Essential Role of A-kinase Anchor Protein 121 for cAMP Signaling to Mitochondria*

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A-kinase anchor proteins (AKAPs) immobilize and concentrate protein kinase A (PKA) isoforms at specific subcellular compartments. Intracellular targeting of PKA holoenzyme elicits rapid and efficient phosphorylation of target proteins, thereby increasing sensitivity of downstream effectors to cAMP action. AKAP121 targets PKA to the cytoplasmic surface of mitochondria. Here we show that conditional expression of AKAP121 in PC12 cells selectively enhances cAMP-PKA signaling to mitochondria. AKAP121 induction stimulates PKA-dependent phosphorylation of the proapoptotic protein BAD at Ser155, inhibits release of cytochrome c from mitochondria, and protects cells from apoptosis. An AKAP121 derivative mutant that localizes on mitochondria but does not bind PKA down-regulates PKA signaling to the mitochondria and promotes apoptosis. These findings indicate that PKA anchored by AKAP121 transduces cAMP signals to the mitochondria, and it may play an important role in mitochondrial physiology.

Binding of extracellular ligand to G-protein-coupled receptors at the cell membrane activates adenylate cyclase and increases cAMP levels at discrete points along the membrane. cAMP binds the regulatory (R) subunits of protein kinase A (PKA),1 dissociating the holoenzyme and releasing free catalytic subunit (C-PKA). Phosphorylation of nuclear and cytoplasmic substrates by PKA controls multiple cell functions (1–7).

PKA is concentrated in particulate membranes and cellular organelles through interaction with a family of distinct but functionally homologous A-kinase anchor proteins (AKAPs) (8–11). Although the preferred ligand is RII/PKAII, several AKAPs also bind RI/PKAI (12–14). AKAPs enhance the efficiency of cAMP signal-transducing pathways by localizing PKA near sites of cAMP generation or at PKA targets (15–26).

S-AKAP84 and AKAP121 derive from alternatively spliced products of the same gene. They are expressed in the male germ cell lineage as well as in several other tissues (27–31). Hormones that activate the cAMP-PKA pathway induce accumulation of S-AKAP84/AKAP121 transcripts and protein (28). This suggests a positive feedback loop between membrane-generated signals and downstream effector molecules of cAMP-PKA signals. All splice variants share the same 525-amino acid NH₂-terminal core, which includes the anchoring domain and the R-binding domain but diverge significantly at the COOH terminus. The first 30 NH₂-terminal residues mediate the targeting of S-AKAP84/AKAP121 to the outer membrane of mitochondria, both in male germ cells and in transfected heterologous cells (27, 30). However, other localization sites have been observed. n-AKAP, an alternative splice product of S-AKAP84 carrying additional NH₂-terminal residues, colocalizes with both mitochondria and endoplasmic reticulum (32, 33). Furthermore, S-AKAP84/AKAP121 also interacts with microtubules and associates with mitochondria in interphase and mitotic spindles during metaphase transition (34). This suggests that the same anchor protein might focus cAMP-PKA signaling to distinct subcellular compartments in a cell cycle-dependent manner.

Its location on the outer surface of mitochondria implies a role for AKAP121 in cAMP-mediated reactions at or within mitochondria. Mitochondria are the seat of a number of major cellular functions, including essential pathways of intermediary metabolism, amino acid biosynthesis, fatty acid oxidation, steroid metabolism, apoptosis, and oxidative energy metabolism (35). Several of these functions are constitutive, whereas others are finely regulated at the post-translational level. BAD is a BH3-proapoptotic Bcl-2 family member that acts at a key nodal point in the mitochondrial apoptotic pathway. Unphosphorylated BAD binds and inactivates antiapoptotic Bcl-2 homologs. This allows release of cytochrome c from mitochondria and consequent activation of the apoptotic pathway (36–41). Phosphorylation by PKA blocks BAD association with BCL-2 and inhibits apoptosis (42–45). Previous observations suggested a role of mitochondria-mounted PKA in the inhibition of apoptosis (46). Treatment with a synthetic peptide spanning the RII-binding domain of thyroid PKA (HT-31) decreased BAD phosphorylation at Ser112 and increased apoptosis of FL12.5 cells. However, it was not clear whether BAD phosphorylation necessitates PKA anchored to mitochondria by AKAP121 or whether it can be carried out by PKA anchored in other membrane compartments (46). To further analyze this mechanism and investigate the role of AKAP121 in the cAMP signaling to the mitochondria, we have established a cond-

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† These authors contributed equally to this work.

‡ These authors contributed equally to this work.

§ These authors contributed equally to this work.

¶ To whom correspondence should be addressed. Tel.: 39-081-7463614; Fax: 39-081-7463252; E-mail: feliciel@unina.it.

The abbreviations used are: PKA, cAMP-dependent protein kinase; AKAP, A-kinase anchor protein; CAT, chloramphenicol acetyl transferase; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; CRE, cAMP-response element; CREB, CRE-binding protein; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3’,5’-cyclic monophosphate; NGF, nerve growth factor; dox, doxycyclin; TPA, 12-O-tetradecanoylphorbol-13-acetate; PIPES, 1,4-piperazinediethanesulfonic acid; RSV, Rous sarcoma virus.

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tional PC12 cell line in which the expression of AKAP121 is regulated. AKAP121 induction stimulates BAD phosphorylation at Ser155 and increases cell survival. Conversely, expression of an AKAP121 mutant that binds to mitochondria but does not anchor PKA activates the mitochondrial caspase pathway and provokes apoptosis.

MATERIALS AND METHODS

Antibodies—Anti-caspase-3 was purchased from Pharmingen; anti-caspase-9, anti-BAD, and anti-phospho-specific BAD antibodies were purchased from Cell Signaling; anti-phospho-ERK, anti-ERK, anti-p21/Waf, anti-RII, and anti-cytochrome c antibodies were purchased from Santa Cruz Biotechnology, Inc.; and anti-manganese superoxide dismutase were purchased from Bender MedSystems. Anti-RII and anti-AKAP121 polyclonal antibodies were purchased from Santa Cruz Biotechnology, Inc.; CREB and anti-phosphoCREB antibodies were purchased from Upstate Biotechnology Inc.

Cell Culture—PC12 wild-type or PC12-tet off (Clontech) cells were maintained in DMEM supplemented with 10% heat-inactivated horse serum, 5% fetal bovine serum, 1 mM glutamine, and 1% penicillin-streptomycin at 37 °C and 5% CO2. PC12-tet off cells stably expressing the doxycyclin-regulated transcription factor (tTA) were grown in the presence of G-418 (100 μg/ml, Invitrogen). Cells were cultured on poly-l-lysine-coated plates. SK-N-BE neuroblastoma cells (American Type Culture Collection (ATCC)) were maintained in RPMI 1640 medium (Invitrogen) containing 16% fetal bovine serum, 100 μg/ml glutamine, 100 μg/ml streptomycin, and 100 units/ml penicillin in 5% CO2 at 37 °C.

Plasmid and Transfections—AKAP121 cDNA was excised from pCEP4-AKAP121 vector using SacII/BamHI restriction enzymes. AKAP121 cDNA was subcloned into pTRE-vector (Clontech) predigested with the same restriction enzymes. Mutant AKAP121 (AKAP121m) was generated by using the QuikChange™ site-directed mutagenesis kit (Stratagene). Oligonucleotide primers containing point mutations are as follows: forward, 5’-GCT GCC TTC CAG CCC ATC TCC CAG GTG ATC CCG GAA GCA ACT-3’; reverse, 5’-AGT TGC TTC CGG GAT CAC CTG GGA GAT GGG CTG GAA GGC AGC-3’. Amplification products were sequenced, and cDNA vectors encoding wild-type or mutant AKAP121 were prepared and purified with Qiagen Plasmid Maxi kit tips columns (Qiagen, Chatworth, CA) and stably transfected in PC12-Tet-off cells (Clontech) using the Lipofectin procedure (Lipofectin reagent, Invitrogen). Positive clones were selected in complete medium containing G-418 (100 μg/ml), hygromycin (200 μg/ml), and doxycyclin (10 ng/ml). Removal of doxycyclin for 2 days induces accumulation of significant amounts of AKAP121. Transient transfections using CRE-CAT or pBD-Elk1/GAL4-CAT (Stratagene) vectors were performed using the calcium phosphate procedure (47). CRE-CAT expression is enhanced by phosphorylated CREB (15, 18). pBD-Elk1 is a fusion protein between the activation domain of Elk1, a substrate of MAPK, and the DNA-binding domain of the yeast GAL4 transcription factor. GAL-CAT is a reporter plasmid carrying CAT downstream to a GAL-4 synthetic promoter. The promoter contains five tandem repeats of GAL4-binding sites. Transfection efficiency was analyzed by cotransfecting RSV-β-galactosidase vector. 24 h after transfection, the cells were washed twice with phosphate-buffered saline and serum-starved overnight. Drug concentrations were: CPT-cAMP (50 or 250 μM),

Fig. 1. Inhibition of apoptosis by cAMP-PKA signaling. A, apoptosis induced by serum deprivation of neuroblastoma cells (SK-N-BE). B, immunoblot analysis for caspase-3 on total proteins from growing (0) or serum-deprived neuroblastoma cells. C, apoptosis induced by serum deprivation of neuroblastoma cells (SK-N-BE) ± CPT-cAMP (100 μM). As shown in D, total extracts from control or serum-deprived neuroblastoma cells ± CPT-cAMP were immunoblotted with anti-caspase-3 antibody. E, apoptosis induced by serum deprivation of PC12 cells ± CPT-cAMP. As shown in F, CPT-cAMP-treated PC12 cells were serum-deprived for 48 h in the presence or absence of the PKA inhibitor, H89 (5 μM), and monitored for apoptosis. Data are expressed as the mean ± S.E. of 3–6 independent experiments made in duplicate.
Apopoptin and Fluorescent-activated Cell Sorting (FACS)—Apopoptin was analyzed by double staining with propidium iodide and annexin (Apopoptin detection kit, Medical and Biological Laboratories). Briefly, cells were harvested at indicated times after treatment, washed twice with 1× PBS, and incubated for 10 min with propidium iodide (50 ng/ml in 1× PBS, Sigma) and annexin. After three washes with PBS, the cells were analyzed by fluorescence microscopy using an Axioscope microscope IX70 (Olympus) or by FACS analysis. For microscopy, apoptoptin was quantified by scoring the percentage of cells stained with propidium iodide (red) and annexin (green) in the adherent cell population. To avoid unbiased counting, plates were coded, and the cells scored blind without knowledge of the treatment performed. Four to six independent experiments made in triplicate were performed for each treatment. For FACS analysis, cells were harvested in 1× PBS containing trypsin and 20 mM EDTA. 3 × 10^6 cells were resuspended in PBS, fixed with cold 100% ethanol, and treated with RNase-DNase-free enzyme (50 μg/ml). Cells were stained with propidium iodide (50 μg/ml) and annexin in a dark room for 20 min and analyzed by flow cytometry using a BD Biosciences FACScan apparatus.

Immunoblot Analyses—Cells were homogenized in lysis buffer (20 mM Tris-HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 0.05% Tween 20, 0.02% sodium azide) containing protease inhibitors (5 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin, and 0.5 mM phenylmethylsulfonyl fluoride) and phosphatase inhibitor (10 mM sodium fluoride, 20 mM β-glycerol-phosphate). Total lysate (0.1 mg) was cleared by centrifugation at 15,000 × g for 15 min, resolved by SDS-PAGE gel electrophoresis, and immunoblotted as described previously (34). Chemiluminescent (ECL) signals were quantified by scanning densitometry (Amersham Biosciences). Cytosolic fractions for anti-cytochrome were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). Proteins were detected with anti-RII or anti-manganese superoxide dismutase antibodies (ABNov, American Biotechnologies). Protein samples were size-fractioned by 8% SDS-PAGE gel as described above. Resolved polypeptides were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). RII probes were labeled with ^32^P]ATP and PKA catalytic subunit (1 unit) (Sigma), as described previously (30).

Immunofluorescence—PC12 cells were rinsed in 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 1× PBS containing 0.1 mg/ml bovine serum albumin for 1 h at room temperature. Double immunofluorescence was carried out with the following antibodies: anti-superoxide dismutase monoclonal antibody (MnSOD) and anti-AKAP121 (dilution 1/1000) or RII (dilution 1/50) polyclonal antibodies. Fluorescein- or rhodamine-tagged anti-rabbit and anti-mouse IgG (Technoclone) were used. Cytosolic and mitochondrial fractions were prepared as described (27).

Immunofluorescence for RII Binding Activity—Protein samples were size-fractioned by 8% SDS-PAGE gel as described above. Resolved polypeptides were transferred to polyvinylidene difluoride membranes (Immobilon P, Millipore). RII probes were labeled with ^32^P]ATP and PKA catalytic subunit (1 unit) (Sigma), as described previously (30).

RESULTS

cAMP-PKA Signaling Suppresses Apoptosis Induced by Serum Deprivation—To analyze the role of PKA in cell survival, we investigated the effects of a cAMP analogue in neuronal cells deprived of growth factors. In human neuroblastoma (SK-N-BE) cells, prolonged serum starvation triggers the apoptotic program that includes stress-activated protein kinases and caspases (48, 49). Cells were grown to subconfluence, starved in medium containing 0.1% serum, and harvested at different times. The percentage of apoptotic cells was determined by fluorescence microscopy (see “Materials and Methods”). As shown in Fig. 1A, serum deprivation induced a time-dependent increase in cell death. As a second monitor of apoptosis, we followed the accumulation of p17, the cleaved active caspase-3 fragment. The kinetics of caspase-3 activity roughly coincided with cellular apoptosis (Fig. 1B). Activation of the cAMP-PKA signal transduction pathway can suppress or promote apoptosis, depending on cell type (50–55). As shown in Fig. 1, A and D, treatment with CPT-cAMP, a potent cAMP analogue, increased survival of serum-deprived SK-N-BE cells and inhibited caspase-3 activation. The protective effects of PKA activation toward trophic factor withdrawal were also demonstrated in the PC12 pheochromocytoma cell line (Fig. 1, E and F).

Conditional Expression of AKAP121 Enhances PKA Targeting to Mitochondria—We wished to determine the role of AKAP
in transmitting cAMP signals to mitochondria. Accordingly, we used the tet-off inducible system to regulate expression of AKAP121, an AKAP that binds and targets PKA to the cytoplasmic surface of mitochondria (Fig. 2A). Doxycyclin down-regulates transcription of a target gene in this system by inactivating the tetA transcription factor. We isolated several PC12 stable transfectants (PC-A121) that expressed AKAP121 when cultured in the absence of doxycycline for 48 h, as shown by RII overlay (Fig. 2B, left panel). Doxycyclin down-regulated AKAP121 accumulation in PC-A121 clones number 3 and 11 to the levels of PC12 controls, as demonstrated by immunoblot analysis with anti-AKAP121 antibody (Fig. 2B, right panel). Expression of the transgene was reversible since readdition of doxycycline to the medium reduced AKAP121 concentrations (data not shown). AKAP121 expressed in PC-A121 localized principally on mitochondria, as shown by double labeling with antibody to manganese superoxide dismutase, a protein that selectively accumulates in mitochondria (Fig. 2C) (56). AKAP121 accumulation coincided with increased targeting of RII subunit to mitochondria, as demonstrated by immunoblot analysis of proteins extracted from partially purified mitochondria (Fig. 2D). The total cellular content of RII was unaffected by AKAP121 expression (Fig. 2D).

**Expression of AKAP121 Protects PC12 Cells Against Apoptosis**—We then assessed the biological effects of AKAP121 accumulation and increased association of PKA with mitochondria. First, we determined the growth rates of PC12 and PC-A121 cells. As shown in Fig. 3A, cells expressing AKAP121 increased more rapidly than controls over a 96-h period. AKAP121 expression did not accelerate the cell cycle since FACS analysis and [3H]thymidine incorporation showed no significant difference in the number of cells in G1, S, or G2/M when compared with controls (data not shown). We thus considered the possibility that AKAP121 might enhance cell viability. We measured the effects of AKAP121 on sensitivity to trophic factor deprivation. Cells were grown to semiconfluence and then serum-starved and harvested at different times. Fig. 3B shows that...
control cultures (PC12 + dox and PC-A121 + dox) became apoptotic more rapidly than the experimental culture (PC-A121 + dox). We conclude that AKAP121 expression protects cells against apoptosis induced by serum deprivation.

To demonstrate that AKAP121 acted through PKA, these experiments were repeated in the presence of the PKA inhibitor, H89. As shown in Fig. 3C, H89 abrogated the enhancement of survival by AKAP121 in serum-deprived cells. Parallel experiments were performed using hydrogen peroxide (H2O2) as a proapoptotic stimulus. About 30% of PC12 and PC-A121 + dox cells treated with H2O2 (200 μM) became apoptotic 1 day after treatment. In contrast, 20% of cells expressing AKAP121 (PC-A121 + dox) showed H2O2-induced apoptosis (Fig. 3D).

The release of cytochrome c from mitochondria is a critical step in the activation of downstream effectors of the apoptotic pathway. The binding of released cytochrome c to Apaf-1 induces the formation of the “apoptosome” complex and the sequential activation of the caspase cascade (38). As shown in Fig. 3E and F, deprivation of trophic factors in PC-A121 + dox cells induced a time-dependent translocation of cytochrome c from mitochondria to cytosol. Expression of AKAP121 (PC-A121 + dox) delayed cytochrome c release (Fig. 3, see 6 and 24 h). Under these conditions, activation of pro-caspase-3 was partly inhibited by AKAP121 expression (data not shown).

AKAP121 Selectively Increases PKA-dependent Phosphorylation of Endogenous BAD at Ser155—These data indicate that the AKAP121-PKA pathway mediates, at least in part, the protective effects of cAMP on cell survival. Based on previous reports, we thought it likely that the downstream effector of PKA was likely to be BAD. BAD, a proapoptotic protein, binds and inactivates Bcl, an antiapoptotic protein located on the outer mitochondrial membrane. Phosphorylation by PKA at Ser155 blocks association of BAD with Bcl (45). To determine the phosphorylation pattern of BAD, total proteins isolated from control or cAMP-treated cells were size-fractionated by denaturing gel electrophoresis and immunoblotted using specific antibodies to BAD phosphorylated at Ser112, Ser136, or Ser155. As shown in Fig. 4A, growing PC-A121 cells contain significant amounts of BAD phosphorylated at one or more of these three sites. After 6 h of serum deprivation, little or no phosphorylated BAD could be detected. Addition of CPT-cAMP 24 h after serum deprivation increased phosphorylation of BAD at Ser155. Phosphorylation could be detected at 15 min and was more extensive at 30 min. Expression of AKAP121 (PC-AKAP121 + dox) stimulated basal and cAMP-induced phosphorylation of BAD at Ser155 at both time points. Under these conditions, phosphorylation of BAD at Ser136 and Ser112 was undetectable, even after a 60-min exposure of cells to CPT-cAMP (Fig. 4, A and B).
We next asked whether expression of AKAP121 modulates BAD phosphorylation induced by other signaling pathways. Using the phospho-BAD specific antibodies described above, we performed immunoblot analysis on total proteins from serum-deprived PC-A121 cells stimulated with neurotrophin (NGF), phorbol ester (TPA), or CPT-cAMP (Fig. 4, C–E). NGF stimulated Ser155 and Ser112 phosphorylation 2-fold in both control cells (H11001 dox) and in cells that expressed AKAP121 (H11002 dox). TPA enhanced Ser112 phosphorylation but had no effect on Ser155. As shown above, CTP-cAMP induced phosphorylation of Ser155 but not Ser112, and this was enhanced by AKAP121 expression. AKAP121 enhancement of BAD Ser155 phosphorylation was detectable even at very low concentrations of CPT-cAMP (50 μM). As expected, CTP-cAMP stimulation of BAD phosphorylation was sensitive to the PKA inhibitor, H89. Ser136 phosphorylation was unaffected by any of the stimuli applied (data not shown).

AKAP121 specifically stimulated phosphorylation of mitochondrial PKA substrates. Fig. 5A shows that the transient increase in phospho-CREB in cells treated with cAMP was unaffected by AKAP121 induction during CPT-cAMP exposure (Fig. 5B), nor did AKAP121 affect NGF-dependent phosphorylation of ERK and activation of the MAPK signaling pathway (Fig. 5, C and D) (55, 57). Furthermore, stimulation of the MAPK signaling pathway by cAMP, as shown by ERK phosphorylation, was likewise independent of AKAP121 (Fig. 5E). These data indicate that expression of AKAP121 selectively up-regulates PKA signaling to the mitochondria without affecting the rate or magnitude of PKA-dependent or MAPK-dependent signaling to the nucleus.

**Fig. 5. AKAP121 does not influence CREB- and MAPK-dependent signaling.** As shown in A, serum-deprived PC-A121 ± dox cells were treated with CPT-cAMP (200 μM) for the indicated times, harvested, and lysed. Total proteins were immunoblotted with either anti-phospho-Ser133 of CREB or anti-CREB antibodies. P-CREB, phospho-CREB. As shown in B, PC-A121 ± dox cells were transiently transfected with a CRE-CAT reporter cDNA vector, serum-deprived for 24 h, and treated with CPT-cAMP (200 μM) for the indicated times. CAT activity is expressed as relative units and represents a mean ± S.E. of three independent experiments. C, immunoblot analysis of total proteins extracted from serum-starved or NGF-stimulated PC-A121 ± dox cells by using anti-phospho-ERK or anti-ERK antibody. P-ERK, phospho-ERK. As shown in D, PC-A121 ± dox cells were transiently co-transfected with pBD-Elk1 and Gal-CAT cDNA vectors, serum-deprived overnight, and stimulated with NGF (100 ng/ml). CAT activity is expressed as relative units and represents a mean ± S.E. of three independent experiments. E, immunoblot analysis of total proteins extracted from serum-starved or cAMP-stimulated PC-A121 ± dox cells by using anti-phospho-ERK or anti-ERK antibody.
growing the cells for 48 h in the absence of doxycyclin. Total cellular proteins were then extracted and assayed for AKAP121 by immunoblot and RII overlay analyses. As shown in Fig. 6A, wild-type and mutant AKAP121 accumulate to comparable levels after doxycyclin removal. As predicted, the affinity of AKAP121m for RII was significantly lower than wild-type AKAP121. The mutant protein remains associated with mitochondria (Fig. 6B). Expression of the mutant protein provokes the movement of PKA from mitochondria to the cytosol without significantly altering the total concentration of the kinase (Fig. 6C and D).

The phenotype of PC12 cells that express AKAP121m is shown in Fig. 7. Expression of AKAP121m correlates with increased apoptosis, as shown by reduced cell viability and activation of mitochondrial pro-caspase 9, and the extent of apoptosis is directly related to the amount of AKAP121m expressed (Fig. 7A). The proapoptotic effects of AKAP121m are evident both in growing cells (Fig. 7B) and in cells deprived of serum (Fig. 7, C–E). Furthermore, AKAP121m impedes cAMP-dependent phosphorylation of endogenous BAD at Ser155 (Fig. 7F). The data indicate that AKAP121m protein acts in a dominant-negative fashion by displacing AKAP121/PKA from mitochondria and down-regulating cAMP signaling to these organelles.

**DISCUSSION**

PC12 cells deprived of serum and trophic factors undergo apoptosis (48, 49). Activation of cAMP-PKA signaling prevents apoptosis and induces their differentiation toward neuronal cells (50–52, 59–61). We have sought to identify the PKA targets involved in this response. Note that PKA phosphorylates and modulates a great variety of cellular substrates localized in distinct cellular compartments. We report here that reversible phosphorylation of PKA substrates on or within mitochondria inhibits apoptosis. This has been achieved by establishing a PC12 line (PC-A121) that conditionally expresses AKAP121, a scaffold protein that anchors PKA to the outer membrane of mitochondria (27–29). AKAP121 induction in PC-A121 cells stimulates translocation of PKA to mitochondria. When the induced cells are treated with cAMP, there is enhanced phosphorylation of BAD at Ser155. Phosphorylation of BAD correlates with inhibition of cytochrome c release from mitochondria and reduced apoptosis. Our system is uniquely suited to analyze the effects of PKA and cAMP on mitochondrial physiology and apoptosis: 1) expression of AKAP121 is efficient, reversible, and temporally modulated; 2) AKAP121 increases PKA targeting to mitochondria without affecting the total concentration of cellular PKA holoenzyme; 3) AKAP121 facilitates PKA-cAMP signaling to mitochondria without affecting cAMP signaling to the nucleus or activation of MAPK signaling by cAMP or NGF. Conversely, a mutant of AKAP121 that does not bind PKA but localizes on mitochondria (AKAP121m) acts as dominant-negative. Induction of AKAP121m activates mitochondrial caspase-9 and promotes apoptosis, even in the presence of trophic factors. The mutant protein displaces endogenous AKAP121-PKA complexes from mitochondria sites, thus impairing physiological flux of cAMP signals from cell membrane to organelles. In particular, cAMP-dependent phosphorylation of endogenous BAD at Ser155 is down-regulated. A similar mechanism has been postulated for the β-adrenergic receptor where expression of an AKAP79 mutant, which does not bind PKA but still associates with the receptor, down-regulates PKA-dependent phosphorylation of the receptor (26). The use of such proline-
derivative AKAP mutants represents a novel and useful approach to dissect and selectively manipulate signaling pathways traveling from cell membrane to target organelles.

Biochemical and genetic studies indicate that Ser155 of BAD is the PKA high affinity site (43–45). However, most of these studies were performed supplying BAD as a substrate. BAD phosphorylation was measured with recombinant protein in vitro or expressing exogenous BAD in vivo. In this work, we have explored site-specific phosphorylation of endogenous BAD following activation of distinct signaling pathways. We found that PKA specifically phosphorylates BAD at Ser155 in intact cells. This effect is potentiated by AKAP121 and inhibited by AKAP121m. Ser 112, another potential phosphorylation site, was efficiently phosphorylated after activation of the MAPK or protein kinase C signaling pathways. No phosphorylation of Ser136 was observed under any of our experimental conditions (43). Different signals converge to inactivate BAD through phosphorylation at various serine resides (42–45). The specificity of the responding serines and the extent to which they are modified may be a critical element to discriminate the pathway activated and the intensity of the signal. In this respect, BAD may be similar to other key signaling molecules where many pathways converge, such as the cyclin-dependent kinase (CDK) inhibitor p27 (62).

Our studies demonstrate that AKAP121-PKA complexes play a unique role in mediating cAMP signaling to mitochondria.

The cAMP pathway influences mitochondrial physiology at multiple points, and AKAP121 appears to be an important multifaceted mediator of these effects. For example, we recently found that AKAP121 binds the 3′-untranslated region of mRNA encoding mitochondrial proteins and that this interaction is stimulated by PKA phosphorylation of AKAP121.2 Thus AKAP121 assembles protein kinases, mRNA, and possibly protein phosphatases on the mitochondrial surface in proximity to heterogeneous PKA substrates and other macromolecules critical for mitochondrial function(s).

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