondrial membrane potential. A. Confocal images showing TMRE fluorescence were collected from HEK293 cells transiently transfected with CMV (a-c) or AKAP121 (d-f) expression vectors. Forty-eight hr after transfection, cells were treated for 30 minutes either with PP2 (10µM) or H89 (10µM) and subjected to TMRE analysis. a, d, control cells; b, e, cells treated with H89 (10µM); c, f, cells treated with PP2. B. Cumulative data are expressed as mean ± S.E. of changes in TMRE fluorescence and represent fold increase over control (CMV, untreated cells) that was set as 100. The intensity of fluorescence was evaluated in single cell by Meta-Morph software analysis. * P<0.05 v.s. CMV transfected cells, **P<0.05 v.s. AKAP121 transfected cells. C. Cumulative data from HEK293 cells stably transfected with CMV or AKAP121 are expressed as mean ± S. E. of changes in TMRE fluorescence and represent fold increase over control (CMV, untreated cells) that was set as 100. PP2 and H89 treatment were performed as in B. * P<0.05 v.s. PCMV transfected cells. ** P<0.05 v.s. AKAP121 transfected cells. Statistical analysis was performed by ANOVA and Newman Keuls methods. Bar, 20 µm.

**Figure 6.** AKAP121 protects cells from chemical hypoxia and serum deprivation. A. Confocal images collected from HEK293 cells transiently transfected with CMV (a-c) or AKAP 121 (d-f). Cells were exposed to chemical hypoxia or serum deprivation. a, d, control cells; b, e, cells treated with 2DG (2mM) and oligomycin (5µg/ml) for 45 min followed by re-oxygenation for twenty-four hr; c, f, cells exposed to serum deprivation for eighteen hr. B. Cumulative data are expressed as mean ± S.E. of changes in TMRE fluorescence and represent fold increase over control (CMV, untreated cells) that was set as 100. * P<0.05 v.s. CMV transfected cells, **P<0.05 v.s. AKAP 121 transfected cells. Bar, 20 µm.
Figure 7. AKAP121 controls oxidative ATP synthesis. A. HEK293 cells were transiently transfected with AKAP121 or control vector (CMV). Twenty-four hr after transfection, cells were treated with PP2 (10 µM), H89 (10 µM) or CPT-cAMP (250 µM) for 30 min, or serum deprived (SD) for eighteen hr and harvested. ATP accumulation was evaluated as described in the “Experimental Procedures”. The data are expressed as fold increase over the control (CMV) that was set as 1 and represent the mean ± S.E.M. of four independent experiments. B. HEK293 cells were transiently transfected with siRNA control vector, or with the vector targeting AKAP121 (siRNA_AKAP121) or a scrambled sequence (siRNA_scrambled). Cells were harvested 24 and 48 hr following transfection. Cell lysates were sequentially immunoblotted with anti-AKAP121 and anti-tubulin antibodies. C. Assay of oxidative ATP synthesis in cells from B harvested at forty-eight hr from transfection. The data represent fold increase over basal value from control cells (siRNA vector) that was set as 1 and are expressed as S.E.M. of three independent experiments. D. Model of AKAP121 effects on oxidative phosphorylation and mitochondrial membrane potential.
References


figure 1
Figure 2
figure 3
Figure 6

Panel A: Images (a-f) showing fluorescence microscopy images with red fluorescence indicating the location of the protein of interest.

Panel B: Bar graph showing the fold increase in ΔΨm under different treatment conditions. The graph includes conditions for CMV, AKAP121, Hypoxia, and SD, with + and - indicating presence or absence of each condition, respectively. The bars are marked with asterisks (*) and double asterisks (**) indicating statistical significance.
Figure 7

Panel A: Bar graph showing ATP levels (Fold increase) under different conditions.

Panel B: Graphs showing AKAP121 protein levels (arbitrary units) over 24h and 48h.

Panel C: Bar graph showing ATP levels (Fold increase) under different siRNA conditions.

Panel D: Diagram illustrating the relationship between oxidative phosphorylation, AKAP121, and ATP.