A-kinase-interacting protein localizes protein kinase A in the nucleus

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The genetic variability and covalent modifications associated with the amino terminus of the protein kinase A (PKA) catalytic (C) subunit suggest that it may contribute to protein–protein interactions and/or localization. By using a yeast two-hybrid screen, we identified a PKA-interacting protein (AKIP1) that binds to the amino terminus (residues 1–39) of the C subunit of PKA. The interaction was localized to the A helix (residues 14–39) of the C subunit and to the carboxyl terminus of AKIP1. AKIP1 thus defines the amino-terminal A helix of PKA as a protein interaction motif. In normal breast (Hs 578 Bst) and HeLa cells, AKIP1 is present in the nucleus as speckles. A nuclear localization signal (Arg-14 and Arg-15) was identified. Upon stimulation with forskolin, HeLa cells expressing AKIP1 accumulated higher levels of the endogenous C subunit in the nucleus. Deletion of the carboxyl terminus of AKIP1 or overexpression of residues 1–39 of the C subunit abolished nuclear localization of the activated endogenous C subunit. Thus, AKIP1 defines a PKA-interacting protein that can contribute to localization by a mechanism that is distinct from A-kinase anchoring proteins that interact with the regulatory subunits.

nuclear retention | speckles

Signaling molecules work as teams and on scaffolds to elicit biological responses on external stimuli. cAMP-dependent protein kinase [protein kinase A (PKA)] is ubiquitous in mammalian cells (1) and is targeted and compartmentalized to specific sites in the cell by a family of proteins that are collectively referred to as A-kinase-anchoring proteins (AKAPs). By binding to the amino-terminal dimerization/docking domain of the regulatory subunits, these diverse proteins assemble PKA and related signaling molecules in close proximity to substrates (2–5).

Although the catalytic (C) subunit is anchored by the regulatory subunits to AKAPs, it could localize independently of them. Although the C subunit of PKA is highly conserved in eukaryotic cells, the amino terminus preceding the conserved kinase core exhibits variability (6–9). This variability is in distinct contrast to the carboxyl terminus (residues 300–350), which is remarkably conserved from yeast to humans. The amino terminus is also subject to a variety of posttranslational modifications, such as myristylation, phosphorylation, and deamidation. These modifications are capable of contributing to localization, further suggesting a unique role for this region. The C subunit is myristylated at the amino-terminal Gly except in some isoforms (9, 10). This myristylation allows the type II holoenzyme to associate with lipid vesicles through the C subunit (11). It has also been shown that deamidation at Asn-2 causes the C subunit to localize in the nucleus (12). There is a PKA phosphorylation site at Ser-10 (13); however, the role of this phosphorylation in localization is currently unknown. These modifications and probably the amino terminus itself may play an essential role in the targeting and localization of the C subunit. Additionally, the amino terminus contributes to isoform diversity by introducing multiple splice variants (14), and there easily could be more that have yet to be recognized. This combined evidence suggests that this region can contribute in unique ways to localization, targeting, and protein–protein interactions, although the biological significance of this segment is unknown.

Although many of the substrates that are phosphorylated by PKA are in the vicinity of the AKAPs (15), nuclear signaling requires the translocation of the free C subunit to the nucleus. Upon production of the second messenger cAMP, the C subunit is released from the regulatory subunits that are in many cases tethered to AKAPs. The C subunit then regulates the expression of various genes by phosphorylating cAMP-response element-binding protein transcription factor (16). Free C subunit can be exported from the nucleus by a mechanism involving the protein kinase inhibitor (PKI). Binding to PKI causes not only the inactivation of the C subunit but also the export of the complex out of the nucleus (17). However, little is known about how the C subunit is retained in the nucleus to carry out gene transcription.

To determine whether there are proteins that bind specifically to the amino terminus of the C subunit and affect its localization, we used the amino terminus (residues 1–39) of the C subunit, including the A helix, as bait in a yeast two-hybrid screen. We describe here an A-kinase-interacting protein (AKIP1) that binds to this region. AKIP1 is highly conserved among mammals, contains several motifs, and is localized to the nucleus. This protein most likely contributes to the integration of signaling by PKA by retaining the C subunit in the nucleus.

Methods
cDNA Yeast Two-Hybrid Screening. Matchmaker GAL4 Two-hybrid System 3 (Clontech) was used to screen for novel proteins that interact with the first 39 residues of the C subunit of PKA. The amino-terminal region of human Cα subunit cloned into a pGBT7 vector was used as a bait to screen a pretransformed human fetal brain library. Approximately a million transformants were screened on a nutritionally selective medium deficient in adenine, His, Leu, and Trp. Thirteen clones positive for α-galactosidase were selected. After rescoring the clones, four remained positive and were sequenced and analyzed by searching BLAST. One of the clones encoded AKIP*. This construct was subcloned into pGEX-4T.

Northern Blotting. Human and mouse tissue blots (MTN blots) were purchased from Clontech. AKIP* DNA was labeled by using the AlkPhos Direct system (Amersham Pharmacia Biosciences) according to the manufacturer’s protocol. After prehybridizing the blots with the hybridization buffer for 15 min, the probe was added and the blot was hybridized overnight at 55°C. The blot was washed twice for 10 min at 55°C with gentle agitation. The blots were further washed at room temperature for 5 min and detected by using the CDP-Star detection reagent (Amersham Pharmacia Biosciences).

Abbreviations: AKIP, A-kinase-interacting protein; C, catalytic; AKAP: A-kinase-anchoring protein; PKI, protein kinase inhibitor; PKA, protein kinase A; YFP, yellow fluorescent protein; NLS, nuclear localization site; His-C, His-tagged C; HA, hemagglutinin.

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RT-PCR and PCR. Tissues were excised from adult male BALB/c mice, and 5 ng was used to isolate mRNA by using a polyA mRNA isolation kit (Qiagen, Valencia, CA). Approximately 20 pg of mRNA was used to set up the reverse transcriptase reaction with a one-step RT-PCR kit (Invitrogen). The RT-PCR was carried out by using the mouse AKIP1-specific (sense, 5'ATG GAC CAA CTG TTT GGC GGC CGC-3'; anti-sense, 5'AGG GAA GAC CAG GTC CAC-3') and GAPDH primers. Reverse transcription was carried out at 50°C for 30 min followed by 40 cycles of 94°C for 15 sec, 55°C for 30 sec, and 68°C for 1 min. For RNA isolation from cells, cells were cultured in 60-mm dishes, and total RNA was isolated by using the Qiagen RNeasy kit according to manufacturer's instructions. Approximately 100 pg of RNA was used in RT-PCR experiments. The PCR was carried out on the Marathon-Ready human fetal brain cDNA library (Clontech) by using the Pfx platinum polymerase (Invitrogen) out on the Marathon-Ready human fetal brain cDNA library according to manufacturer's protocol. The proteins were eluted in 30 µl of elution buffer, and 20 µl was loaded onto SDS/PAGE and subjected to Western blot analysis. The primary antibodies for AKIP1 were used at a dilution of 1:5,000, and the primary antibodies for the C subunit were used at a dilution of 1:10,000.

Immunoprecipitation. pcDNA3-AKIP1a and the hemagglutinin (HA)-tagged C subunit (21) were transfected into 293T cells by using the FuGENE Transfection reagent. After 9–12 h of transfection, the cells were collected and subjected to immunoprecipitation by using the Catch and Release kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's protocol. The proteins were eluted in 30 µl of elution buffer, and 20 µl was loaded onto SDS/PAGE and subjected to Western blot analysis. The primary antibodies for AKIP1a were used at a dilution of 1:5,000, and the primary antibodies for the C subunit were used at a dilution of 1:10,000.

Immunofluorescence. HeLa cells were plated on 35-mm glass-bottom microwell dishes and grown to 50% confluency to obtain asynchronous cells. The cells were then either fixed in 4% paraformaldehyde for 15 min or transfected with the FuGENE reagent for 10–12 h. The cells were washed twice with PBS and then either taken directly for imaging the YFP constructs or fixed and probed with the anti-AKIP1 antibody (1:50 dilution) and the anti-C subunit antibodies (1:150 dilution). For cells that were probed with the antibodies, cells were immunolabeled as mentioned in ref. 22. Donkey anti-rabbit FITC and donkey anti-mouse Cy5 were used as secondary antibodies at a dilution of 1:100.

For studies on retention of the endogenous C subunit, the cells were treated 10–12 h after transfection with 25 µM forskolin and 25 µM inhibitor 3-isobutyl-1-methylxanthine for 0, 5, 15, and 45 min. The cells were then fixed and stained for endogenous C subunit and visualized. The transfected cells were counted and averaged over at least three independent experiments.

Human normal mammary gland Hs 578 Bst cells were grown in Dulbecco's modified media supplemented with 10% FBS and 30 mg/ml epidermal growth factor. For immunostaining, the cells were grown on the microwell dishes, fixed, and stained with the affinity-purified antibodies. The anti-PKII and anti-C subunit antibodies were used at a dilution of 1:100 and probed with the anti-AKIP1 antibody (1:50 dilution). The anti-C subunit antibodies (1:150 dilution). For cells that were transfected with the AKIP1 constructs, the cells were immunolabeled as mentioned in ref. 22. Donkey anti-rabbit FITC and donkey anti-mouse Cy5 were used as secondary antibodies at a dilution of 1:100.

BrUTP Incorporation Assay. The BrUTP incorporation assay was performed by using Hs 578 Bst as described with slight modifications (23) and by Roche Applied Biosciences. Briefly, Hs 578 Bst cells were plated on microwell dishes for 2 days. FuGENE transfection reagent was added to 0.1 volume of 10 mM BrUTP (Sigma–Aldrich) and incubated for 15 min at room temperature. The cells were washed with PBS, and 100 µl of the transfection mix was added to the plates and incubated at 4°C for 15 min. The cells were pulse-chased at 37°C for 15 min with Dulbecco's modified media supplemented with 10% FBS. The cells were fixed and stained with mouse anti-BrUrd antibody at a dilution of 1:50. AKIP1 was detected by using anti-AKIP1 antibody.

Results

Identification, Isolation, and Analysis of AKIP1. Because the amino-terminal region of the various isoforms of the C subunit of PKA are highly variable, we performed a yeast two-hybrid screen by using the first 39 residues of the Cα subunit as bait against a human fetal brain library. One of the clones obtained was designated as AKIP*. When searched in the BLASTN database of GenBank, AKIP* matched with the Pro-rich breast cancer-associated protein BCA3, which has no known function (24). AKIP1 has 6 exons with the coding region starting at the second exon (Fig. L4). There are three putative splice variants, the
full-length AKIP1a; AKIP1b where exon 3 is deleted; and AKIP1c, which lacks exons 3 and 5 (Fig. 1A). AKIP* lacked the first 36 residues at the amino terminus and the third exon. AKIP1 gene is located on human chromosome 11, and the full-length protein has 210 amino acids, whereas the splice variants have 182 and 152 amino acids, respectively (Fig. 1A).

Several putative motifs are found in AKIP1. Analysis by using the PREDICTNLS program reported a potential nuclear localization site (NLS) at the amino terminus, indicating that the protein may be nuclear. The carboxyl-terminal region of the protein is highly conserved and has a predicted PDZ class II binding site (Fig. 1B) (24, 25). There were no known conserved domains when the protein sequence of AKIP1 was scanned in the PFAM database (26). This protein has putative phosphorylation sites for PKA, PKC, casein kinase II, and Tyr kinase (Fig. 1B). AKIP1 is well conserved in mammals but less conserved across other species (25). Human AKIP1a shares 70% identity to the predicted mouse sequence. Mouse AKIP1 is located on chromosome 7 and, unlike human AKIP1, lacks splice variants. Comparisons between human and mouse sequences showed that there is a deletion of a single amino acid in the mouse sequence near the second and third exon junction and an insertion of two residues at the fourth and fifth exon boundaries (Fig. 1B).

Expression of the Human and Mouse AKIP1. Screening of a human fetal multitissue Northern blot with AKIP* showed the presence of mRNA of ~1.35 kb in heart and skeletal muscle (Fig. 2A). RT-PCR analysis showed that HEK 293 cells had products corresponding to AKIP1a, AKIP1b, and AKIP1c. In contrast, HeLa and MCF7 cells had mostly AKIP1a and AKIP1b (Fig. 2B). PCR of the clones from the human fetal brain library yielded two products, AKIP1a and AKIP1b (Fig. 2C). The mouse Northern blot showed that the RNA was present as two transcripts at ~1.35 kb that were highly expressed in the heart, liver, kidney, and testis and poorly expressed in other tissues (Fig. 2D). Similar results were obtained by RT-PCR of tissues.
The C subunit of PKA interacts with AKIP1. (A) Immunoprecipitation with the HA antibody showed that the HA-tagged C subunit specifically coprecipitated AKIP1a. Blots were probed with anti-C and anti-AKIP1 antibody. (B) GST pull down showed direct binding of the C subunit with GST-AKIP1a as analyzed by SDS-PAGE. GST was used as control. (C) AKIP1a, AKIP1b, AKIP1 (residues 1–136), and H6-C subunit were in-vitro-transcribed, translated by using [35S]Met, separated by SDS-PAGE, and analyzed by autoradiography. Ten percent of the mix was loaded to obtain the total protein (Input). Pull down of the proteins with affinity matrix showed that the H6-C subunit interacted with AKIP1a, interacted weakly with AKIP1b, and failed to interact with AKIP1 (residues 1–136). The affinity matrix alone with AKIP1a and AKIP1b was used as control. (D) The C subunit and Δ(residues 1–14) C subunit were in-vitro-translated and pulled down with GST-AKIP* and GST, separated by SDS-PAGE, and autoradiographed. The C subunit and Δ(residues 1–14) C subunit interacted with GST-AKIP*.

Interaction of AKIP1 with the C Subunit of PKA. When FLAG-tagged AKIP1a and the HA-tagged C subunit were cotransfected into HeLa cells and immunoprecipitated by using mouse anti-HA antibody, the C subunit was able to specifically bind AKIP1a (Fig. 3A). Because AKIP1a interacted with the first 39 residues of the C subunit in the yeast two-hybrid screen, GST-AKIP1a protein was used in pull-down experiments with the purified recombinant C subunit to verify this interaction in vitro (Fig. 3B).

To determine the region on AKIP1a that is required for binding to the C subunit, AKIP1b and a carboxyl-terminal deletion construct lacking the last 74 amino acids (AKIP1a, residues 1–136) were in vitro transcribed and translated and used in pull-down assays with in vitro transcribed and translated H6-C subunit. Fig. 3C shows that AKIP1a interacted with the H6-C subunit of PKA. AKIP1b interacted weakly, but AKIP1 (residues 1–136) failed to bind. Therefore, an essential site of interaction resides at the extreme carboxyl terminus, which is the most highly conserved part of the protein. Further experiments have to be done to determine the exact residues involved and the role of the third exon in this interaction.

To define the site of interaction on the C subunit, the first 14 residues of C subunit were deleted [Δ(residues 1–14) C subunit]. This protein was then in vitro translated and used in pull-down assays. As seen in Fig. 3D, Δ(residues 1–14) C subunit still interacted with GST-AKIP* and, thus, by deduction, localized the site of interaction to residues 14–39 of the C subunit.

Subcellular Distribution of AKIP1. Because this protein has not, to our knowledge, been characterized, it was imperative to determine the in vivo localization of endogenous AKIP1. RT-PCR of RNA isolated from HeLa cells had shown the presence of AKIP1 and its splice variants (Fig. 2C). Staining for the endogenous protein with anti-AKIP1 antibody showed that it was present in HeLa cell nuclei as punctuate spots (Fig. 4A). It has been reported that mRNA levels are detectable in normal breast tissues, albeit at low levels, and elevated in breast cancer tissues (24). Therefore, a normal mammary gland cell line (Hs 578 Bst) was used to determine the expression pattern of the endogenous protein. AKIP1 showed a similar punctuate distribution in the nucleus of these cells (Fig. 4A). When the antibody was preincubated with the purified GST-AKIP1a before immunostaining, the speckling was nearly abolished, thereby confirming the specificity of the antibody. The secondary antibody alone showed no background staining (Fig. 4A).

Similarly, when YFP-AKIP1a was transfected into HeLa cells, it
localized to the nucleus. There is a predicted NLS at the amino terminus of AKIP1 (15-RRSLSQRS-21) (Fig. 1B). To confirm that this site in AKIP1 is responsible for nuclear localization, a mutant was engineered where both Arg 14 and 15 were replaced with Ala (RR14,15AA AKIP1a). The mutant was then expressed as both YFP- and FLAG-tagged constructs in HeLa cells. The expressed FLAG-tagged protein was stained with the anti-AKIP1 antibody and observed by epifluorescence microscopy. Fig. 4B shows that both AKIP1a and AKIP1b were enriched in the nucleus, whereas mutation of the two consecutive Arg residues of the putative NLS led to diffused distribution of the protein.

Colocalization of Endogenous AKIP1 in Hs 578 Bst Cells. Because endogenous AKIP1 in the nucleus was present in a speckled pattern, we attempted to determine whether AKIP1 localizes to specific subnuclear structures. The nucleus contains morphologically distinct and dynamic structures that include the nucleoli, Cajal bodies, splicing speckles, Gems, and promyelocytic leukemia bodies (27, 28). Double-labeling of Hs 578 Bst with the AKIP1 and promyelocytic leukemia protein or AKIP1 and splicing factor SC35 (28, 29) antibodies did not show any overlap (data not shown). Because AKIP1 seems to be distributed in very tiny speckles, was it localized to the transcription hot spots in the cells? To address this question, Hs 578 Bst cells were pulse-labeled with BrUPT, which indicates the major sites for RNA synthesis (30), and then double-labeled with anti-BrdUr and anti-AKIP1. As shown in Fig. 5, there was extensive incorporation of BrUPT, and there was a strong suggestion of colocalization of AKIP1 and BrUPT. Double-labeling with anti-TFIIID and anti-AKIP1 antibodies showed similar results.

Because AKIP1 interacted with the C subunit, localization of these two proteins in Hs 578 Bst was also studied. Staining with an antibody raised to the C subunit showed that the protein was diffused in the nucleus, and there was partial overlap between the two proteins (Fig. 5). PKI, the highly specific inhibitor for PKA is also known to be present in the nucleus during certain stages of the cell cycle (31). Labeling with anti-PKI antibody showed that although PKI was present as distinct punctuate spots, it did not overlap with AKIP1 (Fig. 5).

Endogenous C Subunit Translocates into the Nucleus. In cells, PKA is predominantly present as an inactive holoenzyme and, upon stimulation, there is an increase in intracellular cAMP that leads to dissociation and release of the active C subunit (32). However, when HeLa cells were transfected with YFP and then treated with forskolin, which increased the intracellular pool of cAMP, the endogenous C subunit remained mainly excluded from the nucleus. There was no dramatic change in the C subunit localization even after 45 min of stimulation (data not shown). In contrast, in HeLa cells transfected with YFP-AKIP1a, there was a distinct change in the subcellular localization of the endogenous C subunit upon treatment with forskolin (Fig. 6A). At 5 min after forskolin treatment, the C subunit remained in the cytoplasm. However, by 15 min of stimulation, the C subunit localized to the nucleus in some cells, and this nuclear localization persisted even after 45 min. Approximately 50–60% of the cells that harbored YFP-AKIP1a showed the redistribution of the C subunit in contrast to 0–5% of the nontransfected cells or cells transfected with YFP. As demonstrated in Fig. 2C, AKIP (1–136) lacking the carboxyl terminus does not bind C subunit. Upon forskolin stimulation, there was 0–5% change in the localization of endogenous C subunit in cells harboring YFP-AKIP (1–136).
To determine whether the amino terminus of the C subunit was capable of interfering with the translocation of the C subunit, cells were cotransfected with YFP-AKIP1α and HA-tagged amino-terminal residues 1–39 of PKA. This region corresponds to the “haut” thir that was used to isolate AKIP1 in the two-hybrid screen. The translocation of endogenous C subunit to the nucleus in response to forskolin was blocked by HA (residues 1–39) (Fig. 6B). In an independent experiment, cells were cotransfected with the above two plasmids and stained with the anti-HA antibody to show that the construct was expressed in the majority of cells (Fig. 6B Inset).

Discussion
PKA isoforms are differentially expressed and targeted to various subcellular localizations to carry out different functions (33). The PKA isoforms are differentially expressed and targeted to various subcellular localizations. In many cell lines, such as REF52 and PC3M (human prostate cancer cell line), the C subunit migrates to the nucleus upon elevation in cAMP (36, 37). However, in HeLa cells we observed that only ~5% of cells exhibited nuclear C subunit upon stimulation with forskolin. In contrast, ~50–60% of cells overexpressing YFP-AKIP1α exhibited nuclear C subunit in response to forskolin treatment. Nuclear accumulation of C subunit was blocked when the amino terminus (1–39 residues) of the C subunit was coexpressed with YFP-AKIP1α. Deletion of the carboxyl terminus of AKIP1, which harbors the binding site for C subunit, also abolished the endogenous C subunit nuclear localization. Thus, AKIP1α contributes to retention of the C subunit in the nucleus. There was a decrease in nuclear localization of the C subunit after 2 h of forskolin stimulation (data not shown), which could be due to export of the C subunit by PKI (17). Could AKIP1 act as transient anchor for PKA? According to the anchorage-release model, there is a regulated compartmentalization of signaling molecules, such as a PKA, NF-κB, and the glucocorticoid receptor (38). AKAPs play a major role in compartmentalizing PKA outside the nucleus, but less is known about how AKIP1 is retained in the nucleus. Thus, AKIP1 provides a paradigm for C subunit retention in the nucleus.

Endogenous AKIP1 was present as speckles in the nucleus and immunofluorescence showed that these speckles colocalize partially with TFIID and with the RNA transcripts as seen by BrUTP labeling. Although remaining a strong possibility, it remains to be established whether AKIP1 is directly associated with the transcriptional complex. In summary, we have identified a transient PKA-associated protein that interacts with the amino terminus of the C subunit. Although interactions of the C subunit with other proteins, such as the NF-κB and serum amyloid factor (39, 40), have been speculated, this demonstration provides a molecular basis for such an interaction.

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