Expression of A Kinase Anchor Protein 121 Is Regulated by Hormones in Thyroid and Testicular Germ Cells

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Distinct A Kinase Anchor Proteins (AKAPs) immobilize and concentrate protein kinase A II (PKAII) isoforms at specific intracellular locations. AKAP121 binds and targets PKAIIα to the cytoplasmic surface of mitochondria. Mechanisms that control expression of this mitochondrial AKAP are unknown.

We have cloned cDNA for rat AKAP121 and show that AKAP121 protein expression is regulated by thyroid stimulating hormone (TSH) and cAMP. Differentiated thyroid cells (TL5) accumulate AKAP121 upon incubation with TSH or a cAMP analog. Levels of total and newly synthesized AKAP121 mRNA also increased after treatment. AKAP121 mRNA accumulated in the presence of cycloheximide, suggesting that transcription of the anchor protein gene is directly controlled by cAMP and PKA. AKAP121 is induced with similar kinetics when an unrelated, spermatocyte-derived cell line (GC-2) is incubated with 8-chlorophenylthio-cAMP. Thus, AKAP121 concentration may be controlled by hormones that activate adenylate cyclase. This mode of regulation could provide a general mechanism for (a) enhancing the sensitivity of distal organelles to cAMP and PKA, (b) shifting the focus of cAMP-mediated signaling from cytoplasm to organelles.

Signaling mediated by cAMP and activation of protein kinase A (PKA) plays an essential role in the regulation of many important cellular activities, including motility, metabolism, differentiation, synaptic transmission, ion channel activities, growth, and coordination of gene transcription (1–3). The distinctive characteristics of the PKA holoenzymes are largely determined by the structure and properties of their R subunits. Unlike PKAI, which is typically cytosolic, PKAII (α and β) are often targeted to certain subcellular locations by specific anchor proteins (AKAPs) (4–20). Localization of PKAII isoforms are thought to promote specialized aspects of cAMP-activated signal transduction (4, 5, 21). The tethering of PKAII holoenzymes in specific intracellular microenvironments also influences accessibility to upstream and downstream effector molecules of cAMP action (22–24). Intracellular targeting of PKAII isoforms has been linked to cAMP-dependent gene transcription in differentiated and non-differentiated cells. In thyroid cells, displacement of immobilized PKAII from perinuclear sites to the cytoplasm impaired cAMP-regulated transcription of the thyroglobulin gene (25). Overexpression of AKAP75 and C subunit in variant PC12 cells defective in cAMP signaling restored cAMP-activated gene transcription, whereas overexpression of either protein alone was ineffective (26). We recently demonstrated that the assembly of AKAP75/PKAI complexes in the cortical cytoskeleton of HEK293 cells enhanced the propagation of cAMP signals to the nucleus, as shown by increased CREB/CRE-controlled gene transcription (27). These data suggest the possibility that cells could control their sensitivity to hormones and/or developmental factors that activate adenylate cyclase by regulating the content and distribution of specific AKAPs. S-AKAP84 and its splice variant AKAP121, anchor PKAIIα to the cytoplasmic surface of mitochondria. The anchoring of PKAIIα may be involved in the translocation of mitochondria to the site of cytoskeleton assembly in male germ cells (28, 29).

Enrichment and localization of C subunits of PKAIIα in proximity of target molecules on mitochondria/cytoskeleton may regulate the phosphorylation of regulator/effecter proteins involved in the dynamic reorganization of the flagellar cytoskeleton. This ultimately may modulate the motility and/or the fertilization capacity of spermatozoa (28, 29). Disruption of the targeting of PKAII in mammalian sperm may result in a reduction in sperm motility (30). Accumulation of S-AKAP84 and its cognate mRNA are developmentally regulated during sperm development. The anchor protein is expressed de novo during late spermiogenesis, and this is coincident with the maximal expression and subsequent anchoring of RIIα and RIIβ subunits (28).

An important, but still open, question is how hormones and growth or developmental factors promote or regulate expression of AKAPs. Are cAMP, PKAs, and PKA-dependent protein phosphorylation involved in the regulation of the location and
cAMP Increases the Expression of A Kinase Anchor Protein121

EXPERIMENTAL PROCEDURES

Cell Lines—TL5 cells (FRTL5) are a line of epithelial cells derived from normal rat thyroid. Cells were cultured in Coon's modified F-12 medium supplemented with 5% calf serum and a mixture of 6 hormones: TSH (10 nM), insulin (10 μg/ml), hydrocortisone (10 nM), somatostatin (10 ng/ml), transferrin (5 μg/ml), and glycyl-L-histidyl-L-lysine acetate (10 ng/ml) (31). Where indicated, the cells were incubated in serum- and hormone-free Coon's modified F-12 medium supplemented with 0.1% bovine serum albumin.

GC2 (GC2 spd (ts)) cells were generously provided by J. L. Millan and M. C. Hofmann, La Jolla Cancer Research Foundation, La Jolla, CA. GC2 cells were derived from primary mouse preleptotene spermatocytes by stable co-transfection with transgenes encoding SV40 large T antigen and a temperature-sensitive variant of the p53 transcriptional regulator protein (39). Immortalized GC2 cells exhibit morphological and biochemical properties characteristic of developing spermatids when they are grown at temperatures that permit low (37 °C) or high (32 °C) level expression of p53. Growth at 37 °C enables continuous proliferation of GC2 cells, whereas incubation at 32 °C results in withdrawal from the cell cycle and cell death after ~10 passages (39). For studies described in this paper, GC2 cells were grown at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum.

Screening of cDNA Libraries—A rat thyroid expression cDNA library was prepared in the bacteriophage λ Hybri ZAP (Stratagene) using poly(A) RNA from cultured thyroid PC-C13 cells as a template. The library was screened using 32P-labeled RIIβ as a probe by the procedure of Bregman et al. (6) to identify β-galactosidase fusion proteins that bind RII. Two recombinant phage clones containing overlapping cDNA sequences were isolated from 7 × 10^9 plaques. The extreme 5' end of rat AKAP121 was isolated by employing a Taq DNA polymerase chain reaction as described under “Results.” Full-length AKAP121 cDNA was sequenced by the dideoxynucleotide chain termination procedure of Sanger et al. (37).

Preparation of Protein Extracts and Immunoblot Analysis—Fractionation of cell extracts and immunoblot analysis were performed as described previously (25).

Assay for RIIβ Binding Activity—Ligand blot analysis (overlay) was carried out as described (6, 25). The Ht-31 peptide sequence is as follows: Asp-Leu-Ile-Glu-Glu-Ala-Ala-Ser-Arg-Ile-Val-Aep-Ala-Val-Ile-Glu-Glu-Val-Lys-Ala-Tyr-amine (24).

Northern Gel Analysis—Cells were grown to semiconfluence, deprived of serum and hormones for three days, and then treated for the

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Identification and Cloning of Rat AKAP121 cDNA—In rat TL5 thyroid cells, targeting of PKA regulates accumulation of the catalytic subunit of PKA (C) in the nucleus, CREB phosphorylation, and cAMP-induced gene transcription (25). To identify thyroid AKAPs, we used 3P-labeled RIIβ as a probe in an overlay binding assay (6). A 120-kDa RII-binding protein was identified in particulate fractions from rat thyroid and TL5 cells (Fig. 1A, left panel). Treatment with a peptide, Ht-31, which contains a domain that binds RII (24), competitively blocked RIIβ binding to the thyroid AKAP (Fig. 1A, right panel). We then screened a rat thyroid expression cDNA library with 3P-labeled RIIβ (see “Experimental Procedures”). Several cDNAs that encode a homolog of a previously identified AKAP, mouse AKAP121 (24), were isolated. Two overlapping cDNA clones coding for 3' region of rat AKAP121 were further characterized by sequencing. By using oligonucleotide primers for the 5' end of mouse AKAP121 (29) and the 3' end of the rat cDNA, we employed a polymerase chain reaction on a thyroid cDNA template. The resulting full-length rat AKAP121 cDNA encodes a protein composed of 854 amino acids. Alignment of the sequence of rat AKAP121 with mouse AKAP121 and S-AKAP84, and human S-AKAP84 (28, 29) revealed extensive regions of homology. The RII tethering domains of rat (residues 303–322) and mouse (residues 306–325) AKAP121 are 80% identical. Moreover, large aliphatic amino acids that govern the high affinity binding of RII (4, 5) appear at identical positions in both proteins. The N-terminal mitochondrial targeting do-

RESULTS

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indicated times with medium alone or with medium containing the indicated drugs. Northern gel analysis was performed as described (27). Relative amounts of AKAP121 mRNA were determined by scanning densitometry (Molecular Dynamics).

Nuclear Run-on Assay—Elongation rates of nascent transcripts were assayed as described by Quinn et al. (38). Briefly, 2 × 106 TL5 cells were deprived of serum and hormones for three days in F-12 medium containing 0.1% bovine serum albumin and then treated with 1 mM 8-chloro-AMP (CPT-cAMP) for 2 h. Cells were harvested in phosphate-buffered saline and lysed at 4 °C for 5 min in lysis buffer (10 mM NaCl, 3 mM MgCl2, 0.5% Nonidet P-40, 10 mM Tris-Cl, pH 8.3). Nuclei were pelleted (500 g, 5 min) and washed once in cold reaction buffer (50 mM Tris-HCl, pH 8.3, 5 mM MgCl2, 300 mM KCl, 40% glycerol, and 0.5 mM each UTP, GTP, and CTP). The nuclear pellet was resuspended in reaction buffer (cold reaction buffer plus 150 μCi [α-32P]ATP, 3000 Ci/mmol) and incubated for 30 min at 30 °C. Reactions were stopped by adding 40 units of RNase-free DNase and incubation at room temperature for 30 min. The reaction mixture was diluted 5-fold with TE (10 mM Tris, 0.5 mM EDTA) and incubated with proteinase K (25 g/ml) at 37 °C for 30 min. RNA was extracted with phenol:CHCl3:isoamyl alcohol (25:24:1, v/v) and precipitated with isopropanol. The pellet was washed with 70% ethanol, air dried, and resuspended in TE. Plasmids containing or lacking AKAP121 cDNA were applied on N-Hybond membranes using a Life Technologies, Inc. slot blot manifold. Each plasmid (20 μg of DNA) was resuspended in 6× SSC and applied to presoaked filters (6× SSC) by vacuum. DNAs were UV-cross-linked to the filters (Stratalinker™, Stratagene). Filters were prehybridized for 4 h and hybridized with 32P-labeled transcripts for 3 days at 42 °C in 40% formamide, 5× SSC, 2× Denhardt’s solution, 0.1% SDS, 100 μg/ml salmon sperm DNA, 25 μg/ml yeast tRNA. Next, filters were washed once for 15 min at room temperature in 2× SSC, 0.5% SDS, and twice for 15 min at 55 °C in 1× SSC, 0.1% SDS. Filters were air dried and analyzed by autoradiography.

Fig. 3. TSH and cAMP promote the rapid accumulation of AKAP121 in TL5 cells. TSH, CPT-cAMP, insulin, or TPA were added to the medium of serum-deprived TL5 cells at indicated concentrations. Cells were harvested at indicated times after stimulation. In panel A, total cell-proteins (120 μg) (see “Experimental Procedures”) were fractionated on a denaturing gel (8% polyacrylamide) and then immunoblotted with anti-mouse AKAP121 serum. The lower part of the gel was probed with anti-MAPK antibodies as a control. A representative set of autoradiograms is shown. In panel B, the relative abundance of AKAP121 was quantified by scanning densitometry. Levels of AKAP121 are presented as arbitrary densitometric units (ADU) and represent a mean ± S.E. from three independent experiments that yielded similar results. In panel C, TL5 cells were deprived of TSH for three days and then stimulated with the indicated concentration of CPT-cAMP. AKAP121 expression was assayed by immunoblotting as described above. Panel D shows an immunoblot analysis of extracts (100 μg protein) from control cells and cells treated with insulin or TPA. Cells were harvested at indicated times (h). The autoradiogram was overexposed (1 min) relative to panels A and C.
main (residues 1–30) and KH domains (residues 565–613), previously identified in the mouse homolog, are also highly conserved in rat AKAP121 (residues 1–30 and residues 562–610), respectively (29). Coimmunoprecipitation experiments with antibodies directed against mouse AKAP121 and RII-binding assays confirmed that the major thyroid RII-binding protein is AKAP121 (data not shown). Northern blot analysis detected expression of a 4.3-kbp AKAP121 mRNA in several tissues including: thyroid, brain, liver, and heart (data not shown).

**TSH and cAMP Elicit the Accumulation of AKAP121 in TL5 Cells**—Thyroid cell growth and differentiation are dependent upon TSH. Binding of ligand to the plasma membrane TSH receptor activates Gs-coupled adenylase cyclase and increases the intracellular levels of cAMP. Physiological effects of cAMP are mediated by activation of PKA (31–35). To determine whether AKAP121 is regulated by hormone, we performed RII-binding and Western blot analyses on “particulate” (P) and “cytosolic” (S) proteins extracted from TL5 cells. Cells were incubated in standard, serum-free medium containing 0.1% bovine serum albumin for three days and then were treated with a mixture of TSH, insulin, and serum. Serum-starved TL5 cells contain low levels of AKAP121 polypeptide and RII-binding activity (Fig. 2, A and B) (29). Incubation with serum, insulin, and TSH increased AKAP121 levels in a time-dependent fashion. Accumulation of the anchor protein was first evident 12 h post-stimulus, and the level increased over the next 24 h. AKAP121 was recovered in the particulate fraction. To
A representative set of autoradiograms is shown. Run-on experiments were performed as described under “Experimental Procedures.” A representative set of autoradiograms is shown.

Further define the signaling pathway governing induction of AKAP121, TL5 cells were treated with TSH or with CPT-cAMP, a stable and potent cAMP analog. TSH or CPT-cAMP elicited substantial increases in AKAP121 content (Fig. 3, A and B). CPT-cAMP induced AKAP121 12–15-fold, whereas a 5–6-fold increase in anchor protein ensued after treatment with TSH. AKAP121 accumulation was first detected 3 h post-stimulus and reached a maximal value at 6 h with both CPT-cAMP and TSH. Induction of AKAP121 by CPT-cAMP was also observed in TL5 cells incubated for 3 days in medium containing 5% calf serum and 5H (no TSH; see “Experimental Procedures”). Thus, the presence of serum growth factors and hormones had little effect on CPT-cAMP-mediated accumulation of AKAP121 in TL5 cells (Fig. 3C). Treatment with insulin or the phorbol ester TPA, failed to elevate AKAP121 content in TL5 cells (Fig. 3D). Because TSH and a cAMP analog induce AKAP121 accumulation, it appears that PKA activation regulates the concentration of AKAP121.

TSH/cAMP Increases AKAP121 mRNA Levels and Gene Transcription in TL5 Cells—Quiescent TL5 cells contain a very low level of AKAP121 mRNA (Fig. 4A). TSH treatment increased AKAP121 mRNA abundance within 6 h; the AKAP121 mRNA concentration peaked 12 h post-stimulus, reaching a concentration of 6–7-fold above the initial level. The level of the AKAP121 transcript then declined over the next 12 h. CPT-cAMP provoked a more rapid and robust response. A substantial increase in AKAP121 mRNA was detected 3 h after exposure of cells to CPT-cAMP. AKAP121 mRNA concentration peaked at 6 h (10–15-fold over basal levels) and then declined slowly during the succeeding 18 h. Accumulation of AKAP121 mRNA was evident over a wide range of TSH or CPT-cAMP concentrations (Fig. 4, C and D). To determine whether accumulation of AKAP121 transcripts by cAMP requires new protein synthesis, we added CPT-cAMP in the presence of the protein translation inhibitor, cycloheximide. Treatment with CPT-cAMP for the indicated time periods. Total cell proteins (50 μg) were immunoblotted with antibodies directed against mouse AKAP121 after denaturing electrophoresis. In panel A, representative autoradiograms are shown. Filters were exposed for 8 s. In panel B, the relative abundance of AKAP121 was quantified by scanning densitometry. Levels of AKAP121 are presented as arbitrary densitometric units (ADU) and represent a mean ± S.E. from four independent experiments that yielded similar results.

Induction of AKAP121 by cAMP in Spermatocyte-derived GC2 Cells—Thyroid cells depend on TSH for both growth and differentiation. To investigate the generality of the effects of PKA/cAMP on AKAP121 expression and separate the induction process from growth and differentiation, we assayed spermatocytes (GC2 cells) that were immortalized by SV40 T antigen (39). Basal expression of AKAP121 in GC2 cells is higher than in TL5 cells (Fig. 6). Nevertheless, CPT-cAMP promoted a 3–4-fold increase in AKAP121 protein accumulation in GC2 (Fig. 6, A and B). AKAP121 levels increased 3 h post-stimulus and reached a maximal value 6–9 h after exposure to CPT-cAMP. No further increase was detected at 24 h post-stimulus (data not shown). Thus, PKA activation increases the abundance of AKAP121 in distinct cell systems. This suggests a potential general mechanism whereby hormonal regulation of specific anchor proteins may modulate the sensitivity of cell compartments to the cAMP signal transduction pathway.

**Discussion**

PKA isoenzymes are tethered and targeted to discrete intracellular microenvironments by AKAPs (4, 5). Co-localization of PKA with adenylate cyclase may reduce effects of cAMP diffusion and augment PKA signaling when modest physiological levels of hormones encounter target cells (27). The proximity of released catalytic PKA subunits to certain target effectors also influences the propagation of cAMP signals in cells and focuses signals on specific effector molecules (22–27). A centrally important aspect of cAMP-PKA signaling is that spatially regulated accumulation of AKAPs, achieved by altering the intra-
cellular distribution of PKAI isoenzymes, could influence cAMP signal dissemination to distal organelles. In granulosa cells, follicle-stimulating hormone induces a cytosolic translocation of PKAI coincident with an increase in the levels of an uncharacterized cytosolic RII-binding protein (36). In spermatozoids, S-AKAP84 accumulates at the outer membrane of mitochondria at a late phase of development, the beginning of nuclear condensation and tail elongation (28). At present, molecular mechanisms underlying developmentally or hormone-regulated accumulation of anchor proteins are poorly understood.

In differentiated thyroid cells, the binding of TSH to its cognate transmembrane receptor activates adenylyl cyclase, which, in turn, increases the level of intracellular cAMP. Reversible phosphorylation of nuclear transacting factors by PKA is linked to transcriptional regulation of several genes (33–35). We have discovered that AKAP121 expression is regulated by the cAMP signal transduction pathway in thyroid-derived TL5 cells. TSH and/or cAMP induce AKAP121 gene transcription and accumulation of AKAP121 mRNA and anchor protein. The transcriptional induction of AKAP121 mRNA is unaffected by cycloheximide, suggesting the presence of target sequences for transcriptional induction of AKAP121 mRNA is unaffected by and accumulation of AKAP121 mRNA and anchor protein. The occurrence of a positive feed-back loop between membrane- and nuclear condensation and tail elongation (28). At present, molecular mechanisms underlying developmentally or hormone-regulated accumulation of anchor proteins are poorly understood.

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