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Inhibition of T Cell Activation by Cyclic Adenosine 5'-Monophosphate Requires Lipid Raft Targeting of Protein Kinase A Type I by the A-Kinase Anchoring Protein Ezrin

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cAMP negatively regulates T cell immune responses by activation of type I protein kinase A (PKA), which in turn phosphorylates and activates C-terminal Src kinase (Csk) in T cell lipid rafts. Using yeast two-hybrid screening, far-Western blot, immunoprecipitation and immunofluorescence analyses, and small interfering RNA-mediated knockdown, we identified Ezrin as the A-kinase anchoring protein that targets PKA type I to lipid rafts. Furthermore, Ezrin brings PKA in proximity to its downstream substrate Csk in lipid rafts by forming a multiprotein complex consisting of PKA/Ezrin/Ezrin-binding protein 50, Csk, and Csk-binding protein/phosphophosphoprotein associated with glycosphingolipid-enriched microdomains. The complex is initially present in immunological synapses when T cells contact APCs and subsequently exits to the distal pole. Introduction of an anchoring disruptor peptide (Ht31) into T cells competes with Ezrin binding to PKA and thereby releases the cAMP/PKA type I-mediated inhibition of T cell proliferation. Finally, small interfering RNA-mediated knockdown of Ezrin abrogates cAMP regulation of IL-2. We propose that Ezrin is essential in the assembly of the cAMP-mediated regulatory pathway that modulates T cell immune responses. The Journal of Immunology, 2007, 179: 5159–5168.

Cyclic AMP and protein kinase A (PKA) mediate intracellular signals from a wide variety of hormones, neurotransmitters, growth factors, and cytokines (1). cAMP and PKA signaling affect a range of cellular processes, strongly suggesting that this signaling pathway has the capacity to provide rapid and precise signaling with the required sensitivity and specificity. Specificity can be achieved by tissue and cell type-specific expression of PKA isoforms with different biochemical properties. In addition, A-kinase anchoring proteins (AKAPs) target PKA isoforms to defined subcellular sites in close proximity to relevant substrates (2). AKAPs may also tune the sensitivity of the signal pathway by recruiting PKA into multiprotein complexes that may include phosphodiesterases and protein phosphatases as well as other signal proteins (3).

There are two major isoforms of PKA in eukaryotes, PKA type I and II. These isoforms can be distinguished by their regulatory subunits labeled RI and RII. Activation occurs upon binding of cAMP to the R subunits, followed by the release of the active catalytic subunit. PKA type II is mainly particulate and associated with AKAPs, whereas PKA type I is both soluble and particulate. During T cell activation, PKA type I is redistributed during formation of the immunological synapse and localizes with the TCR-CD3 complex (4). PKA type I inhibits T cell activation by activation of C-terminal Src kinase (Csk) through phosphorylation of serine 364 in Csk (5). Active Csk subsequently phosphorylates the C-terminal inhibitory tyrosine residue of Lck and thereby acts as a negative regulator of TCR signaling. Although the molecular details of this process are well understood, it is not known how PKA type I is localized to lipid rafts during T cell activation.

Ezrin is a 78-kDa protein belonging to the Ezrin-Radixin-Moesin (ERM) family of proteins that play structural and regulatory roles in the assembly and stabilization of specialized plasma membrane domains by linking microfilaments to the membrane. ERM proteins have a highly homologous N-terminal 4.1/ezrin/radixin/moesin (FERM) domain and bind directly to a number of transmembrane proteins, including CD44, the transmembrane protein Na/H exchanger NEH1, CD43, and ICAMs (6–10), in addition to indirect binding to other membrane proteins via the scaffolding proteins ERM-binding phosphoprotein 50 (EBP50) and sodium-hydrogen exchanger 3 kinase A regulatory protein. The
FERM domain also binds to signaling molecules in the Rho pathway, including Rho guanine dinucleotide dissociation domain and Dbl (11, 12). Most of Ezrin’s interactions are dependent on conformational activation of the molecule. In a dormant state, binding sites for interaction partners are masked due to an intramolecular interaction between the FERM domain and the C terminus. Upon phosphorylation of a C-terminal threonine (T567) by protein kinase C or p kinase, the intramolecular bond is released and other interactions can occur (13).

Previous reports have suggested that Ezrin recruits type II PKA to the secretory canaliculi in gastric parietal cells when stimulated by gastrin (14). Ezrin has also been proposed to mediate interaction between the cystic fibrosis transmembrane conductance regulator (CFTR) and PKA type II (15). CFTR is an epithelial chloride channel, the activity of which is enhanced by PKA-dependent phosphorylation. However, the requirement of PKA anchoring to Ezrin for CFTR phosphorylation has never been tested experimentally. In this study, we have identified Ezrin as the dual-specificity AKAP responsible for targeting PKA type I to the TCR-CDS3 complex during T cell activation. Moreover, Ezrin also binds EBP50, a linker protein that binds to Csk-binding protein (Cbp)/phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG). Cbp/PAG is an adaptor protein that binds Csk (16, 17). Thus, Ezrin, EBP50, and Cbp/PAG act as a scaffold that assembles the cAMP/PKA/Csk pathway in lipid rafts of the plasma membrane during T cell activation. Disruption of PKA binding to Ezrin or Ezrin knockdown eliminates PKA type I from lipid rafts and prevents cAMP/PKA type I-mediated inhibition of T cell activation. These findings provide functional evidence that PKA type I regulation of T cell responses is dependent on AKAP anchoring by Ezrin.

Materials and Methods

Yeast strains and medium
Saccharomyces cerevisiae yeast strains were grown at 30°C in standard liquid yeast-peptone-dextrose medium or minimal synthetic defined medium with appropriate supplement amino acids (BD CloneTech).

Yeast two-hybrid screening
The Matchmaker Gal4 two-hybrid system (BD CloneTech) was used to screen a normal lymphocyte library. The full-length Rho subunit of PKA was subcloned into pAS2.1 and cotransformed together with the cDNA library (BD CloneTech catalog no. HL4014AB) into Y90 yeast cells. HIS-positive clones were further selected by colony lift filter assay for β-galactosidase activity. Plasmid DNA was recovered from yeast cells in Escherichia coli DH5α and retransformed into yeast reporter strain H7F7c (genotype: MATa, ura3–52, his3–200, try1–901, leu2–3, 112, gal4–542, gal80–538, LYS::GAL-HIS3, URA3::(GAL4-17mers)-CYC1-lacZ from BD CloneTech) with plasmid pGPT-PBR to test for histidine prototrophy and β-galactosidase activity (BD CloneTech, mammalian). The cDNA inserts from the positive clones were then sequenced.

Yeast two-hybrid interaction analysis
Ezrin constructs were cloned into the EG202 bait vector, whereas RI was subcloned into pAS2.1 and cotransformed together with the cDNA inserts from the positive clones were then sequenced. The cDNA inserts from the positive clones were then sequenced. The Matchmaker Gal4 two-hybrid system (BD Clontech) was used to identify Ezrin as the dual-specificity AKAP responsible for targeting PKA type I to the TCR-CDS3 complex during T cell activation. Moreover, Ezrin also binds EBP50, a linker protein that binds to Csk-binding protein (Cbp)/phosphoprotein associated with glycosphingolipid-enriched microdomains (PAG). Cbp/PAG is an adaptor protein that binds Csk (16, 17). Thus, Ezrin, EBP50, and Cbp/PAG act as a scaffold that assembles the cAMP/PKA/Csk pathway in lipid rafts of the plasma membrane during T cell activation. Disruption of PKA binding to Ezrin or Ezrin knockdown eliminates PKA type I from lipid rafts and prevents cAMP/PKA type I-mediated inhibition of T cell activation. These findings provide functional evidence that PKA type I regulation of T cell responses is dependent on AKAP anchoring by Ezrin.

Purification, culture, and transfection of human peripheral blood T cells and Jurkat T cells

The human leukemic T cell line Jurkat Tag was cultured and transfected, as described elsewhere (5). Purification of peripheral blood CD3+ T cells by negative selection from buffycoats of normal healthy donors (Ullevaal University Hospital Blood Center) and T cell proliferation assays was conducted, as described in detail elsewhere (20). For transfections of peripheral blood T cells, cells (5 × 10⁶) in 0.1 ml of Nucleofection solution (Amaza) were mixed with 1200 nM siRNA and subjected to electroporation in a Nucleofector (Amaza) following the manufacturer’s protocol. The cells were expanded in complete medium and incubated for 20 h at 37°C. A total of 4 × 10⁵ T cells was treated with 10 μM, 50 μM, or without cAMP (15 min (37°C)) and subsequently activated by addition of 8 μl of anti-CD3/CD28 beads (Dynal Biotech). After 20-h incubation at 37°C, the level of secreted IL-2 was determined by ELISA. The H31 expression vector was used as a gift from J. Scott (Howard Hughes Medical Institute, Vollum Institute, Oregon Health Sciences University, Portland, OR).

Fractionation of detergent-resistant glycosphingolipid-enriched membrane microdomains (lipid rafts) by density gradient ultracentrifugation

Primary T cells or Jurkat T cells (3 × 10⁶ cells) were washed in PBS, resuspended in 1 ml of MES containing 80 μg/ml cytochalasin D, and pelleted after 30 min at 37°C. T cells were resuspended in 1 ml of ice-cold lysis buffer (50 mM MES (pH 6.5), 100 mM NaCl, 5 mM EDTA, 0.7% Triton X-100 with 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM sodium pyrophosphate, and 50 mM sodium fluoride) and 0.7% Triton X-100, and lysed 10 min on ice. After 10 strokes with a Dounce glass homogenizer, the lysate was mixed 1:1 with 80% (v/v) sucrose and placed at the bottom of a 5.2-m1 polyallomer centrifuge tube (Beckman Instruments). The tubes were overlaid with 2.0 ml of 30% (v/v) sucrose in MNE buffer and finally with 1 ml of 5% (v/v) sucrose in MNE buffer (containing 25 mM MES (pH 6.5), 150 mM NaCl, and 5 mM EDTA).

Centrifugation was performed at 4°C in a Beckman SW55Ti rotor (200,000 × g, 20 h). Twelve 0.4-ml fractions were collected gradually from the top of the gradient, and proteins were next separated by SDS-PAGE and analyzed by immunoblotting.

Abs and fluorescent probes
Polyclonal Ab EZ10 to Ezrin was described previously (10, 21), and Ezrin 3C12 mAb (catalogue no. MS-661-P1) was from LabVision. mAbs directed against human RhoA, human RhoB, AKAP79, AKAP149, Csk, linker for activation of T cells (LAT), and phospholipase C-γ (catalogue no. P53620, P55120, 610351, A59820, C14520, L611108, and P12220, respectively) were from BD Biosciences, whereas human RhoA mAb clone 4D7 was described previously (4, 22). Anti-PKA C subunit polyclonal Ab (catalogue no. SC-286) and anti-CDS3 mAb (UCH-T1) were from Santa Cruz Biotechnology, and anti-CDS5 (MEM-43) was from AbCam. Anti-EPP50 mAb was a gift from V. Horejsi (Institute of Molecular Genetics, Academy of Science of the Czech Republic, Prague, Czech Republic), and rabbit polyclonal Ab to Lex A was a gift from E. Golemis (Fox Chase Center, Philadelphia, PA; to O. Carpen). Anti-HA mAb (catalogue no. 12CA5) was from Boehringer Mannheim. Anti-rabbit and anti-mouse IgG HRP-conjugated secondary Abs were from Jackson ImmunoResearch Laboratories. Alexa Fluor 488 goat anti-rabbit IgG, Alexa Fluor 647 anti-mouse IgGl, and Alexa Fluor 546 anti-mouse IgG2a were from Molecular Probes.

Immunoprecipitation and Western blotting

A total of 1–5 × 10⁶ of Ab and 25 μl of protein A/G plus agarose (SC-2003; Santa Cruz Biotechnology) was added to T cell lipid raft fractions. Precipitates were washed three to four times with lysis buffer containing 0.1%–1% Triton X-100, resolved by 10% SDS-PAGE under reducing conditions, and transferred by semidy electroblotting to polyvinylidene difluoride membranes. Blots were blocked with 5% dry milk in PBS-T (PBS/0.1% Tween 20), before incubation with the indicated Ab. After extensive washings with PBS-T, blots were incubated with either HRP-conjugated goat anti-mouse or goat anti-rabbit IgG, washed, and developed with ECL (Supersignal Pierce).

Immunofluorescence analysis and T cell activation by capping or Ag presentation

For fluorescence microscopy experiments, resting cells were washed in PBS and attached to poly(l-lysine)-coated coverslips (Sigma-Aldrich) by incubation on ice for 45 min (2 × 10⁵ cells/slip). Capping of the TCR was achieved by incubating cells with 10 μg/ml anti-CD3 mAb (UCH-T1) in
PBS/0.1% BSA for 30 min on ice, followed by incubation with 5 μg/ml Alexa Fluor 647 goat anti-mouse IgG1 (Molecular Probes) in PBS/0.1% BSA for 30 min on ice, and then 20 min at 37°C. Cells were next fixed with 3% paraformaldehyde in PBS for 30 min at room temperature, attached to coverslips, and permeabilized with 0.1% Igepal 630/PBS for 5 min before incubation with 2% BSA/0.01% Tween 20/PBS for 30 min, incubation with primary Ab in 2% BSA/0.01% Tween 20/PBS for 30 min, followed by incubation with fluorescein-conjugated secondary Ab for 30 min. Confocal microscopy was performed with a Zeiss LSM 510 META confocal microscope with a Plan-Apochromat 63 × 1.4 Oil DIC objective lens, using laser excitation at 488, 543, and 633 nm. The widths of Alexa Fluor 488, 546, and 633 emission channels were set such that bleed through across channels was negligible. In addition, sequential scanning was used.

Cell activation with Ag by APCs was achieved by pulsing superantigen-presenting Raji B lymphoma cells for 15 min by 10 μg/ml staphylococcal enterotoxin B (Sigma-Aldrich) at 37°C. T cells were then added at a T cell/APC ratio of 2, and the incubation was continued for 1 and 30 min, respectively. Cells were attached to poly(t-Lysine)-coated coverslips by centrifugation at 30 × g for 1 min at 4°C and fixed with 3% paraformaldehyde in PBS for 30 min at room temperature, and immunofluorescence was performed as above.

RI/RII overlay

Ezrin fragments fused to GST were produced in E. coli BL21 cells following stimulation with isopropyl β-thiogalactoside (IPTG) (3 h). BL21 cells were pelleted from 10 ml of broth and lysed, and after removal of insoluble pellet, glutathione beads were added for 1 h at 4°C. The beads were then washed and finally boiled in SDS-PAGE buffer. Proteins were insoluble pellet, glutathione beads were added for 1 h at 4°C. The beads were then added at a T cell/APC ratio of 2, and the incubation was continued for 1 and 30 min, respectively. Cells were attached to poly(t-Lysine)-coated coverslips by centrifugation at 30 × g for 1 min at 4°C and fixed with 3% paraformaldehyde in PBS for 30 min at room temperature, and immunofluorescence was performed as above.

GST-precipitation assay

Fragments of Ezrin or full-length Ezrin mutants TS67D and TS67A fused to GST were expressed in E. coli BL21 cells, induced using 0.4 mM IPTG, and purified on glutathione-Sepharose (Amersham Biosciences). A total of 2.5 μg of purified Ezrin fragments and R subunits immobilized on 1:1 ratio (50 μl each) in 100 μl of pulldown buffer (300 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 1 mM EDTA, 5 mM benzamidine, 5 mM DTT, 10 μg/ml antipain, chymostain, leupeptin, and pepstatin A) overnight at 4°C. Glutathione-Sepharose beads equilibrated in pulldown buffer with 1% BSA were added and incubated with rotation for 30 min at 4°C. The beads were washed with pulldown buffer five times for 5 min and boiled in SDS-PAGE buffer. Proteins were resolved on an 8–16% SDS-polyacrylamide gel and analyzed by immunoblotting for the presence of R subunits.

Peptide loading of peripheral T cells

To facilitate uptake of peptides into cells, we used liposome-mediated peptide loading. Peptide dissolved in 10 mM HEPES (pH 7.4) was mixed with 3 μl of liposomes (DOTAP; Boehringer Mannheim) and brought to a total volume of 10 μl before being added to the cells (7.5 × 10^6 in 90 μl). The optimal concentrations of DOTAP and anti-CD3 Ab were titrated carefully to maintain membrane stability and normal TCR/CD3-dependent activation of the T cells. Cells were incubated for 20 h with DOTAP/peptide, after which they were cultured in the absence or presence of 8-(4-chlorophenylthio)-cAMP (8-CPT-cAMP) (6.25 μM) and induced to proliferation via TCR/CD3 activation.

Results

Cloning of T cell AKAPs for PKA types I and II

T cell AKAPs were identified by a tandem strategy that used separate screens for PKA type I (RI) and type II (RII) interacting proteins. To look for RI-interacting proteins in T cells, we used the yeast two-hybrid system, a method that has previously been used to successfully identify proteins that bind RI and RII (25, 26). Screening of a human lymphocyte yeast two-hybrid library using the full-length RIIa subunit as bait identified a different set of AKAPs in T cells than the RII-overlay screening did. This was confirmed by hybridization of the full-length RIIa subunit with an RIIa RNA probe that hybridized to a different set of AKAPs in T cells than the RII-overlay screening did.

Identification of AKAPs associated with T cell lipid rafts

We next performed sucrose gradient fractionation of Jurkat T cell lysates and R overlay to identify AKAPs that can anchor the pool of PKA type I present in T cell lipid rafts. For this purpose, we used radiolabeled PKA RIIs subunit because most RI-binding AKAPs identified to date have been shown to be dual specific, binding both RI and RII, and because only the RII subunit has an autophosphorylation site allowing radiolabeling to high specificity. As shown in Fig. 1A, R-binding proteins of ~80 and 150 kDa were identified. Parallel sucrose gradient fractionations of lipid rafts were examined by immunoblot analysis using Abs to known AKAPs. The 80-kDa R-binding protein that was present in lipid rafts was identified as AKAP150, which was consistent with the report by Carr and coworkers (29) on the PKA type II AKAPs in T cells. The screening with RIIs as bait identified a different set of AKAPs in T cells than the RI-I overlay screening did.

FIGURE 1. Identification of AKAPs in T cell lipid rafts. A, Jurkat T cells were lysed on ice in lysis buffer containing 0.7% Triton X-100 and fractionated on sucrose gradients, as described in Materials and Methods. Fractions were resolved on SDS-PAGE, blotted to polyvinylidene difluoride (PVDF) membranes, and subjected to RII-overlay assay using radiolabeled RII (left lanes, longer exposure). Arrows indicate R-binding proteins detected. B, Lipid rafts were purified from T lymphocytes by sucrose gradient centrifugation following incubation in the absence or presence of cytochalasin D (80 μg/ml). Fractions were resolved on SDS-PAGE, blotted to PVDF membranes, and probed with the indicated Abs. Immunodetection of LAT-Ab was used as a marker for lipid rafts (indicated by bars). MOBs of m.w. markers are indicated.
rafts (fractions 3–5) was identified as Ezrin (78 kDa) (Fig. 1B, left panel). In contrast, AKAP79, which is of approximately the same molecular mass, was not detected in lipid rafts (data not shown). Lipid raft fractions, identified by immunoblot analysis with the lipid raft marker LAT, were also shown to contain PKA RIα and C subunits (Fig. 1B), as earlier reported (5).

Because Ezrin typically associates with transmembrane proteins via its globular domain, the possibility existed that PKA would mainly be associated with active Ezrin attached to T cell lipid raft proteins such as CD43 or CD44. Furthermore, the Ezrin-PKA complex could be tethered to the cortical actin cytoskeleton in T cells through the actin binding site in Ezrin. To optimize separation of the lipid raft-associated pool of Ezrin and examine association with PKA, we prepared lipid rafts from T cells both in the absence and presence of the actin-depolymerizing drug cytochalasin D (Fig. 1B, left and right panels, respectively). The rationale behind this approach was to prevent the artificial removal of Ezrin complexed with dense cytoskeleton from lipid raft fractions during sucrose density gradient centrifugation. When the actin cytoskeleton was disrupted, we observed a 50% increase in lipid raft-associated Ezrin (Fig. 1B, right panel). Also, the relative amount of PKA RIα was somewhat enriched (30%) in raft fractions, as opposed to nonraft fractions, after cytochalasin D treatment, as assessed by densitometric scanning.

**Mapping and characterization of the R-binding domain of Ezrin**

Deletional mapping analysis of determinants for interactions with RIα and RIIα was performed using a yeast two-hybrid system and assessed both by growth on drop-out medium and β-galactosidase activity. The smallest fragment of Ezrin that interacted with PKA was comprised of aa 363–470, which encompasses parts of the α-helical region of Ezrin (Fig. 2A, left and middle). The β-galactosidase reporter activity was higher when Ezrin fragments 278–585, 280–470, and 363–470 interacted with RIα than in interaction with RIIα, which indicates that Ezrin may have a higher affinity for RIα in situ. Further deletional mapping to a smaller fragment (aa 404–445) containing an amphipathic helix earlier predicted as a putative R-binding domain (14) showed little or no binding in the two-hybrid system, but showed some weak binding vs RIα using GST-Ezrin in an in vitro precipitation assay (Fig. 2A, right columns). Furthermore, both the results from the GST-Ezrin precipitations (Fig. 2A) and immunoprecipitation of tagged proteins from yeast cell lysates with the bait and prey proteins (Fig. 2B) verified the observations made in the yeast two-hybrid system. However, full-length Ezrin containing both the FERM and C-terminal domains did not bind PKA, as also demonstrated by surface plasmon resonance experiments (data not shown). To test the possibility that the conformation of full-length Ezrin may affect PKA binding, we mutated the C-terminal threonine (T567) to aspartate (T567D) to asparagine to mimic the phosphorylation-dependent activation of Ezrin. As seen from GST-pulldown experiments in Fig. 2C, Ezrin T567D binds PKA readily. In contrast, the T567A mutant, which should not affect the dormant state of full-length soluble Ezrin, does not. One conclusion from these experiments was that the PKA binding site in Ezrin may be inaccessible for PKA binding due to steric hindrance (Fig. 2D) when Ezrin is in the closed conformation (30).

The fragment of Ezrin (aa 280–470) identified in the two-hybrid screenings was inserted in sense and antisense orientation in a GST-expression vector. Expression was induced with IPTG, and expressed proteins were subjected to R-overlay using either RIα detected by Ab or radiolabeled RIIα. Both RIα and RIIα bound to the same fragment expressed in the sense orientation (Fig. 3A). Control experiments showed that proteins expressed from the antisense GST vector did not bind either R subunit. The same fragment of Ezrin was purified, cleaved to remove GST, and examined for binding to the RIα subunit of PKA immobilized on a cAMP chip by surface plasmon resonance (Fig. 3B). This analysis, using a range of concentrations of Ezrin, indicated a nanomolar affinity (K_D 25 nM) for the interaction of Ezrin with RIα, whereas similar experiments with RIIα demonstrated a ~10-fold higher affinity (K_D 1.4 nM; Fig. 3C). The low nanomolar affinities and the difference in affinity between RI and RII due to a faster off-rate of RI from different AKAPs are in agreement with what is normally observed for AKAPs (31, 32). Furthermore, binding of both RIα (Fig. 3D) and RIIα (data not shown) was fully displaced with 10 μM Hi31 competitor peptide, whereas no effect was seen with the proline-substituted Hi31P control peptide in agreement with earlier studies (33).
PKA and Ezrin colocalize in T cell lipid rafts

Immunofluorescence studies of the localization of PKA type I and Ezrin in resting, normal peripheral blood T cells revealed that whereas some PKA type I (RIα) appeared to be cytoplasmic, a fraction of RIα also appeared to colocalize with more peripherally localized Ezrin in the vicinity of the cell membrane (Fig. 4, A–C). Furthermore, when CD3 was cross-ligated at 37°C to allow patching on the TCR/CD3 complex (Fig. 4, D and I), lipid raft markers such as CD59 were also observed in patches at the cell periphery (Fig. 4E) that appeared to colocalize with CD3. Costaining of Ezrin with CD3 (Fig. 4, F and G) and CD59 (Fig. 4H) revealed a membrane-near distribution of Ezrin consistent with its role in organizing cortical actin and with overlap with both CD3 and the lipid rafts marker CD59. In addition, labeling of RIα, Ezrin, and CD3 in CD3-patch T cells (Fig. 4, I–L) demonstrated that a pool of Ezrin and PKA type I (RIα) appeared to colocalize in the proximity of the plasma membrane (Fig. 4M). Lastly, when Raji B lymphoma cells loaded with staphylococcal enterotoxin B were used as APCs to activate T cells, colocalization of RIα, Ezrin, and CD3 was observed in immunological synapses at 0–2 min (Fig. 4, N–R). Due to high levels of Ezrin in APCs, Ezrin distribution toward the synapse in the T cells could only be seen as a somewhat thicker decoration at the T cell:APC contact site (P–R; see also contact site in U–X). However, as reported earlier (34), we could observe Ezrin exit the synapse and move to the distal pole of the T cell following the T cell:APC contact from 5 to 30 min (Fig. 4, S–X). Interestingly, this was accompanied by a redistribution of some of the RIα staining to the distal pole (T and X) consistent with the role of Ezrin as an AKAP for PKA type I.

In summary, we show that a fraction of both Ezrin and RIα overlaps with CD3 and CD59, and that there is a partial colocalization of Ezrin and RIα in the vicinity of the plasma membrane, in CD3 patches and immunological synapses upon T cell activation, and at distal poles postactivation. This is in agreement with the observation by lipid raft purification that a fraction of both proteins localizes to rafts (see Fig. 1).

Ezrin, EBP50, and Cbp/PAG serve as a scaffold for the PKA-Csk signaling pathway in T cell lipid rafts

Immunoprecipitations from cytochalasin D-treated preparations of T cell lipid rafts using RIα or RIα Abs coprecipitated both Ezrin and the PKA C subunit (Fig. 5A). Conversely, immunoprecipitation with Ezrin Abs coprecipitated RIα and C as well as smaller amounts of RIα (Fig. 5B). This indicates that although Ezrin is able to bind both RIα and RIα, the dominant isoform of PKA associated with Ezrin in T cell lipid rafts appears to be PKA type I (RIα2, C2).

Previous observations indicate that Ezrin and Cbp/PAG can independently associate with the linker protein EB50. We have earlier reported that the lipid raft pool of Csk is almost exclusively localized to Cbp/PAG (5, 35). Immunoprecipitation of the Csk-Cbp/PAG complex by anti-Csk Abs revealed that EB50 (as earlier reported (36)) as well as Ezrin were present in the Csk immunoprecipitates (Fig. 5C). Furthermore, both Csk and the PKA C subunit were detected by immunoblot analysis of EB50 immunoprecipitates (Fig. 5D). Lastly, immunoprecipitation of Csk coprecipitated PAK C and vice versa (Fig. 5E). This indicates that all components of a peptide containing a proline residue in the AKAP interaction site (H31P) was injected with Ezrin, which was not able to compete for the Ezrin/R subunit interaction.
PKA/Ezrin/EBP50-Cbp/PAG signaling complex can be detected in the same precipitates, and that both kinases can be associated with the same population of the scaffold. One implication of these observations is that such a multiprotein complex may function to position PKA in close proximity to Csk for phosphorylation and regulation of Csk activity (Fig. 5F).

Disruption of PKA type I anchoring in lipid rafts reverses the inhibitory effect of cAMP on T cell immune functions

Cell-soluble peptides or expressed fragments containing the PKA binding domain of AKAP-Lbc (Ht31) were next used to compete the localization of PKA type I and to abolish the effect of anchored PKA type I in T cell lipid rafts. The Ht31-anchoring disruptor containing the R-binding site from AKAP-Lbc is well established and widely used to compete localization of PKA (23). Although Ht31 has a higher affinity for PKA type II, it is also effective in disrupting the interaction of PKA type I with Ezrin, as shown in Fig. 3D, as well as interaction with other PKA type I AKAPs (31, 32). Indeed, when T cells were transfected with a construct containing PKA/Ezrin/EBP50-Cbp/PAG/Csk signaling complex can be detected in the same precipitates, and that both kinases can be associated with the same population of the scaffold. One implication of these observations is that such a multiprotein complex may function to position PKA in close proximity to Csk for phosphorylation and regulation of Csk activity (Fig. 5F).

FIGURE 4. Colocalization of Ezrin, PKA RIα, and the lipid raft marker CD59 in CD3-patched and APC-conjugated T cells. Peripheral blood T cells were immunostained using polyclonal rabbit Abs to Ezrin (A, F, K, P, and U) and mAbs to RIα (B, J, O, and T), CD3 (D, I, N, and S), and the lipid raft-specific protein CD59 (E) and species and isotype-specific Alexa Fluor-conjugated secondary Abs. Merged images show overlapping subcellular distribution that appears yellow (C, H, M, R, and X) or blue (G, L, Q, and V). Patching or capping of the TCR was achieved by binding anti-CD3 Ab, followed by secondary Ab, and incubating for 20 min at 37°C before fixation and immunofluorescent staining (D–M). Activation of T cells by APC was performed by incubating T cells with staphylococcal enterotoxin B-loaded Raji cells for 1 (N–R) or 30 min (S–X) before fixation and immunostaining and identifying representative conjugates. Scale bar, 5 μm.

PKA/Ezrin/EBP50-Cbp/PAG/Csk signaling complex can be detected in the same precipitates, and that both kinases can be associated with the same population of the scaffold. One implication of these observations is that such a multiprotein complex may function to position PKA in close proximity to Csk for phosphorylation and regulation of Csk activity (Fig. 5F).

Disruption of PKA type I anchoring in lipid rafts reverses the inhibitory effect of cAMP on T cell immune functions

Cell-soluble peptides or expressed fragments containing the PKA binding domain of AKAP-Lbc (Ht31) were next used to compete the localization of PKA type I and to abolish the effect of anchored PKA type I in T cell lipid rafts. The Ht31-anchoring disruptor containing the R-binding site from AKAP-Lbc is well established and widely used to compete localization of PKA (23). Although Ht31 has a higher affinity for PKA type II, it is also effective in disrupting the interaction of PKA type I with Ezrin, as shown in Fig. 3D, as well as interaction with other PKA type I AKAPs (31, 32). Indeed, when T cells were transfected with a construct containing PKA/Ezrin/EBP50-Cbp/PAG/Csk signaling complex can be detected in the same precipitates, and that both kinases can be associated with the same population of the scaffold. One implication of these observations is that such a multiprotein complex may function to position PKA in close proximity to Csk for phosphorylation and regulation of Csk activity (Fig. 5F).

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directing the expression of the Ht31 fragment (37), this displaced both PKA types I and II from lipid rafts (Fig. 6A).

Next, normal peripheral blood CD3⁺ T cells were incubated with increasing concentrations of Ht31 peptide. Peptides were mixed with liposomes before incubation with T cells to facilitate uptake. Cells were incubated for 20 h with DOTAP/peptide, and incubations continued in the absence or presence of 8-CPT-cAMP. Subsequently, cells were activated to proliferation by stimulation of TCR/CD3. T cell immune responsiveness was assessed as [³H]-thymidine incorporation (cpm) and average ± SEM (n = 10) (C) or duplicate measurements (average ± half range) (E) are shown.

FIGURE 6. Expression of Ht31 competes anchored PKA RIα from lipid rafts and disrupts cAMP inhibition of T cell proliferation. A, Jurkat T cells were transfected with a mammalian expression vector encoding Ht31 and incubated for 16 h at 37°C. T cells were lysed on ice in lysis buffer containing 0.7% Triton X-100 and fractionated on sucrose gradients, as described in Materials and Methods. Fractions were resolved on SDS-PAGE, blotted to PVDF membranes, and probed with mouse mAb against RIα. B, Reversal of cAMP-mediated inhibition of TCR/CD3-stimulated T cell proliferation by the use of competitor peptide to compete the TCR/CD3-associated anchoring of RIα subunit of PKA type I in T cells. TCR/CD3-stimulated proliferation of peripheral blood CD3⁺ T cells from normal healthy blood donor following treatment with liposomes alone (mock) or with increasing concentrations (25–100 μM) of a competitor peptide (Ht-31) that competes anchoring to PKA (left panel) or a control peptide (right panel; Ht31-P). Note: reduced sensitivity to cAMP following loading with liposomes alone (mock) or with increasing concentrations of Ht31 competitor peptide, but not with the control peptide (Ht-31P). C, TCR/CD3 stimulated proliferation of peripheral blood CD3⁺ T cells in the presence of increasing concentrations of 8-CPT-cAMP. Normalized levels of proliferation of T cells incubated with liposomes loaded with Ht31-P control peptide (●, continuous line) or Ht31 peptide (35 μM) to compete anchoring of PKA type I (○, dotted line) were assessed as [³H]-thymidine incorporation after 48 h during which [³H]-thymidine was added for the last 18 h. Note: right shift of the IC₅₀ (arrow) from 1.8 to 4.8 μM in the presence of 8-CPT-cAMP.

FIGURE 7. Knockdown of Ezrin eliminates PKA type I from T cell lipid rafts and disrupts cAMP-mediated inhibition of T cell immune function. Purified T cells were transfected with Ezrin-specific (Ez799, Ez1245) siRNAs or triple G/C-mismatched control siRNAs (Ez799M3, Ez1245M3), immunoblotted to verify Ezrin knockdown, and examined for effect on cAMP-mediated inhibition of IL-2 secretion. A, Effect of Ezrin knockdown on lipid raft localization of Ezrin and PKA RIIα. B and D, Ezrin knockdown in one representative experiment corresponding to data shown in C and E. C and E, Effect of Ezrin knockdown on cAMP-mediated inhibition of IL-2 secretion. Forty-eight hours posttransfection, cells were pretreated with 8-CPT-cAMP (0, 10, or 50 μM) and either kept unstimulated or stimulated for 20 h with anti-CD3/anti-CD28-coated beads (bead-to-cell ratio 1:1). Then supernatants were harvested and assessed for IL-2. Levels of IL-2 secretion are shown relative to those of anti-CD3/anti-CD28-stimulated cells. Average ± SEM (n = 4–9) (C) or duplicate measurements (average ± half range) (E) are shown.
right-shifted inhibition curve by cAMP was observed with an apparent IC₅₀ of 4.8 μM, a shift that would have physiological impact on T cell function based on our earlier studies (20).

To specifically interfere with Ezrin-mediated PKA anchoring, peripheral blood T cells were transfected by nucleoelection (Amaxa) with siRNA against Ezrin. Under optimal conditions (1200 nM siRNA, 48 h posttransfection), we achieved 70–80% knockdown of Ezrin at the protein level with two different siRNAs (Fig. 7, A, B, and D). As seen from A, extinction of Ezrin expression by siRNA strongly reduced the levels of PKA type I (RIα) in T cell lipid rafts. Next, transfected and control-transfected cells were either left untreated, activated by anti-CD3/CD28-coupled beads, or pretreated with increasing concentrations of cAMP that produced 80% of maximal inhibition of IL-2 production, and subsequently activated and cultured for 20 h, after which IL-2 levels secreted to the supernatant were assessed. Results from these experiments show that T cells with knockdown of Ezrin were less sensitive to cAMP/PKA-mediated inhibition of IL-2 production (Fig. 7, B and D). In fact, IL-2 production in the presence of cAMP increased 2- to 4-fold after knockdown of Ezrin.

Discussion

A substantial body of evidence suggests that regulation by cAMP is determined by compartmentalization of PKA by AKAPs, which direct PKA to specific substrates and organize multiprotein signaling units (1–3). Most AKAPs preferentially bind PKA type II low nanomolar affinities; however, dual-specific and type I-specific AKAPs have been identified, typically with somewhat lower affinity due to a faster off rate (25, 26, 38, 39). This latter group may be particularly important for immune function. PKA type I is essential in the negative regulation of TCR/CD3-induced immune responses in T cells through activation of Csk. Csk subsequently inhibits Lck by phosphorylation of the C-terminal regulative tyrosine (Y505) (5). PKA type I resides in the lipid raft fraction of the plasma membrane in resting T cells as well as in the soluble fraction (5). T cell activation leads to a rapid polarization of the cell with redistribution of PKA type I (and a plethora of other signaling proteins involved in the signaling process) to the immunologic synapse (4). Targeting of PKA type I to this domain is likely to involve an anchoring protein. Using a combination of yeast two-hybrid screen of a normal lymphocyte cDNA library and far-Western analyses of lipid raft preparations from T cells, we were able to identify two anchoring proteins, Ezrin and AKAP149, in the lipid raft fraction of activated T cells. Both proteins were originally identified as AKAPs that target PKA type II (14, 40, 41), although AKAP149 was later shown to be dual specific (25). In this study, we now identify Ezrin as a functionally important binding partner of the RIα subunit in T cell lipid rafts and immunologic synapses. In contrast, we were not able to show any functional significance of PKA type I anchoring to AKAP149 with respect to T cell activation (data not shown).

Mapping studies of Ezrin reveal that the RI-binding sequence is located in the helical region between the FERM domain and the C terminus. This region is analogous to the RI binding region of Merlin, a close homologue of the ERM proteins (42). The smallest fragment sufficient for interaction with RIα in the yeast two-hybrid system contained aa 363–470. However, a smaller, 41-aa-long fragment inside the 363–470 region that encompasses aa 404–445 showed a weak interaction exclusively with the RIα subunit in the more sensitive GST-pulldown assay. Furthermore, our recent bioinformatic and peptide array analysis of RI- and dual-specific AKAPs revealed a consensus RI binding domain corresponding to aa 406–421 of Ezrin (43), which is shifted some 3 aa to the N terminus compared with the binding site reported for RIι in Ezrin (14). In contrast to the deletional mapping that indicated an apparent higher affinity for RIι, kinetic analysis of the interaction with RIα and RIι showed higher affinity for RIι. This apparent discrepancy might be explained by the Biacore analysis mainly measuring interaction of the R subunits with the amphipatic helix region of Ezrin. Additional experiments will be necessary to determine whether the region spanning residues 363–470 contains an independent RIα binding domain or provides additional determinants that augments RI binding to the amphipatic helix region within aa 404–445, as suggested by our RI binding site analysis (43). Alternatively, the preference for RI in situ despite a higher affinity for RIι could reflect the availability of PKA type I and II inside T cells.

The full-length Ezrin expressed in yeast interacted neither with RI nor with RIι. This is consistent with previous reports suggesting that Ezrin and other ERM proteins exist in two conformations, as follows: an open, active form in which the C terminus interacts with F-actin linking the cytoskeleton to the plasma membrane and a closed conformation in which intramolecular interactions mask the binding domains. The open configuration of Ezrin is induced by protein kinase C phosphorylation on threonine 567 and subsequent 4,5-bis-phosphate binding (44–46). Conversely, Ezrin dephosphorylation induces the closed conformation (13, 47). When actin polymerization was abrogated by pretreatment with cytochalasin D, the amount of Ezrin in the lipid raft fraction increased. Thus, the binding to F-actin appears to be sufficiently strong to survive the lipid raft fractionation so that a portion of Ezrin in the active state remains bound to the cytoskeleton. To date, the F-actin binding site and sites for interaction with EB5 and Rho guanine dinucleotide dissociation domain have been shown to be masked in the dormant, inactive monomer (48). Presumably, the closed conformation also masks the PKA binding site because PKA did not interact with full-length Ezrin in the absence of actin, whereas a TS67D mutation of Ezrin to mimic phosphorylation allowed for PKA binding.

Interestingly, T cell activation through the TCR leads to activation of protein kinase Cθ in lipid rafts that could phosphorylate and activate Ezrin (49, 50). T cell activation also triggers phospholipase Cγ1, which yields 4,5-bis-phosphate, which would further facilitate a conformational change of any folded Ezrin into the active open conformation (51). This would allow PKA to bind and could serve to establish a negative feedback loop. However, although this may be occurring, we did not observe any increase in the Ezrin-associated pool of PKA type I upon T cell activation (data not shown). On the contrary, PKA and Ezrin appeared to be constitutively associated, which could mean that the majority of Ezrin is activated, bound to actin, and accessible for PKA binding in T cells, or that even though dormant Ezrin is activated upon T cell activation, one of its partners, PKA, actin, or membrane-anchoring molecules, is limiting further complex formation. In agreement with our observations, the only change in Ezrin phosphorylation that has been observed following T cell activation is a rapid dephosphorylation of a C-terminal Thr, the dephosphorylation of which correlates with the export of Ezrin from immunologic synapses correlating with a more efficient conjugate formation (52, 53). However, because PKA type I also seems to redistribute with Ezrin to the distal pole, it may be that PKA and/or EB5 stabilize the active conformation of Ezrin even when dephosphorylation occurs.

Both CD59 and RI are located in lipid rafts and associated with patched and capped TCR/CD3 and immunologic synapses (4, 5, 54). Immunofluorescence studies in normal T cells revealed that a fraction of Ezrin colocalized with these two proteins. Furthermore, immunoprecipitation studies of the complex consisting of Csk and
Cbp/PAG demonstrated interaction with both Ezrin and EBP50, and both Csk and the catalytic subunit of PKA were present in immunoprecipitates of EBP50. Lastly, Csk and PKA C coprecipitated, indicating their association to the same population scaffold proteins. Together, this demonstrates formation of a lipid raft-associated complex consisting of PKA, Ezrin, EBP50, Csk/PAG, and Csk, and suggests a model in which Ezrin is bound via CD43, CD44, or ICAMs to the plasma membrane and that EBP50 links Ezrin to Cbp/PAG (Fig. 5F). Thus, Ezrin positions PKA in close proximity to its substrate Csk that is bound to Cbp/PAG. The role of EBP50 would appear to be scaffolding, and stabilization of the complex as EBP50 is an adaptor protein that binds Cbp/PAG via its N-terminal PDZ domain (36) and activated Ezrin via a defined C-terminal region (47), but has no membrane-targeting domain. In support of this, we have earlier shown that PKA phosphorylation of Csk occurs in lipid rafts (5) and that PKA phosphorylates the pool of Csk anchored to PAG/Cbp (35). This, together with the observations that downstream effector functions modulated by the PKA-Csk pathway are released from cAMP inhibition by knockdown of Ezrin argues that the PKA regulation of Csk is discretely coordinated and spatiotemporally restricted to this supramolecular complex. Interestingly, two recent papers have reported the Cbp/PAG knockout showing that Cbp is dispensable for T cell function (55, 56). However, although the two reports agree on the lack of any effect of the Cbp null mutation on T cell function, one report states that Cbp is dispensable also for Csk localization and suggests that other adaptor proteins might compensate (55), whereas the other report states that the amount of Csk that localizes to the lipid rafts is greatly reduced in the absence of Cbp (56). Anyhow, the possibility of redundancy and the probable presence of other Csk-anchoring proteins for Csk opens up the possibility that Csk is only regulated by PKA in the context of the Ezrin/EBP50/Cbp/PAG scaffold and may escape regulation by PKA if anchored elsewhere.

CD43 plays a negative role in T cell signaling (57–59) and is actively excluded from the immunological synapse during T cell activation (60) in an ERM-dependent manner (34). Expression of a dominant-negative Ezrin mutant, in which the domain that binds to CD43 is deleted, inhibits CD43 movement. The main hypothesis derived from these findings was that the negative effect of CD43 on T cell activation is due to steric hindrance of intercellular interaction by its large heavily glycosylated extracellular domain forming a barrier for interaction. However, recent data support that it is rather the intracellular domain of CD43 that is involved in the negative regulation of T cell responsiveness because expression of a CD43-extracellular domain fusion protein failed to reverse the hyperactivated state of CD43 null mutant mice. In support of this, reversal of the hyperactivated state was achieved with a fusion protein containing the intracellular domain of CD43 and the transmembrane domain of CD7 (61). These data argue against the barrier hypothesis. The mechanism whereby the intracellular domain of CD43 conveys the inhibitory function on T cell activation is not known. Our data suggest that this may be due to recruitment of Ezrin to the plasma membrane and the establishment of an inhibitory signaling unit that includes PKA type I, Csk, and Cbp/PAG. The possibility of colocalization and cimation of PKA type I with CD43 out of lipid rafts will be interesting to pursue in future studies because Ezrin and PKA type I appear to colocalize at the distal pole postactivation.

PKA type I plays an important role in the negative regulation of T cell function by mediating the effect of paracrine and hormonal cues that increases CAMP, the most prominent of which is PGE2 that at physiological levels generates intracellular cAMP levels sufficient to inhibit effector T cell functions (62). Interestingly, we also recently found that regulatory T cells may mediate their suppressive effect by secretion of PGE2 (63). In this study, we demonstrate that disruption of targeting of PKA to endogenous AKAPs in T cells using Ht31 and siRNA-mediated knockdown of Ezrin eliminates PKA type I from lipid rafts and perturbs the inhibitory effect of cAMP on T cell activation. In conclusion, our data suggest that Ezrin via EBP50 is the essential link between cytoskeletal F-actin and a lipid raft-associated multiprotein complex that includes Cbp/PAG, Csk, and PKA type I that negatively regulates immune responses upon T cell activation.

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Disclosures

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References


