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A-Kinase Anchoring Proteins Interact with Phosphodiesterases in T Lymphocyte Cell Lines

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The cAMP protein kinase A (PKA) pathway in T cells conveys an inhibitory signal to suppress inflammation. This study was performed to understand the mechanisms involved in cAMP-mediated signaling in T lymphocytes. A-kinase anchoring proteins (AKAPs) bind and target PKA to various subcellular locations. AKAPs also bind other signaling molecules such as cyclic nucleotide phosphodiesterases (PDEs) that hydrolyze cAMP in the cell. PDE4 and PDE7 have important roles in T cell activation. Based on this information, we hypothesized that AKAPs associate with PDEs in T lymphocytes. Immunoprecipitation of Jurkat cell lysates with Abs against both the regulatory subunit of PKA (RIIα) and specific AKAPs resulted in increased PDE activity associated with RIα and AKAP95, AKAP149, and myeloid translocation gene (MTG) compared with control (IgG). Immunoprecipitation and pull-down analyses demonstrate that PDE4A binds to AKAP149, AKAP95, and MTG, but not AKAP79, whereas PDE7A was found to bind only MTG. Further analysis of MTG/PDE association illustrated that PDE4A and PDE7A bind residues 1–344 of MTG16b. Confocal analysis of HuT 78 cells stained with anti-PDE7A showed overlapping staining patterns with the Golgi marker GM130, suggesting that PDE7A is located in the Golgi. The staining pattern of PDE7A also showed similarity to the staining pattern of MTG, supporting the immunoprecipitation data and suggesting that MTG may interact with PDE7A in the Golgi. In summary, these data suggest that AKAPs interact with both PKA and PDE in T lymphocytes and thus are a key component of the signaling complex regulating T cell activation.


Cyclic AMP is a ubiquitous second messenger in the cell. Several studies have documented its potent anti-inflammatory role through inhibition of T cell activation (1, 2). However, the details of the biochemical mechanisms that control the concentration of cAMP in the cell and the downstream events initiated by an increase in cAMP are still emerging. cAMP activates protein kinase A (PKA), a broad specificity tetrameric serine/threonine protein kinase that is capable of phosphorylating many different substrates. PKA is comprised of two regulatory and two catalytic subunits. Binding of two cAMP molecules to each regulatory subunit promotes dissociation and activation of the catalytic subunit. The catalytic subunit then catalyzes the phosphorylation of local substrates. Specificity of PKA phosphorylation is achieved via anchoring of PKA through the interaction of the regulatory subunit type II (RII) of PKA with A-kinase anchoring proteins (AKAPs) (3).

AKAPs are a family of structurally diverse but functionally similar proteins containing an amphipathic helix that functions to bind the N termini of the PKA-RII dimer, and a targeting domain that determines the subcellular localization of the PKA-AKAP complex in the cell. Such targeting of AKAPs ensures that PKA is exposed to cAMP gradients locally generated by adenyl cyclases and phosphodiesterases (PDEs) thus allowing for appropriate substrate selection (4). Approximately 30 different AKAPs have been identified so far (3). Using Western blot and PCR analyses of a Jurkat cell library, our laboratory has identified over 15 different AKAPs in this cell line alone, including myeloid translocation gene (MTG) 16b, a novel AKAP that targets PKA to the Golgi of T lymphocytes (5). In addition to binding PKA, AKAPs are also known to bind other signaling molecules such as other kinases and phosphatases. In recent studies, Dodge et al. (6) have found that the muscle-selective AKAP (mAKAP) directly binds PKA and a splice variant of the cAMP-specific type 4 PDE4D3 in cardiac myocytes. Additionally, in Sertoli cells of the testis, Tasken et al. (7) have discovered that AKAP450 forms a multiprotein complex with PKA and PDE4D3.

PDEs are hydrolytic enzymes that play a crucial role in maintaining cAMP homeostasis in the cell. They cleave cAMP at the 3′ phosphodiester bond yielding inactive 5′ monophosphate. The PDE enzyme family currently is comprised of 11 members and over 30 isoforms that are distributed in different tissues with varying levels. The primary isoforms that have been detected in T cells include PDE1, PDE2, PDE3, PDE4, PDE5, PDE7, and PDE8 (8). Studies by Li et al. (9) have revealed that isoforms of PDE such as PDE7A are up-regulated when T lymphocytes are stimulated. As PDEs are involved in all aspects of cellular regulation, selective PDE inhibitors have been used clinically to combat cancer, depression, inflammatory conditions, asthma, and sexual dysfunction (10–13). Inhibitors of PDE4 have been proposed as novel anti-inflammatory agents for T cell-mediated diseases (8). For example, rolipram, a PDE4 inhibitor, has been documented to suppress TNF-α and IFN-γ production in animal models of rheumatoid arthritis and has been suggested to have chondroprotective effects (14). Clinical trials with piclamilast, also a...
PDE4 inhibitor, induced significant symptom relief in patients with rheumatoid arthritis along with decreased levels of IL-6 and C-reactive protein (15). Additionally, PDE4 inhibitors have been suggested to inhibit HIV replication in activated or memory T lymphocytes (16).

In this study we investigated the interactions of AKAPs with PDE4 and PDE7, two isoforms that have been reported to play a role in T cell activation (9, 17–22). The PDE4 enzyme family consists of at least 16 different isoforms encoded by four different genes, PDE4A, 4B, 4C, and 4D. Alternative splicing and transcription initiation of these genes results in long and short PDE4 isoforms that vary in the N-terminal sequence and consequently in their intracellular localization and activation by physiological stimuli. Each PDE4 gene (4A, 4B, 4C, and 4D) has a unique C terminus, which remains constant for splice variants of that gene (e.g., PDE4A-18, -22). PDE7 is a rolipram-insensitive cAMP-specific PDE consisting of two genes PDE7A and PDE7B that are 70% homologous in the catalytic region. PDE7A is known to have alternative splice variants A1, A2, (25) and A3 (22).

As discussed, various isoforms of PDE are involved in the maintenance of cellular homeostasis and are believed to be compartmentalized thereby enabling distinct cellular effects. In addition, because AKAPs appear to regulate cAMP levels by regulating PDE/PKA activity in other cells, we reasoned that AKAPs might interact with PDE in T lymphocytes to regulate T cell activation. In this study we present the evidence for the interaction of specific AKAPs with discrete isoforms of PDE in T lymphocytes.

Materials and Methods

Antibodies

The following Abs were purchased from BD Transduction Laboratories (San Diego, CA) for immunoblotting: mouse monoclonal AKAP149 (250 μg/ml), 1/250 dilution for Western blots; mouse monoclonal AKAP95 (250 μg/ml), 1/250 for Western blots; mouse monoclonal AKAP79 (250 μg/ml), 1/250 dilution for Western blots; mouse monoclonal P62-AKA (250 μg/ml), and mouse monoclonal GM130 (250 μg/ml), 1/100 dilution for immunofluorescence. Rabbit polyclonal Ab against MTG (1.1 mg/ml) was custom synthesized by Zymed Laboratories (South San Francisco, CA) as described in Schillace et al. (5) and used at 1/2000 dilution for Western blots and 1/60 for immunofluorescence. This Ab was generated against a conserved region of the MTG family of proteins, including MTG7B and 9b, MTG16a and 16b, and R1. Therefore, when referring to experiments using this Ab, the whole family of proteins is labeled simply MTG, whereas in experiments using recombinant protein (i.e., pull-down experiments) we refer to the specific isoform, MTG16b. Rabbit polyclonal Abs for PDE4A (Fabgennix, Shreveport, LA) were used at 5 μg for immunoprecipitation. Goat polyclonal Ab to PDE7A (200 μg/ml) and PDE7B blocking peptide (10 μg/ml), Santa Cruz Biotechnology, Santa Cruz, CA) were used at 5 μg for immunoprecipitation. Additionally, rabbit polyclonal Ab to PDE7A was custom made by Zymed Laboratories using peptide 469–482 Cys-ELSNQQQLQENRSL-COOH as Ag. The Ab was purified on Sulfolink columns according to the manufacturer’s protocol (Pierce, Rockford, IL). The PDE7A antibody (0.24 mg/ml) was used at 5 μg for immunoprecipitation, a dilution of 1/1000 for Western blots and 1/100 for immunofluorescence. Rabbit polyclonal anti-GST-HRP (10 μg/ml; Sigma-Aldrich, St. Louis, MO) conjugate and S protein-HRP conjugate (Novagen, Madison, WI) were used at 1/3000 dilution for Western blots. Peroxidase-conjugated goat anti-mouse and goat anti-rabbit were used at 1/5000 (Santa Cruz Biotechnology). FITC-conjugated secondary goat anti-mouse, goat anti-rabbit, and rabbit Texas Red-conjugated goat anti-mouse, goat anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at 1/100 dilution for immunofluorescence.

Jurkat cell and HuT 78 lytase preparation and immunoprecipitation

Jurkat cell clone E6-1 was purchased from American Type Culture Collection (ATCC, Manassas, VA) and maintained in culture with RPMI 1640, 1% antibiotic/antimycotic and 10% FBS (Invitrogen Life Technologies, Gaithersburg, MD) at 37°C in a 95% air/5% CO2 incubator. For immunoprecipitations, 15 million Jurkat cells per sample were collected and lysed in 0.5 ml of lysis buffer (1% Triton X-100, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 2 mM EGTA, 1/100 protease inhibitor mixture (Sigma-Aldrich), 1 mM benzamidine, and 10 μg/ml soybean trypsin inhibitor) and incubated with rotation at 4°C for 30 min. The mixture was centrifuged at 13,000 × g for 15 min at 4°C and the supernatant was preclarified for 30 min at 4°C with 20 μl of 50% protein A-Sepharose slurry master buffer. The preclarified supernatant was incubated for 2 h at 4°C with 5 μg of human IgG, or Abs to RIIα, AKAP95, AKAP149, MTG, PDE4A, or PDE7A based on the immunoprecipitations experiment. After adding 20 μg of protein A or 75 μl of protein A/G slurry, the mixture was rotated for 30 min at 4°C. The beads were washed with lysis buffer by centrifugation at 13,000 × g for 4 min at 4°C, boiled for 5 min in 5 × SDS sample buffer (60 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS w/v, 5% 2-ME, 0.05% bromophenol blue). The bound proteins were analyzed by SDS-PAGE and Western blotting (5). For immunoprecipitation with HuT 78 cells, the cell line was purchased from ATCC, cultured in IMDM with 20% FBS (ATCC) and cultured at 37°C in a 95% air/5% CO2 incubator. The HuT 78 cells were processed for immunoprecipitations in the same manner as Jurkat cells.

PDE activity assay

To assay for PDE activity, immunoprecipitations with Abs to RIIα, AKAP95, AKAP149, or MTG16b were performed as outlined for immunoprecipitations. The beads were washed and then used for the PDE activity assay using 1 μM CAMP as a substrate according to the method described by Beavo et al. (26). Antibodies were used in 50% PDE buffer A (100 mM MOPS pH 7.5, 4 mM EDTA, 1 mg/ml BSA) and 50 μl of PDE buffer B (100 mM MOPS pH 7.5, 75 mM MgAc, 100,000 cpm of [3H]cAMP; DuPont-NEN, Boston, MA) in a total volume of 250 μl. The reaction was terminated by boiling followed by addition of 10 μl of snake venom (2.5 mg/ml). The samples were then transferred to an ion exchange column and [3H]nucleoside was eluted into scintillation vials. Scintillation fluid was added to the eluted samples, mixed and counted in a scintillation counter.

Cloning of PDE constructs

For construction of PDE into GST and S-tag expression vectors, PDE4A (full length, GenBank accession number L36467) was amplified from rat PDE4A cDNA. The amplified cDNA was cloned into pGEX (Amersham, Arlington, VA) expression vector. MTG16b (full length, GenBank accession number L36467) was amplified from rat MTG16b cDNA by PCR using the forward primer 5′-GGA TCC GCG TTC CTG CAA TGC CAA GTC G-3′ and reverse primer 5′-CTG CGG-3′. The amplified cDNA was cloned into pET30a (Novagen, Madison, WI) expression vector. AMAP7A (full length, GenBank accession number L36467) was amplified from rat AMAP7A cDNA by PCR using the forward primer 5′-GGA TCC GCG TTC CTG CAA TGC CAA GTC G-3′ and reverse primer 5′-CTG CGG-3′. The amplified cDNA was cloned into pET30a (Novagen, Madison, WI) expression vector. PDE7A1 (full length, GenBank accession number L36467) was amplified from rat PDE7A1 cDNA by PCR using the forward primer 5′-GGA TCC GCG TTC CTG CAA TGC CAA GTC G-3′ and reverse primer 5′-CTG CGG-3′. The amplified cDNA was cloned into pET30a (Novagen, Madison, WI) expression vector.

Cloning of AKAP constructs

AKAP149 was amplified from Jurkat cell cDNA by PCR using the forward primer 5′-CCG GAA TTC ATG ATG GCA ATC CAG TTC CG-3′ and reverse primer 5′-ATA AGA ATG CGG CCG CGG TGA AGG GAA AAA GGA TCA GG-3′ were used to amplify the fragment. The restriction enzyme sites are underlined in all the primers that were used to amplify the various fragments. The fragment was digested with restriction enzymes BamHI and NotI and then subcloned into GST tagged pGEX. To subclone PDE4A into S-tagged pET30a forward primers 5′-CCG GAA TTC ATG AGG ATG GCA ATC CAG TTC CG-3′ and reverse primer 5′-ATA AGA ATG CGG CCG CAT CCA GGA GGC ATG CTT TTC C-3′, which attach BamHI and NotI restriction sites (respectively) to the fragment. PDE7A1 (full length) was then subcloned into pGEX-5X-1.

Cloning of AKAP constructs

AKAP149 was amplified from Jurkat cell cDNA by PCR using the forward primer 5′-CCG GAA TTC ATG ATG GCA ATC CAG TTC CG-3′ and reverse primer 5′-ATA AGA ATG CGG CCG CGG TGA AGG GAA AAA GGA TCA GG-3′ were used to amplify the fragment. The restriction enzyme sites are underlined in all the primers that were used to amplify the various fragments. The fragment was digested with restriction enzymes BamHI and NotI and then subcloned into GST tagged pGEX. To subclone PDE4A into S-tagged pET30a forward primers 5′-CCG GAA TTC ATG AGG ATG GCA ATC CAG TTC CG-3′ and reverse primer 5′-ATA AGA ATG CGG CCG CAT CCA GGA GGC ATG CTT TTC C-3′, which attach BamHI and NotI restriction sites (respectively) to the fragment. PDE7A1 (full length) was then subcloned into pGEX-5X-1.
ACG CTC-3'. The PCR product was then cloned into pET30a with EcoR I and HindIII restriction enzymes; MTG 1510–2000 was generated by PCR using forward primer 5'-CGCGAAATTCGAGCGCTCCGAAGATTGG-3' and reverse primer 5'-CCCATATGCTTGCCCCGCTTTTGGCCGC-3' and cloned into pbLueScript by blunt cutting with Sfi I restriction enzyme. Primers contained an EcoRI site and a HindIII site (respectively) that were used to digest sequence out of pbLueScript and to subclone into pET30a.

Expression of plasmid

All constructs were sequenced to ensure no PCR-induced mutations were created. The DNA from a single colony was transformed into BL21 DE3 pLysS competent bacteria for protein expression and grown in 10 ml Miller Luria Broth at 37°C using an orbital shaker (Forma Scientific, Marietta, OH). At 16 h with appropriate antibiotic (50 mg/L kanamycin or 100 mg/L ampicillin). After 16 h the 10 ml culture was added to 500 ml prewarmed medium/antibiotic and grown for ~2 h until OD = 0.6–1.0. When confluence was reached optimal protein expression was induced with 0.1 mM isopropyl β-D-thiogalactoside (Sigma-Aldrich) for PDE7A/pET30a cultures and 1 mM isopropyl β-D-thiogalactoside for all other cultures. The cultures were incubated an additional 1.5 h and then transferred to 50 ml conical tubes, pelleted at 3000 × g for 15 min and supernatant removed and pellets were frozen at ~80°C.

In vitro pull-down assay for PDE4A/AKAP interactions

For pull-down assays, extracts were prepared from bacterial pellets thawed on ice, resuspended in 300 μl of cold bacterial lysis buffer (50 mM Tris pH 8.0, 2 mM EDTA, 0.05% aprotinin (5 mg/ml), 0.5% soybean trypsin inhibitor (5 mg/ml)) and sonicated, 10 bursts at 35% power (Branson Sonifier 450; Branson, Danbury, CT). The sonicated mixture was combined with equal volume (300 μl) of bacterial lysis buffer and 2% Triton X-100 (Sigma-Aldrich) for PDE samples and 0.5% Triton X-100 for AKAP samples and rotated at 4°C for 45 min then spun at 13,000 × g for 15 min at 4°C, and the supernatant was removed. To analyze interaction of PDE4A with AKAP95, AKAP79, or MTG16b, S-tagged AKAP95 and PDE4A were incubated with S protein beads (Novagen, Madison, WI). For experiments examining PDE4A/AKAP4194 interaction, GST-tagged AKAP4194 lysate was incubated with glutathione-Sepharose fusion protein beads (Amersham Biosciences, Uppsala, Sweden) (glutathione-Sepharose beads were previously washed five times in cold PBS, spun at 1000 × g for 1 min in between washes) for 1 h while rotating. The beads were then spun at 1000 × g, for 1 min, supernatant was discarded, and the beads were washed five times with cold PBS (spun 1000 × g, 1 min each wash). For pull-down assays determining AKAP4194/PDE4A interaction PDE4A/pET30a lysates were added. For analysis of AKAP95 interaction with PDE, PDE4ApGEX lysates were added. To analyze interaction of PDE with MTG16b or AKAP97, PDE4ApGEX and AKAP97/pGEX lysates were added. The exact quantity depended on induction strength determined by earlier S protein or GST Western blot of bacterial lysates. pET30a or GST was added to a separate tube as a binding control. Equal volumes of Blotto (2% BSA and 5% nonfat milk) were added to all lysis samples. The beads were then rotated at 4°C for 2 h and centrifuged at 1000 × g for 1 min, supernatant was discarded, and the beads were washed five times with cold PBS (spun 1000 × g, 1 min each wash). The beads were then boiled for 5 min with SDS sample buffer. Protein sample lysates were processed as earlier discussed. pET30a or pGEX lysate, PDE4ApGEX lysate, pET30a lysate, or PDE4A lysate, was incubated with glutathione-Sepharose fusion protein beads for 1 h while rotating. The beads were then centrifuged at 1000 × g for 1 min, supernatant discarded, and the beads were washed five times with cold PBS (spun 1000 × g, 1 min each wash). Then MTG16b/pET30a fragment protein lysates were added. The exact quantity depended on induction strength determined by earlier S-tag Western blot of bacterial lysates. S-tagged pET30a was added to a separate tube as a binding control. Blotto (TBS with 0.1% BSA and 5% nonfat milk) at a ratio of 1:1 was added to the lysates of all samples except MTG16b full length and MTG16b 1510–2000 that require 2:1 proportions to block nonspecific protein binding to beads. The beads were then rotated at 4°C for 2 h, washed twice with PBS, twice with 0.5 M salt and PBS and followed by one wash with PBS. The beads were centrifuged at 1000 × g, for 1 min, and the supernatant was discarded and processed for SDS-PAGE as described in the PDE4A/AKAP pull-down analysis. Dilutions of protein lysates were prepared with PBS and SDS sample buffer, boiled for 5 min, and centrifuged for 30 s at 13,000 × g. The resulting supernatants were analyzed with SDS-PAGE and S protein Western blot analyses.

Immunofluorescence and confocal analysis

HuT 78 cells were allowed to adhere on coverslips previously coated with 1% poly-L-lysine (Sigma-Aldrich) by centrifuging at 100 × g for 5 min. The cells were fixed at room temperature with 3.7% formaldehyde and 120 mM sucrose in PBS for 15 min and processed for immunofluorescence as described in Schillace et al. (5). Confocal microscopy images were obtained using a Leica TCS-NT confocal imaging system (Deerfield, IL) with a ×40 objective.

Results

AKAPs associate with PDE

To determine whether AKAPs interact with PDE in T lymphocytes, we initially analyzed PDE activity that copurified with the regulatory subunit of PKA (RIαs). We have previously shown that T lymphocytes contain many different AKAPs (5) and other reports have shown that T lymphocytes contain many different isoforms of PDE (8, 17). By immunoprecipitating with Abs to RIαs, which presumably binds to all cellular AKAPs, and then analyzing for PDE activity, we reasoned we would be able to detect all AKAP/PDE interactions via the ternary RIα/AKAP/PDE complex (Fig. 1A). PDE activity of Jurkat cell lysates immunoprecipitated with Abs against the RIα subunit of PKA was significantly (p < 0.05) higher than IgG control (Fig. 1B). This data suggested that PKA and PDE were present in the same signaling complex. To further investigate the direct interaction of specific AKAPs with PDEs, Jurkat cell lysates were immunoprecipitated with Abs to AKAP95, AKAP149, and MTG (Fig. 1, C and D). Immunoprecipitation of AKAP95, AKAP149, and MTG resulted in a significant fold increase, (2.7, 7.6, and 5.6, respectively) of PDE-associated activity, compared with control. As we have not monitored the relative amount of AKAP immunoprecipitated from each sample, the different fold increases do not necessarily reflect affinity of interaction. However, these data do suggest that lymphocytes contain several AKAPs that interact with PDEs. Immunoprecipitation with Abs to PDE4A followed by immunoblot analysis revealed that AKAP95, AKAP149, and MTG, but not AKAP79, coprecipitate with PDE4A but not with control IgG. These data suggest that there is interaction between PDE4A and selective AKAPs in T lymphocytes.

In vitro evidence for direct interactions of AKAPs with PDEs

To evaluate the direct interaction of AKAPs with PDEs, in vitro pull-down experiments were performed. To perform these experiments, PDE4A was cloned into two bacterial expression vectors, pGEX (resulting in a PDE4A-GST fusion protein) and pET30a (resulting in a PDE4A-S-tag fusion protein). Additionally, other constructs including AKAP149-GST, MTG-GST, AKAP79-GST (all in pGEX) and AKAP95-S-tag in pET30a (kindly provided by Dr. Coghlan) were used in these experiments. In vitro pull-down experiments were performed using the bacterially expressed proteins and the resulting samples were subjected to SDS-PAGE and immunoblotted with either S protein or Ab to GST (Fig. 2, A, C, and E). Incubation of PDE4A with AKAP95 and AKAP149 (previously attached to beads) resulted in a detectable band of appropriate size (Fig. 2B, lane 2 and 2D, lane 2, respectively), whereas no bands were detected in the beads-alone control (Fig. 2B, lane 1 and 2D, lane 1). Lysate loading controls (Fig. 2B, lane 3 and 2D, lane 3) are shown. Incubation of AKAP79 or MTG with PDE4A (previously attached to beads) resulted in a detectable band of appropriate size for MTG-GST (Fig. 2F, lane 2) but no detectable
band for AKAP79-GST (Fig. 2F, lane 1). To ensure that PDE4A was not interacting with the GST portion of the fusion proteins, a GST control was also incubated with PDE4A (Fig. 2F, lane 3). Lysate loading controls (Fig. 2F, lanes 4–6) are examined. These data suggest PDE4A interacts with AKAP95, AKAP149, and MTG16b but not with AKAP79.

**MTG interacts with PDE7A**

So far we have shown that three AKAPs interact with PDE4A. However, as there are many T cell AKAPs and PDE isoforms, the number of possible AKAP/PDE interactions is potentially daunting. Therefore, we focused our attention on a PDE7A an isoform known to play an important role in T cell activation (9). Recently, variants of PDE7 such as PDE7A3 and PDE7A1 have been discovered in T cells and are up-regulated following stimulation (22).
Immunoprecipitation of Jurkat cells was performed with Abs to PDE7A (Santa Cruz Biotechnology) and subjected to immunoblotting analysis with Abs to AKAP95, AKAP149, AKAP79 and MTG. We could not detect binding of PDE7A with AKAP95, AKAP149 or AKAP79 (Fig. 3A) even though these AKAPs were detectable in the Jurkat cell lysate. However, we discovered that MTG proteins did coimmunoprecipitate with PDE7A but not with the IgG control (Fig. 3A, bottom row). To confirm this interaction, the immunoprecipitation experiment was repeated with the addition of a control antigenic peptide. MTG did not coprecipitate with control IgG (Fig. 3B, lane 2) but coprecipitated with PDE7A (Fig. 3B, lane 3) and the binding was significantly reduced when a peptide Ag for the PDE7A Ab was added to the immunoprecipitation (Fig. 3B, lane 4). These results suggest that the interaction of PDE7A appears to be very specific for MTG.

Characterization of binding domains in MTG/PDE interaction

To further characterize the interaction of MTG16b with PDE, we performed in vitro pull-down experiments to identify the region of MTG16b that is necessary for interaction with PDE7A and PDE4A. PDE7A and PDE4A were expressed as GST-fusion proteins in the bacterial expression vector pGEX. The samples were lysed and subjected to SDS-PAGE and analyzed using an anti-GST Western blot. The PDE-GST fusion proteins were then loaded onto glutathione beads.

To determine the binding domains, bacterial lysates of MTG16b fragments (Fig. 4, B and C, lanes 1–5) 1–160, 160–344, 344–432, and 432–600 were expressed in PET30 expression vector and pull-down assays were performed. The MTG16b fragments encompassing 1–160 (Fig. 4, B and C, lane 1), 160–344 (lane 2), and 160–432 (lane 3) all showed increased binding to PDE7A or PDE4A compared with GST beads alone (comparison shown as + or −). Fragment 344–432 (Fig. 4, B and C, lane 4) did not bind to PDE7A or PDE4A and fragment 434–600 (lane 5) bound equally or even more intensely to GST beads alone, suggesting that neither of these fragments contributes to the interaction between MTG16b and PDE7A. Lysate loading controls (Fig. 4, B and C, end lane) are shown to indicate the size of each fragment and to demonstrate that a sufficient quantity of each fragment was incubated with the PDE/GST beads. Collectively, the results suggest that the full-length PDE7A and PDE4A protein binds residues 1–344 of MTG and suggests there may be multiple binding domains within this

![Diagram](https://via.placeholder.com/150)

**FIGURE 3.** PDE7A interacts with a specific AKAP in T cells. A, To study the interaction of AKAPs with PDE7A, Jurkat cell lysates were immunoprecipitated (IP) with Abs to IgG (lane 2), and PDE7A (lane 3) (Santa Cruz Biotechnology). Lysates from Jurkat cells (lane 1) were processed in the same blot to demonstrate that sufficient quantities of AKAP proteins were available for immunoprecipitation. The samples were separated on SDS-PAGE, transferred to Immobilon-P membranes, and probed with Abs to AKAP79, AKAP95, AKAP149, and MTG. B, To confirm the interaction of MTG with PDE7A, Jurkat cell lysates were immunoprecipitated (IP) with Abs to IgG (lane 2), and PDE7A (Santa Cruz Biotechnology) in the absence (lane 3) or presence of PDE7A blocking peptide (Santa Cruz Biotechnology) (lane 4). The samples were separated on SDS-PAGE, transferred to Immobilon-P membranes and probed with Abs to MTG.

![Diagram](https://via.placeholder.com/150)

**FIGURE 4.** Identification of MTG16b binding domains that interact with PDE7A and PDE4A. A, Schematic of amino acid fragments used for MTG16b/PDE7A and MTG16b/PDE4A in vitro binding assays is shown. The region of MTG16b that was found to interact with PDE (■) and the binding site for PKA (▲) are shown. The size of each MTG fragment and its location within the linear sequence of the protein are shown. The first and last amino acids of each fragment are also indicated. B and C, To map the binding domains of MTG16b responsible for interaction with PDE, in vitro pull-down assays were performed using fragments of MTG16b (lanes 1–5, labeled lysates) expressed in S-tagged PET30 and full-length PDE7A (B) or PDE4A (C) expressed in GST-tagged pGEX. Fragments of MTG S-tag were incubated with glutathione beads bound to GST alone (−) or with glutathione beads bound to PDE7A/GST or PDE4A fusion proteins (+). In all cases the amount of GST bound to the beads exceeded the amount of PDE/GST bound to the beads as detected by Coomassie stain of the beads following SDS-PAGE (data not shown). The interaction between MTG fragments and PDEs was analyzed using an S protein Western blot analysis (lanes 1–5, pull-down). The above result is one representative blot of three independent experiments.
region for PDE7 and PDE4A. This is similar to other AKAPs, such as AKAP220, which has multiple binding domains for PP1 (27).

**Intracellular location of PDE7A in HuT 78**

We generated a polyclonal Ab to PDE7A using peptide 469–482 Cys-ELNSOLQPENRLSC dood as Ag (custom synthesized by Zymed Laboratories). The Ab was purified on Sulfolink columns according to the manufacturer’s protocol (Pierce). The Ab was characterized in Fig. 5. Western blot analysis using Ab to PDE7A469–482 as a probe detected a single molecular mass of 56 kDa PDE7A protein in HuT 78 (Fig. 5A, lane 1) and Jurkat cells (lane 2). The 56-kDa PDE7A protein band was blocked when incubated with the PDE7A469–482 blocking peptide (compare Fig. 5A, lanes 1 and 2 with lanes 3 and 4) in both cell lines. Immunoprecipitation experiments on HuT 78 cells were performed to confirm the interaction of PDE7A with MTG (Fig. 5B). We used HuT 78 cells for this purpose as they have been documented (25) to express high levels of PDE7A protein. MTG proteins in the lysate (Fig. 5A, lane 1) did not bind to control IgG (lane 2) but immunoprecipitated with Abs to PDE7A (Fig. 5A, lane 3) and to MTG (lane 4).

The location of PDE7A in T lymphocytes was then determined using immunofluorescence analysis. Immunofluorescence experiments on HuT 78 cells were performed with Abs to PDE7A469–482. Confocal analysis of the staining patterns suggests PDE7A is located in the Golgi apparatus (Fig. 6A). To confirm this hypothesis, HuT 78 cells were costained with the Golgi marker GM130 (Fig. 6B). An overlay of these two staining patterns (Fig. 6C) results in a significant amount of overlap (Fig. 6, yellow), evidence that PDE7A is indeed located in the Golgi. A blocking peptide for the PDE7A469–482 Ab specifically inhibited the staining of PDE7A (Fig. 6, D–F). Schillace et al. (5) have demonstrated that MTG is also localized to the Golgi. Our staining of HuT 78 cells with Abs to MTG and GM130 confirmed these observations (Fig. 6, G–I). Thus, we have shown that PDE7A coimmunoprecipitates with MTG from T lymphocytes and that both of these proteins are located in the Golgi apparatus.

These data provide evidence that MTG and PDE7A form a complex in the Golgi of T lymphocytes and may play an important role in modulating cAMP-initiated signal transduction pathways.

**Discussion**

To understand the mechanisms mediating cAMP signaling in the T lymphocyte, we explored the role of two mediators in the cAMP-signaling pathway, namely PDEs and AKAPs. Both of these signaling molecules have important roles in regulating T cell functions. Williams (28) has shown that AKAPs are involved in maintaining resting T cells in an inactive state and that PKA/AKAP interaction is required for cAMP-mediated responses in T cells. Beavo and his colleagues (9) have provided evidence that PDE7 induction and consequent suppression of PKA activity is required for T cell activation and that selectively reducing PDE7 expression with a PDE7 antisense oligonucleotide-inhibited T cell proliferation. Using immunoprecipitation, pull-down, and confocal analysis, we have now established that AKAPs interact with PDEs in T lymphocyte cell lines.

We have identified three AKAPs (AKAP95, AKAP149, and MTG) that appear to associate with PDE4A and one AKAP (MTG) that immunoprecipitates with PDE7A. T cells contain many AKAPs (5) and express several isoforms of PDE (8, 17). PDE4A is expressed in both CD4 and CD8 lymphocytes (17). AKAP95 has been identified in the nucleus of T lymphocytes (5) and has been reported to bind AMY-1 (c-Myc binding protein) in the cytoplasm of HeLa cells (29). AKAP149 was originally identified in the mitochondria but recent studies have shown that AKAP149 is also required for nuclear lamina assembly (30). Schillace et al. (5) have identified AKAP149 in the cytoplasm of T lymphocytes. AKAP149 is also known to bind PPI in HeLa cells (29). PP1 is known to form a complex with mAKAP in heart cells along with PDE4D and the ryanodine receptor (6, 31, 32). Thus, one hypothesis might be that AKAP149 also forms a complex between PDE4A and PP1 in T cells. In addition to AKAP95 and AKAP149, MTG16b, a recently discovered AKAP in T cells that targets PKA to the Golgi, also binds PDE4A.

cAMP has been known as a potent immunosuppressor for over 30 years. Extensive research has been performed on PDEs in T lymphocytes to exploit their potential for treating clinical conditions such as: inflammatory disorders, chronic obstructive pulmonary diseases, cancer, multiple sclerosis, and other neurological disorders (8, 15). However, because PDEs are ubiquitous, general inhibitors of PDE often have severe side effects (33, 34). To combat such limitations, isofrom-specific inhibitors have been explored as potential therapeutic agents. In mammals, 19 different genes encode PDE and each gene contains several distinct transcriptional units that give rise to different regulatory domains joined to a common catalytic domain (11). Many cells contain multiple isoforms of PDE, which would allow for targeting to a variety of subcellular locations and subtle differences in sensitivities to different signals. Our findings that certain AKAPs associate with specific PDE isoforms is a potentially exciting discovery as AKAPs play a pivotal role in cAMP/PKA signaling pathway and PDE isoforms are potential targets for clinical treatments. For instance, Erdogan and House lay (35) have found that a chronic elevation of cAMP levels in Jurkat cells caused a marked increase in PDE4 activity, with an increase in induction of splice variant PDE4D1, but a decline in the expression of isoform PDE4A. In contrast to Jurkat cells, treatment of monocyte cell lines with cAMP elevating agents caused an increase in expression of PDE4A (36). Such observations indicate cell specific differences in expression of various PDE isoforms in response to elevation of cAMP concentration implying cell-specific adaptive mechanisms that have specific functional consequences (35).
In addition to PDE4, we performed experiments with PDE7 as it plays a critical role in T cell activation. A CD3- and CD28-dependent induction of PDE7 is required for T cell activation (9). PDE7A associated with MTG16 but not other AKAPs, once again indicating the specificity of AKAP/PDE interactions. Many studies have documented proteins binding PDE4, however this is the first study to report on a protein interacting with PDE7A. Mapping studies of the PDE-AKAP interactions suggest that PDE7A and PDE4A bind to amino acids 1–344 in MTG16b. The RIIα binding domain on MTG16b has been mapped to residues 344–432 (5). This indicates that PDE and PKA bind different sites on the AKAP, which is consistent with other reports. For instance, in heart cells, PDE4D3 binds mAKAP on domains distinct from the RIIα binding region (6). Based on the results discussed, our studies suggest that MTG16b scaffolds PDE7A and PKA or PDE4A and PKA in the same regulatory complex, possibly for maintaining cAMP homeostasis.

An additional novel finding in this study is the subcellular localization of PDE7A in HuT 78 cells. Confocal analysis of PDE7A staining was consistent with Golgi staining patterns suggesting that...
PDE7A is localized to the Golgi. HuT 78 cells cotained with the Golgi marker GM130 and PDE7A had distinct and overlapping staining patterns providing evidence that PDE7A is indeed present in the Golgi. The staining of PDE7A protein was specific as evidenced by disruption of Golgi staining patterns when cells were incubated with PDE7A blocking peptide. Recent reports (37) have published the detection of PDE7A in T lymphocytes, but our study is the first to show the specific subcellular location of the protein. MTG is also targeted to the Golgi (5). These data support a model in which PDE7A interacts with MTG in the Golgi to form a regulatory module in the T cell.

In summary, our studies show that the interaction between different isoforms of PDEs and specific AKAPs may play an important role in regulating pools of cAMP in the cell. A model depicting this interaction is displayed in Fig. 7. In the T lymphocyte, binding of ligands such as prostaglandins and catecholamines activates G-stimulatory protein-coupled adenyl cyclase resulting in cAMP formation. cAMP binds and activates PKA and is metabolized by PDE. Anchoring proteins located in subcellular compartments of the T lymphocyte such as MTG, AKAP95, and AKAP149 interact with PKA and specific isoforms of PDE such as PDE4A and PDE7A to regulate cAMP homeostasis and may contribute to specific cellular functions. We know that PKA/AKAP interaction is required to maintain resting T cells in an inactive state per Williams (28). Presumably, the functional consequence of AKAPs scaffolding of both PKA and PDE in T lymphocytes is to establish a local feedback regulation to control cAMP concentration. For example, in cardiac myocytes, the scaffolding of PKA and PDE4D3 (a splice variant of PDE4D) by AKAPs establishes a feedback regulation whereby cAMP activates PKA, which in turn phosphorylates and activates PDE4D3 (6). The resulting decrease in cAMP favors reformation of the PKA holoenzyme. In T lymphocytes, cAMP has potent immunosuppressive properties and regulating its concentrations may influence T cell activation. Given the importance of PKA and PDE in the regulation of cAMP homeostasis, we speculate that the specific interaction of AKAPs with PDE and PKA may be a key regulator of T cell activation.

**References**


