Characterization of an A-Kinase Anchoring Protein

in Human Ciliary Axonemes

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ABSTRACT

Although protein kinase A (PKA) activation is known to increase ciliary beat frequency (CBF) in humans the molecular mechanisms involved are unknown. We demonstrate that PKA is associated with ciliary axonemes where it specifically phosphorylates a 23 kD protein. Because PKA is often localized to subcellular compartments in close proximity to its substrate(s) via interactions with A-kinase anchoring proteins (AKAPs), we investigated whether an AKAP was also associated with ciliary axonemes. These studies have identified a novel AKAP that is highly enriched in airway axonemes. The mRNA for this 28 kD AKAP (AKAP28) is upregulated as primary airway cells differentiate and is specifically expressed in tissues containing cilia and/or flagella. Additionally, both western blot and immunostaining data show that AKAP28 is enriched in airway cilia. These data demonstrate that we have identified the first human axonemal AKAP, a protein that likely plays a role in the signaling necessary for efficient modulation of CBF.

INTRODUCTION

Mucociliary clearance is an innate host defense mechanism dependent on the coordinated beating of cilia lining the conducting airways. Ciliary beat is achieved by coupling the hydrolysis of ATP by dynein, the molecular motor of the axoneme, to microtubule sliding. To achieve the precise waveform and beat frequency necessary for efficient clearance, a cilium requires the cooperative action of the 200+ proteins that compose this organelle. A number of pharmacological studies have demonstrated that ciliary beat frequency (CBF) is a highly regulated process (reviewed in Wanner et al., 1996 and Satir and Sleigh, 1990); however, very little is known about the molecular events underlying these regulatory mechanisms.
The influence of cAMP on ciliary motility has been studied in a variety of organisms. Studies of classical non-mammalian systems such as Paramecium, Tetrahymena and Mytilus gill cilia show a strong correlation between increased cAMP levels, subsequent activation of PKA and increased axonemal motility (Stommel and Stephens, 1985; Hamasaki et al., 1989; Christensen et al., 2001). In mammalian airway tissues, a significant increase in CBF results when intracellular cAMP levels are artificially raised in ciliated cells (Tamaoki et al., 1989; Di Benedetto et al., 1991; Wyatt et al., 1998). This increase is blocked by the addition of kinase inhibitors, suggesting a role for both cAMP and PKA in the modulation of CBF.

In human tissue, Morse et al. have recently demonstrated that a sustained receptor-mediated increase in CBF is dependent on the production of cAMP (Morse et al., 2001). In these studies application of adenosine and its non-metabolizable analog 5’(N-ethylcarboxamido)-adenosine (NECA) to nasal explants generated a concentration-dependent, sustained increase in CBF. Application of an adenylyl cyclase inhibitor greatly diminished the NECA effect. Using additional pharmacological agents, the effects of adenosine and NECA were shown to be mediated through the A2B receptor, which couples to adenylate cyclase through Gs. Similar results were obtained in hamster oviduct where Morales et al. also demonstrated that adenosine stimulated CBF in an adenylyl cyclase dependent manner (Morales et al., 2000).

These pharmacological data suggest a role for PKA in the regulation of CBF; however, little is known about the molecular events underlying this phenomenon. We are interested in determining if the compartmentalization of signaling molecules plays a key role in the efficient modulation of human CBF. Specifically, we want to determine whether PKA is anchored in human ciliary axonemes in close proximity to a substrate whose phosphorylation state modulates CBF.
A diverse family of functionally related proteins called A-kinase anchoring proteins (AKAPs) target PKA to discrete locations within a cell (For review see Colledge and Scott, 1999). AKAPs bind to the regulatory subunit dimer of the PKA holoenzyme. Most characterized AKAPs bind to type II (RII) regulatory subunits; however, a few AKAPs are known to bind type I (RI) subunits or bind either subtype of regulatory subunit. In most instances this interaction is mediated via an amphipathic helix at the surface of the AKAP. Each AKAP also contains unique subcellular targeting information. Through a protein-protein and/or protein-lipid interaction AKAP and PKA are specifically localized to a particular organelle or membrane within the cell.

In our studies, we have found that a pool of PKA is compartmentalized in human ciliary axonemes. We have also determined the molecular identity of AKAP28, a novel AKAP that is highly enriched in airway cilia. We propose that AKAP28 anchors the PKA holoenzyme in the axoneme in close proximity to its substrate(s).

MATERIALS AND METHODS

Cell Culture: Human bronchial epithelial (HBE) cells from normal subjects or cystic fibrosis patients were obtained from excess surgical tissue under protocols approved by the University of North Carolina Institutional Review Board. Passage 1 HBE cells were grown at an air-liquid interface on semi-permeable supports as previously described (Gray et al., 1996; Bernacki et al., 1999; Zhang et al., 2002). HeLa cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS).

Isolation and Phosphorylation of Axonemes: Axonemes were isolated from well-differentiated HBE (WD-HBE) cells using a modified calcium shock method (Hastie et al., 1986; Reed et al., 2000; Zhang et al., 2002). To remove membranes, Triton X-100 was added to
a final concentration of 0.5% and samples were incubated on ice for 15-25 minutes. Cilia were re-pelleted and supernatant/membrane fraction removed. For select experiments, axonemes were resuspended in 1.0% Triton X-100 and incubated on ice for 25 minutes. All samples were immediately used or frozen on dry ice and stored at -80°C until needed.

Preparations of freshly isolated axonemes were phosphorylated in buffer containing 20 mM HEPES, pH 7.0, 1mM MgCl₂, 10 µM IBMX (3-Isobutyl-1-methylxanthine, Sigma, St. Louis, MO) and a 1X dilution of phosphatase inhibitor cocktail 1 (Sigma P2850, St. Louis, MO). Axonemes were incubated under five different reaction conditions: (1) γ-P³² labeled ATP (1 µCi, 10 mCi/mL NEN Easytides; Boston, MA) alone; (2) ATP + 10 µM cAMP (Sigma); (3) ATP, cAMP + 10 µM PKI (6-22) amide (Biomol, Plymouth Meeting, PA); (4) ATP + exogenous PKA catalytic subunit (Promega, Madison, WI); and (5) ATP, exogenous PKA catalytic subunit + PKI (6-22) amide. Reactions were incubated at 30°C for 30 minutes. Laemmeli loading dye was added to a final concentration of 1X and samples were resolved by SDS-PAGE. Gels were dried and imaged using a STORM-840 PhosphorImager (Molecular Dynamics).

**Radiolabeled RII Overlays:** Axonemal proteins (30 µg/lane) were resolved on 15% SDS-PAGE gels, transferred to nitrocellulose, and renatured in block [5% nonfat dry milk, 0.1% bovine serum albumin in 1X TBS (50mM Tris, 75mM NaCl, pH 7.5)] overnight at 4°C. Recombinant murine RIIα was phosphorylated by PKA catalytic subunit in the presence of γ-³²P labeled ATP and unincorporated label was removed using a desalting column. Renatured blots were incubated with 250,000 cpm of ³²P-RII/mL in block for four hrs at room temperature, washed in 1X TBS (5x15 min) and exposed to a phosphorimager screen overnight. For competitive peptide experiments, ³²P-RII was pre-incubated with either 1µM competitive peptide Ht31 (DLIEEAASRIVDAEVQKAAGAY), 1 µM negative control peptide Ht31PP
(DLIEEAASRPVDAVPEQVKAAGAY) or with no peptide for 30 min prior to adding probe to the renatured blot. Both Ht31 and Ht31PP were synthesized by the University of North Carolina Custom Peptide Synthesis Facility.

**Cloning of the Novel AKAP:** We screened a λZAP cDNA expression library (Stratagene, La Jolla, CA) generated from mRNA isolated from WD-HBE cultures for clones containing RII-binding proteins. Recombinant RII was purified from induced bacteria with cAMP agarose beads (Gray et al., 1997). Purified RII was biotinylated using standard methods and used as probe for non-radioactive library screens (Trotter et al., 1999). XL1 Blue MRF’ E. coli infected with λZAP phage were plated and incubated at 42° until plaques began to form, approximately 4 hrs. Nitrocellulose filters wet in 1 mM IPTG (isopropyl b-D-1-thiogalactopyranoside) were laid on plates and incubated for 5 hrs at 37° for protein collection. Filters were removed from plates, blocked in 5% nonfat dry milk, probed overnight with RII-biotin prebound to streptavidin-alkaline phosphatase, and developed using standard methods. Positive plaques were cored, tumbled and re-screened. At least three rounds of plaque purification were performed to isolate pure phage encoding RII-binding domains. Positive clones were excised from phage by in vivo excision, and inserts were sequenced by the University of North Carolina Sequencing Facility. One novel 0.3 kb cDNA sequence was isolated from the library screen. To obtain full length sequence we performed both 5’ and 3’ RACE using SMART-RACE (Clontech, Palo Alto, CA) human trachea cDNA generated from RNA according to the manufacturer’s protocol. RACE products were cloned into pTAdv (Clontech) and sequenced. Appropriate primers were designed and used to amplify full-length cDNAs of three predicted splice variants from trachea and testis. PCR products were again cloned into pTAdv and sequenced.
**Identification of the RII Binding Site:**  cDNAs encoding full-length (aa 1-197) and fragments (1-68 and 64-197) of AKAP28 were generated by PCR using the full-length cDNA of AKAP28 as template. PCR products were subcloned into the pET28c expression vector (Novagen, Madison, WI). After confirming the sequence of each insert, plasmids were transformed in to *E.coli* BL21 Codon Plus (Stratagene, La Jolla, CA) and expressed as His₆ fusion proteins. Fusion protein expression was induced overnight at 28°C and the insoluble fusion proteins were purified according to the Novagen protein purification protocol. Post-purification, fusion proteins were dialyzed into PBS and subsequently resolubilized by boiling in 1% SDS in PBS. Protein concentration was determined by a bicinchoninic acid (BCA) protein assay (Pierce Chemical Company, Rockford, IL). Equal moles of each fusion protein were resolved by SDS-PAGE, transferred to PVDF, overlayed with biotinylated RII, probed with streptavidin conjugated horseradish peroxidase (HRP) and visualized using enhanced chemiluminescent (ECL) detection.

The predicted RII-binding site was mutated in the pET28c construct encoding amino acids 1-68. Amino acid substitutions were made using the QuikChange™ XL Site-Directed Mutagenesis kit (Stratagene). After sequence confirmation of the mutagenesis, the plasmid was expressed in bacteria as described above. The soluble mutant fusion protein was purified on a nickel column according to the manufacturers instructions (Novagen).

**Heterologous Expression of AKAP28 and Co-Immunoprecipitation with PKA Subunits:** Recombinant adenovirus encoding AKAP28 was generated for heterologous expression assays. A cDNA encoding AKAP28 fused to the hemagglutin (HA) epitope was subcloned into the adenovirus shuttle vector pAdTrack-CMV (pAdTrack-CMV.HAAKAP28). Following the protocol of He et al. (He et al., 1998) the Vector Core Facility at UNC-Chapel Hill generated recombinant adenoviruses, AdTrack-CMV (expressing GFP, from pAdTrack-CMV alone) and
AdTrack-CMV-HAAKAP28 (expressing GFP and AKAP28 epitoped tagged with HA at its carboxy-terminus, from pAdTrack-CMV.HAAKAP28).

For heterologous expression, 100 mm dishes of 80% confluent HeLa cells were either mock infected or infected with AdTrack-CMV or AdTrack-CMV-HA-AKAP28. After a 2 hr incubation at 37°, cells were washed and the growth media was replaced. Infected cells were harvested 24 hrs post-infection.

For immunoprecipitation experiments, dishes of infected or mock infected cells were placed on ice and rinsed two times with ice cold PBS (50 mM NaH2PO4· H2O, 150 mM NaCl, pH 7.4). Cells were overlayed with 0.4 mL of ice cold lysis buffer (20 mM HEPES, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% IGEPAL, plus protease inhibitors) and removed by scraping. Lysates were passed through a 27 gauge needle several times and left on ice for 30 min. The resulting homogenate was centrifuged at 14,300 rcf for 10 minutes at 4° to precipitate insoluble material. Protein (300 µg) was diluted to 0.5 mL in dilution buffer (lysis buffer without IGEPAL) and incubated with 5 µg of HA 1.1 mAb (Covance, Denver, PA) or isotype matched IgG (R&D Systems, Minneapolis, MN) while tumbling overnight at 4°. Immunocomplexes were collected on protein G agarose and washed extensively with dilution buffer. Bound proteins were analyzed by western blotting using the appropriate antisera or by RII overlay.

**Northern Blot Analysis of AKAP28:** Total RNA from differentiating, code-matched human airway cultures was isolated using the RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer’s instructions. RNA samples were resolved on a 1.5% formaldehyde/agarose gel following standard methods. After electrophoresis, samples were transferred to a Nytran membrane (Schleicher & Schuell, Keene, NH) by capillary blotting and cross-linked using a Stratalinker (Stratagene). Both the time course blot and a multiple tissue expression (MTE)
array (Clontech) were probed with $^{32}$P-labeled random-primed cDNA corresponding to nucleotides 98-400 of AKAP28 using standard methods. All blots were analyzed using a STORM-840 PhosphorImager and ImageQuant software.

**Antisera Generation And Immunoblot Analysis:** Rabbit antisera directed against a GST fusion protein containing amino acids 5-105 of AKAP28 were generated by Covance Research Products. Antisera were affinity purified on a column containing His$_6$AKAP28 fusion protein and subsequently used for both western blotting and immunohistochemistry. Pre-immune sera was purified on an affi-gel blue column (Pierce Chemical Company) to remove complement proteins.

For western blots, proteins from isolated axonemes or WD-HBE whole cell lysates were resolved by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked for at least one hr in 10% non-fat dry milk and incubated in affinity purified rabbit anti-AKAP28 IgG (1 ug/mL), pre-immune IgG (1 µg/mL), affi-gel blue purified anti-EBP50/NHERF IgG (0.2 ng/mL) or commercially available antibodies specific for PKA catalytic subunit (0.1 ug/mL, Santa Cruz Biotechnology, Santa Cruz, CA), PKA type I regulatory subunit (0.1 ug/mL, Santa Cruz Biotechnology), PKA type II regulatory subunit (0.1 ug/mL, Santa Cruz Biotechnology) or type IV β tubulin (1:2000, BioGenex, San Ramon, CA). Blots were incubated overnight at 4° or at room temperature for 1 hr. Blots were washed, incubated with appropriate HRP-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA) and visualized using ECL.

**Immunohistochemistry (IHC):** Histological sections were prepared from paraffin-embedded, 4% paraformaldehyde-fixed blocks of WD-HBE cells. For the analysis of AKAP28 protein, sections were deparaffinzed and rehydrated to water. Sections were rinsed with TBS and then incubated for 30 minutes at 85°C in preheated, diluted (1.5 mL concentrated stock in 160 mL
water) antigen unmasking solution (Vector Laboratories, Burlingame, CA). Sections were washed in TBS and permeabilized in TBS with 0.3% TritonX-100 (TBST) for 30 min. Sections were blocked in 2% normal goat serum, 1% cold water fish gelatin in TBST for 30 min. Primary antibodies diluted in block (5 μg/mL) were incubated with sections overnight at 4°C. Slides were washed in TBS and processed using the Vectashield Elite ABC kit (Vector Laboratories). After final washes, sections were developed using DAB (3,3'-Diaminobenzidine tetrahydrochloride), counterstained with light green SF yellowish (Chroma-Gesellschaft), dehydrated and mounted.

RESULTS

PKA Co-Purifies with Human Ciliary Axonemes and Phosphorylates a 23 kD substrate

To determine if PKA is associated with human ciliary axonemes, we employed both kinase activity assays and western blotting techniques. We isolated axonemes from well-differentiated, human bronchial epithelial (WD-HBE) cells using a modification of the calcium shock method (Hastie et al., 1986; Reed et al., 2000; Zhang et al., 2002). As shown by the electron micrograph (Figure 1A), the 9+2 microtubule structure characteristic of the axoneme is preserved during isolation and most axonemes are free of membranes. To determine if PKA was present in our axonemal preparations, we treated isolated axonemes with [γ-32P]ATP alone or [γ-32P]ATP plus 10 μM cAMP, in order to specifically activate PKA (Figure 1B). Phosphatase inhibitors were added to each reaction to inhibit known serine/threonine phosphatases and L-isozymes of alkaline phosphatase. A protein with an apparent molecular weight of ~23 kD is specifically phosphorylated in the presence of 10 μM cAMP (lane 2), but is not detected under basal conditions (ATP alone, lane 1). The phosphorylation of the 23 kD band is significantly reduced by the addition of PKI, a PKA specific inhibitor (lane 3) (Scott et al., 1986).
Interestingly, the addition of exogenous PKA catalytic subunit results in the phosphorylation of many axonemal proteins whereas endogenous PKA specifically phosphorylates the 23 kD protein (compare lanes 2 and 4).

To determine the isoform(s) of PKA present in the axoneme, we used western blotting techniques to detect specific PKA subunits in isolated human axonemes (Figure 1C). Axonemal preparations (5 µg, lane 1) were compared to whole cell lysates from WD-HBE cultures (5 µg, lane 2). As controls to confirm the purity of the axonemes, antibodies for type IV β tubulin (β-tubIV), the axonemal specific isoform of β tubulin (Renthal et al., 1993), and EBP50/NHERF, a non-axonemal, cytosolic protein (Short et al., 1998; Mohler et al., 1999) were also used. As expected type IV β tubulin is highly enriched in the axonemal fraction and EBP50/NHERF is not detectable in isolated axonemes, but is present in whole cell lysates. The catalytic subunit of PKA (C), as well as both type II (RII) and type I (RI) subunits, are detectable by western blot in ciliary axonemes.

To demonstrate that the signal for PKA was due to the presence of the axonemes and not residual ciliary membranes, we extracted our axonemal preparation with either 0.5% or 1.0% Triton X-100 (Figure 1D). Pelleted fractions (AXO), presumably completely demembranated axonemes, and detergent extracted membranes (MEM) were compared by western blot with the PKA catalytic subunit antibody. The catalytic subunit remains tightly associated with the axoneme pellet in the presence of detergent and is not detectable in the membrane fraction. Since the regulatory subunits of PKA are generally thought to target the holoenzyme, it is likely that all PKA subunits remain with detergent extracted axonemes.

These experiments demonstrate that PKA co-purifies with demembranated human ciliary axonemes and preferentially phosphorylates a single substrate. Therefore, PKA is associated
with the axoneme as either a structural component or as a constituent that strongly adheres to the axoneme through the purification process. Additionally, PKA is likely anchored in close proximity to its preferred 23 kD substrate.

**An Axonemal RII-Binding Protein is Detectable by Overlay**

To determine how PKA is anchored within the axoneme, we assessed whether PKA-binding proteins were also contained within axonemes. In particular, we assayed for RII-binding proteins by performing overlays using recombinant RII as probe (Hausken et al., 1998). In radiolabeled RII overlay assays of isolated cilia from WD-HBE cells, we repeatedly detect two prominent RII-binding proteins (Figure 2A). Competitive inhibitor peptide Ht31, but not Ht31PP, a negative control peptide, specifically blocked RII-binding to these proteins. One of the detected proteins has an apparent molecular weight of 50 kD. This protein may be RII dimerizing with itself since RII is detectable at this molecular weight by western blot of cilia preparations (Figure 1C). The other detected RII-binding protein is 28 kD in size.

As seen with PKA (Figure 1D), the RII-binding protein adheres tightly to the axoneme and is not extractable with detergent (Figure 2B). In RII overlays comparing poorly differentiated human airway cells lacking cilia with WD-HBE cells, we find that the 28 kD RII-binding protein is not detectable in undifferentiated cells (Figure 2C), but is easily detected in differentiated cells. At longer exposures, other RII binding proteins are faintly detected in whole cell lysates (data not shown), however, the predominant proteins detected by RII overlay in WD-HBE cells are 50 and 28 kD in size. Additionally, the putative AKAP is significantly enriched in the axonemal fraction of the cell compared to whole cell lysate (Figure 2D). These data suggest that the putative AKAP specifically targets the axoneme. Since no AKAPs of 28 kD have been
previously characterized, we designed experiments to determine the molecular identity of this protein.

**Determination of Axonemal AKAP Identity**

To determine the identity of the axonemal AKAP, we screened a cDNA expression library generated from mRNA isolated from WD-HBE cultures using recombinant, biotinylated RII as probe. Three known AKAPs were identified in our screen: AKAP350, a multiply spliced AKAP targeted to the centrosome or Golgi complex of cells (Schmidt *et al.*, 1999; Takahashi *et al.*, 1999); S-AKAP84 which co-localizes with mitochondria in sperm flagella (Lin *et al.*, 1995); and AKAP220, an AKAP that is targeted to testicular peroxisomes (Lester *et al.*, 1996). We also isolated one novel sequence from the library based on its ability to bind RII. Sequence analysis revealed the insert contained ~0.3 kb of a novel cDNA with homology to a rat cDNA encoding TAKAP80, a sperm fibrous sheath protein (Mei *et al.*, 1997).

5’ and 3’ rapid amplification of cDNA ends (RACE) and analyses of available expressed sequence tag (EST) and genomic databases were utilized to identify the full-length sequence for the novel AKAP. This gene, which maps to a region of chromosome Xq24 when searched against the human genome database, appears to encode at least three splice variants (submitted to NCBI as AF514780, AF514781 and AF514782). Full-length transcripts of all three splice variants are present in both trachea and testis as determined by RT-PCR. The full-length cDNA we chose to characterize further (accession #AF514780) encodes a protein of 197 amino acids that migrates at 28 kD by SDS-PAGE (see below). Following the tradition of the AKAP community, we have named this protein AKAP28 for AKAP of 28 kD.

The novel clone found in the screening of the well-differentiated airway cell cDNA expression library has no obvious sequence homology with any previously characterized human
proteins. The protein encoded by the ORF of the AKAP cDNA does, however, share 50% identity and 67% conserved homology to a 159 amino acid segment of TAKAP80 (amino acids 340-499), a rat testis-specific, developmentally regulated AKAP present on the fibrous sheath of sperm (Figure 3) (Mei et al., 1997). The predicted RII-binding region of TAKAP80 is contained in the 159 amino acid stretch that shares significant homology to AKAP28.

**AKAP28 contains a single RII-binding site**

To map the location of the RII-binding site of AKAP28, we performed RII overlay assays on purified, recombinant His₆ fusion proteins containing amino acids 1-197 (FL), amino acids 1-68 (NT), and amino acids 64-197 (CT) of AKAP28 (Figure 4A). Both FL and NT fusion proteins bind to biotinylated RII by overlay, while the CT fusion protein does not (Figure 4B). Moreover, pre-incubation of probe with the competitive peptide HT31 blocks RII binding to His₆–FL and NT proteins while the negative control peptide HT31PP does not. Hence, RII binding to AKAP28 is specific and the binding motif is contained within the first 68 amino acids of AKAP28.

In all but one previously characterized AKAP (Diviani et al., 2000), the RII-binding motif is an amphipathic helix that binds to the RII subunit dimer. An amphipathic helix is predicted to form between amino acids 35-52 of AKAP28. Upon further analysis we found that amino acids 35-52 can be aligned with other AKAP RII-binding motifs and shares all of the 8 semi-conserved positions predicted by Vijayaraghavan et al (Vijayaraghavan et al., 1999). The binding motif is defined by:

\[ X\{L,I,V\}X3\{A,S\}X2\{L,I,V\}\{L,I,V\}X2\{L,I,V\}\{L,I,V\}X2\{A,S\}\{L,I,V\}, \]

where X is any amino acid and single letters in brackets represent the conserved amino acids that are favored at a particular position.
To provide experimental evidence that amino acids 35-52 define the RII-binding motif of AKAP28 we mutated residues 43L and 47V of the His$_6$–NT fusion protein to prolines (His$_6$–ΔP). The double mutant is similar to the substitutions made in AKAP-Lbc in order to completely abolish RII-binding (Diviani et al., 2001). We predicted that these mutations would disrupt the amphipathic helix and abolish RII binding in AKAP28. The mutated protein runs aberrantly large by SDS-PAGE and is unable to bind to RII as His$_6$–NT does (Figure 4B). These data provide evidence that AKAP28 is an AKAP that contains a single RII-binding region located between residues 35-52.

**AKAP28 Associates with PKA in HeLa Cells**

To determine if AKAP28 associates with PKA in human cells as well as binding to RII by overlay, we expressed HA-tagged AKAP28 in HeLa cells using recombinant adenovirus. Infected cells were lysed and co-immunoprepitation experiments were performed with either HA antisera or control IgG. Immune complexes from mock infected (mock), control infected (expressing GFP), and infected (expressing GFP and HA-AKAP28) cells were either overlayed with $^{32}$P-RII to detect AKAP28 or blotted to detect the presence of PKA subunits (C, RI, and RII) (Figure 4C). PKA subunits were not detectable by western in control IgG precipitants (Figure 4C, lanes 2, 4, & 6) or from mock and control infected lysates treated with HA antisera (Figure 4C, lanes 1 & 5). In contrast, both RII and C subunits of PKA associate with HA-AKAP28 as determined by western blot (Figure 4C, lane 3). RI, however, does not appear to bind HA-AKAP28 under these conditions (Figure 4C, lane 3). AKAP28 preferentially binds type II PKA when heterologously expressed in a human cervical adenocarcinoma cell line and is able to co-purify with PKA catalytic subunit. This suggests that AKAP28 functions as a type II PKA anchor within cells.
Northern Blot Analysis of AKAP28

The mRNA expression of axonemal proteins is often upregulated as ciliated cells differentiate (Andrews et al., 1996; Reed et al., 2000; Zhang et al., 2002). To determine if AKAP28 mRNA expression also follows this profile, we examined the temporal expression of AKAP28 in differentiating HBE cells. Initially after plating, the HBE cells are a monolayer of undifferentiated cells (Figure 5A). Over time, the cultures differentiate into a pseudostratified epithelium similar to native tissue. Total RNA isolated from differentiating HBE cells at 3, 10, 15 and 30 days post-plating was analyzed by northern blot using $^{32}$P-labeled, random primed AKAP28 cDNA (nts 98-400) as probe (Figure 5B). Message for AKAP28 is not readily detected at early stages of differentiation. However, AKAP28 gene expression (~0.8 kb message) is upregulated as cells differentiate and begin to display a mucociliary phenotype (days 10 & 15). The absence of AKAP28 message in undifferentiated cells and its upregulation during differentiation suggests a functional role for AKAP28 in differentiated HBE cells and is consistent with the expression pattern observed for axonemal proteins.

We also probed a human multiple tissue array to determine the tissue distribution of AKAP28 (Figure 5C). We found that AKAP28 is predominantly expressed in trachea and testis; faint message was also detected in whole adult and fetal lung. No other tissues were positive for the message. Trachea, which contains ciliated columnar cells, and testis, with both developing sperm and ciliated cells of the ductuli efferentes of the epididymis, are the only tissues on the blot that contain axoneme-based organelles. The tightly restricted pattern of AKAP28 expression suggests that this AKAP has a function specific for cells containing axonemes.

Localization and distribution of AKAP28
To determine the subcellular distribution of AKAP28, we generated rabbit polyclonal antisera directed against a GST fusion protein containing amino acids 5-105. Antisera were affinity purified and subsequently used for western blotting and tissue staining.

Proteins from isolated axonemes were analyzed with the AKAP28 antisera. Parallel lanes of a single gel transferred to PVDF membrane were either overlayed with RII or blotted with AKAP28 antisera. As seen in figure 6A, the 28 kD protein detected by RII overlay and AKAP28 as detected by western co-migrate by SDS-PAGE. An equal amount of purified pre-immune IgG does not detect a 28 kD protein. Thus, it is highly likely that the protein detected by RII overlay is AKAP28. Additionally, as with the RII overlay (Figure 2B) detergent extraction of the ciliary preparation did not remove AKAP28 from the axoneme (Figure 6B).

We used immunohistochemistry to determine the distribution of AKAP28 in WD-HBE cells (Figure 6C). Intense staining for AKAP28 is seen in the cilia of columnar cells, but absent from goblet and basal cells. Thus, AKAP28 localizes to airway cilia where it likely plays a role in anchoring PKA near its axonemal substrate.

DISCUSSION

Several pharmacological studies have identified signaling pathways involved in the regulation of ciliary beat frequency. To better understand human deficiencies in mucociliary clearance, it is necessary to delineate the molecular mechanisms underlying these pathways. Specifically, we are interested in understanding the molecular events linking the production of cAMP, the subsequent activation of PKA and the associated increase in CBF. Our studies provide the first biochemical data showing that a pool of PKA is localized within human ciliary axonemes (Figure 1). Under conditions that should inhibit known serine/theronine phosphatases and L-isozymes of alkaline phosphatase, this pool of enzyme is responsible for the specific
cAMP-dependent phosphorylation of a 23 kD substrate (Figure 1B). Additionally, compartmentalization of type II PKA in the axoneme is likely mediated via AKAP28, a novel axoneme-specific anchoring protein.

Changes in ciliary beat frequency are likely mediated via a cascade of phosphorylation/dephosphorylation events that alter the activity of axonemal proteins. In our studies of isolated human ciliary axonemes, we have identified one major 23 kD protein that is phosphorylated in a cAMP-dependent manner (Figure 1B). Parallels may be drawn to other axonemal systems. For example, Salathe et al. studied the in vitro cAMP-dependent phosphorylation of ovine axonemes and found that a protein with an apparent molecular weight of 26 kD was consistently phosphorylated (Salathe et al., 1993). Similar results were obtained in studies of Paramecium (29 kD), mussel gill (27 kD) and Tetrahymena (34 kD) axonemes (Stommel and Stephens, 1985; Hamasaki et al., 1989; Chilcote and Johnson, 1990).

Further studies in Paramecium revealed that p29, the axonemal protein consistently phosphorylated in this species, co-purified with the 22S outer arm dynein (Bonini and Nelson, 1990; Hamasaki et al., 1991). Additionally, cAMP-dependent phosphorylation of p29 has been linked to both increased swimming velocity of permeabilized Paramecia and increased velocity of microtubules gliding across surfaces coated with dynein isolated from phosphorylated axonemes (Barkalow et al., 1994). In studies of Tetrahymena, it has been demonstrated that its PKA substrate, p34 also co-purifies with 22S dynein (Christensen et al., 2001). In a number of biochemical tests, Christensen et al. elegantly demonstrate that p34 is the functional ortholog of the Paramecium 22S regulatory light chain p29. Functionally it seems the regulation of ciliary motility by PKA involves the phosphorylation of a dynein regulatory light chain. To date, however, the primary sequence of this regulatory light chain has not been determined.
Both type I and type II isoforms of PKA are detectable by western blot in human ciliary axonemes (Figure 1C). While the kinetic and enzymatic specificities of the three catalytic subunits (Ca, β, γ) of PKA are indistinguishable, different regulatory subunits (RI and RII) display different cAMP binding affinities and are differentially located within the cell. RI (α, β) is mainly cytoplasmic; however, at times it is compartmentalized. For example, RI holoenzyme is tightly bound to plasma membranes of erythrocytes and is enriched at the neuromuscular junction (Rubin et al., 1972; Imaizumi-Scherrer et al., 1996). Additionally, RI is associated with activated B cell receptors and accumulates at the “cap” site when lymphocytes are stimulated with anti-CD3 antibodies (Skalhegg et al., 1994; Levy et al., 1996). In contrast, RII (α, β) often tightly associates with cellular structures and organelles. No studies determining the specific roles of RI and RII within the axoneme have been conducted. Electron microscopic studies of axonemes with RI and RII specific antibodies will be necessary to determine where each PKA isotype is localized within this structure. Moreover, we are uncertain if type I PKA and type II PKA serve overlapping or distinct roles in the axoneme. Pharmacological experiments utilizing isotype specific agonists will be necessary to delineate the roles of RI and RII in the regulation of CBF.

In this study, we have focused on identifying the protein responsible for anchoring type II PKA in the axoneme. We have determined the molecular identity of AKAP28, an AKAP highly enriched in airway cilia. Due to the insolubility of the axoneme, we are unable to co-immunoprecipitate AKAP28 with its binding partners from primary airway cells. Using a heterologous system, however, we have been able to co-immunoprecipitate epitope-tagged AKAP28 with both RII and PKA catalytic subunit from HeLa cells (Figure 4C). We are unable to detect RI in the same immune complexes. This experimental evidence for preferential binding
to RII is in agreement with the RI/RII binding predictions made by Miki and Eddy in Ala- and Val-scanning mutagenesis studies of AKAP binding domains (Miki and Eddy, 1999). These data show that AKAP28 preferentially binds type II PKA and likely serves as its anchor in the axoneme.

Since type I PKA also co-purifies with axonemes (Figure 1C), RI-specific anchoring proteins are also likely to be present. Unlike RII-binding proteins, RI-specific AKAPs are not detectable by overlay (Miki and Eddy, 1999). Identifying RI-binding AKAPs in the axoneme is difficult as the insolubility of this organelle prohibits the co-immunoprecipitation experiments necessary for the detection of RI-binding proteins.

Sequence analysis indicates that AKAP28 is most closely related to a rat testis-specific, developmentally regulated AKAP, TAKAP-80 (Mei et al., 1997). A 159 amino acid stretch (residues 340-499) at the carboxy-terminus of TAKAP-80 shares 50% identity and 67% conserved homology with amino acids 33-192 of AKAP28 (Figure 3). In contrast, no similarity is found in the N-termini of these proteins. According to Mei et al., TAKAP-80 is exclusively expressed in testis where mRNA expression is upregulated during a time interval that corresponds to the initiation of spermiogenesis (Mei et al., 1997). In the studies by Mei et al., total RNA from whole lung was probed; however, no message was detected. Either TAKAP-80 is not present in rat lung or the percentage of RNA from ciliated cells was too low to detect TAKAP-80 mRNA expression on the blot. Since tracheal RNA was not tested, we are uncertain whether TAKAP-80 is specifically expressed in rat testis or whether TAKAP-80 is the rat ortholog of human AKAP28. TAKAP-80 protein was detected by western blot in fibrous sheath preparations from rat sperm, but no staining of rat tissues has been published. In contrast, our staining of airway sections with AKAP28 specific antibodies indicates that AKAP28 is enriched
in airway cilia. It is also detectable by western blot in axoneme preparations. We have not determined the localization of AKAP28 in testis and sperm. Since many components of ciliary and flagellar axonemes are conserved, it is likely that a protein targeted to ciliary axonemes would also localize to the axoneme of sperm flagella. Another possibility, however, is that AKAP28 contains both fibrous sheath and axoneme targeting information. In ciliated airway cells, where fibrous sheaths do not exist, the protein would be compartmentalized in the axoneme. In sperm, AKAP28 could preferentially target the fibrous sheath. Further study of AKAP28 in both sperm and testis is needed to resolve these questions.

In addition to AKAP28, we have identified two smaller splice variants expressed from the same gene (NCBI accession #AF514781 and AF514782). The predicted splice variants were initially detected by 5' and 3' RACE of human tracheal cDNA and by the examination of EST databases. Their presence was confirmed in both trachea and testis by RT-PCR and the subsequent sequencing of amplified products. All three splice variants of this gene are detected by RT-PCR in both tissues. To date, we have not detected the predicted AKAP28 isoforms by western blot of axonemes or in whole cell lysates of WD-HBE cells. Either the predicted splice forms are never translated into protein or are expressed at low levels that currently prohibit detection.

Axonemal AKAPs have also been detected in *Chlamydomonas* flagella. However, unlike RII overlays of human ciliary axonemes where RII-binding proteins of 50 and 28 kD are detected (Figure 2A), AKAPs of 240 kD and 97 kD are detected in *Chlamydomonas* flagellar axonemes (Gaillard *et al.*, 2001). Using genetic mutants of *Chlamydomonas*, Gaillard et al. found that AKAP240 is a component of the central pair apparatus and that AKAP97 is part of the radial spoke stalk. The molecular identity of AKAP240 has not been determined; however,
AKAP97 has been defined molecularly as radial spoke protein 3 (RSP3). To date the human ortholog of RSP3 has not been characterized. While both human and *Chlamydomonas* axoneme systems contain AKAPs, based on size, the AKAPs readily detected in each system do not appear to be the same proteins. One possibility is that human RSP3 may be a lower affinity AKAP than AKAP28 or found at a lower concentration than AKAP28 in human axonemal preparations. Also, this difference could be due to species variations or in differences between flagella and cilia. In *Chlamydomonas*, agents that reduce cAMP concentrations or inhibit the activity of PKA increase axoneme motility (Hasegawa *et al.*, 1987). The inhibitory effect of cAMP on *Chlamydomonas* axonemes is opposite of the stimulatory effect seen in other organisms. Moreover, unlike the relatively small phosphoproteins detected in other axoneme-based systems in response to cAMP, two proteins of greater than 270 kD are phosphorylated in response to cAMP in *Chlamydomonas* (Hasegawa *et al.*, 1987). While many axonemal proteins are conserved across species, specific signaling pathways and the compartmentalization of particular components vary.

In summary, we have presented the first biochemical data demonstrating the co-purification of PKA with human ciliary axonemes. Additionally, we have identified the first human A-kinase anchoring protein targeted to the axoneme. We propose that AKAP28 localizes PKA to a position in the axoneme where it is able to readily interact with its substrate. This compartmentalization likely plays a role in the regulation of outer dynein arm activity and the control of ciliary beat frequency. Future experiments will be designed to examine the physiological role AKAP28 plays in human cilia.
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REFERENCES


FIGURE LEGENDS

Figure 1. Detection of PKA in human airway cilia (A) A representative electronmicrograph of isolated human airway axonemes. (B) Freshly isolated axonemes were incubated under the following conditions: lane 1, γ-P32 ATP alone; lane 2, γ-P32 ATP + 10 µM cAMP; lane 3, γ-P32 ATP + 10 µM cAMP + 10 µM PKI; lane 4, γ-P32 ATP + catalytic subunit of PKA; and lane 5, γ-P32 ATP + catalytic subunit of PKA + 10 µM PKI. Treated samples were resolved by SDS-PAGE and phosphorimaged. (C) Isolated axonemes (5 µg, lane 1) and WD-HBE cell lysates (5 µg, lane 2 or 40 µg, lane 3) were resolved by 10% SDS-PAGE and transferred to a PVDF membrane. Membranes were probed with antibodies specific for either the catalytic subunit of PKA (C); the type II regulatory subunit of PKA (RII); the type I regulatory subunit of PKA (RI); β-tubulin type IV, the axonemal specific isoform of β-tubulin; or EBP50/NHERF, a non-axonemal, cytosolic protein. Membranes were washed and incubated with appropriate secondary antibodies conjugated to HRP. Westerns were developed by enhanced chemiluminescence. All data are representative of multiple independent experiments. (D) Isolated axonemes were incubated with either 0.5% or 1.0% Triton X-100 for 25 minutes on ice. Detergent extracted axonemes (AXO) and membrane fractions (MEM) were resolved by SDS-PAGE, transferred to immobilon and probed with an antibody specific for the catalytic subunit of PKA. The membrane fraction is 2/3 the amount of axoneme fraction loaded.

Figure 2. Detection of RII-binding proteins in airway axonemes (A) Isolated axonemes (30 µg/lane) were resolved by 15% SDS-PAGE, transferred to nitrocellose and blocked overnight in
5% non-fat dry milk, 0.1% BSA in 1X TBS. Membranes were incubated with $^{32}$P-labeled RII probe either pre-blocked with no peptide, 1 μM competitive Ht31, or 1 μM negative control Ht31PP. (B) Isolated axonemes were incubated with either 0.5% or 1.0% Triton X-100 for 25 minutes on ice. Detergent extracted axonemes (AXO) and membrane fractions (MEM) were resolved by SDS-PAGE, transferred to PVDF, blocked and incubated with $^{32}$P-labeled RII probe. The membrane fraction is 2/3 the amount of axoneme fraction loaded.

(C) Equal protein from whole cell lysates of undifferentiated (UNDIF) and well-differentiated (DIF) human primary airway cells were overlayed with radiolabeled RII. (D) Equal protein from isolated axonemes and differentiated airway cell lysates were also overlayed with radiolabeled RII. All data are representative of multiple independent experiments.

**Figure 3. Alignment of AKAP28 with TAKAP-80** The amino acid sequences of AKAP28 and TAKAP-80 were aligned by Clustal W. Identical residues are shaded in black, while similar charged residues are shaded gray. The predicted RII binding site is marked with a dotted line.

**Figure 4. Analysis of PKA binding to AKAP28** (A) Schematic representation of full-length and truncation His$_6$-AKAP28 proteins. All proteins are drawn to scale and the amino acid numbers are shown. (B) Equal moles of His$_6$ fusion proteins were resolved by SDS-PAGE, transferred to PVDF membrane, probed with biotinylated RII, subsequently incubated with streptavidin-HRP and developed with ECL. Biotinylated RII was either pre-blocked with no peptide, 1 μM competitive Ht31 or 1 μM negative control Ht31PP. A representative Ponceau stained membrane is also shown. (C) HeLa cells were either mock infected (MOCK) or infected with a control adenovirus expressing GFP (GFP), or an adenovirus expressing both GFP and HA-tagged AKAP28 (AKAP28). Cells lysates (300 μg) were incubated with monoclonal HA.11 or mouse IgG1a. Immunocomplexes were collected on protein G and subsequently
analyzed by western blot or RII overlay as indicated. Inputs (1/5th of total) for all cell lysates are shown. Lanes 1, 3 and 5 were incubated with HA antisera, while 2, 4 and 6 were incubated with mouse IgG1a. All data are representative of multiple independent experiments.

**Figure 5. Northern blot analysis of AKAP28** (A) Code-matched HBE cultures were differentiated on semi-permeable supports. Cultures were fixed post-plating at day 3, 10, 15 and 30. Cells were sectioned and stained with H&E. (B) Total RNA isolated from differentiating HBE cells at 3, 10, 15 and 30 days post-plating (20 µg/lane for samples 3, 10 and 15; 6 µg/lane for 30) was analyzed by northern blot using 32P-random primed cDNA corresponding to nucleotides 98-400 of AKAP28 cDNA. The same blot was probed with G3PDH as a loading control. Similar results were obtained on two separate blots. (C) A multiple tissue expression array (MTE) was probed with the same AKAP28 probe used in (B). A selected panel is shown; all tissues not shown were negative for the signal.

**Figure 6. Characterization of AKAP28 localization in WD-HBE cells** (A) Isolated axonemes were analyzed with affinity purified AKAP28 anti-sera. Parallel lanes of a single gel transferred to PVDF membrane were either overlayed with radiolabelled RII (RII) or western blotted (αAKAP28). An equal amount of purified pre-immune IgG (PRE) was used as a negative control. (B) Isolated axonemes were incubated with either 0.5% or 1.0% Triton X-100 for 25 minutes on ice. Detergent extracted axonemes (AXO) and membrane fractions (MEM) were resolved by SDS-PAGE, transferred to PVDF and probed with αAKAP28 antibody. The membrane fraction is 2/3 the amount of axoneme fraction loaded. (C) Sections of WD-HBE cells were deparaffinized and rehydrated to water. Sections were treated with an antigen unmasking solution, washed, permeabilized and blocked. Affinity purified AKAP28 antisera and non-immune IgG were incubated with sections overnight at 4°C. Sections were processed
using the Vectastain Elite ABC kit, developed using DAB, counterstained with light green SF yellowish, dehydrated and cover-slipped.
AKAP28  33  EDELTQVALALVEDVINTYAVKTVEE
TAKAP 80  340  DERMNIEIARTYVEGVEAAASVQFVEE
AKAP28  58  ERNPKNKIKWTHGEFTVEKGLQIQI
TAKAP 80  365  ARNPKNKIKWTHGEFTAEGGRQIQI
AKAP28  83  DLKFKCVSKKCMAGVVEFYERKDL
TAKAP 80  390  EKFKVTWFEQNCRWVYVYADFIKEDL
AKAP28  108  IHSFLVYYYMVWSISTDLPVVARIS
TAKAP 80  415  IHSFLVYYYVRWSAPFARVARWSV
AKAP28  133  AGTVFKMKVKKIKKPPDAPIVVSVVG
TAKAP 80  440  ANAYFTIKFKNKSKPAOPVDSYVF
AKAP28  158  DHQALVRHPGMVRFRQWQKNDLF
TAKAP 80  465  ENSELPRPCRIFREQLRDETET
AKAP28  183  KYSFMEFPPF  192  IDENTICAL RESIDUES
TAKAP 80  490  KHELISIPF  499  SIMILAR RESIDUES
-------- = RII BINDING DOMAIN