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Direct Interaction between Protein Kinase Cθ (PKCθ) and 14-3-3τ in T Cells: 14-3-3 Overexpression Results in Inhibition of PKCθ Translocation and Function†

NAHUM MELLER,[1,2] YUN-CAI LIU,[3] TASSIE L. COLLINS,[1] NATHALIE BONNEFOY-BÉRARD,[1]‡ GOTTFRIED BAIER,[4] NOAH ISAKOV,[2] AND AMNON ALTMAN[1]*

Divisions of Cell Biology[1] and Immunobiology,[3] La Jolla Institute for Allergy and Immunology, San Diego, California 92121; Department of Microbiology and Immunology, Ben Gurion University of the Negev, Beer Sheva 84105, Israel[2]; and Institute for Medical Biology and Human Genetics, University of Innsbruck, A-6020 Innsbruck, Austria[4]

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Recent studies have documented direct interactions between 14-3-3 proteins and several oncogene and proto-oncogene products involved in signal transduction pathways. Studies on the effects of 14-3-3 proteins on protein kinase C (PKC) activity in vitro have reported conflicting results, and previous attempts to demonstrate a direct association between PKC and 14-3-3 were unsuccessful. Here, we examined potential physical and functional interactions between PKCθ, a Ca^{2+}-independent PKC enzyme which is expressed selectively in T lymphocytes, and the 14-3-3τ isoform in vitro and in intact T cells. PKCθ and 14-3-3τ coimmunoprecipitated from Jurkat T cells, and recombinant 14-3-3τ interacted directly with purified PKCθ in vitro. Transient overexpression of 14-3-3τ suppressed stimulation of the interleukin 2 (IL-2) promoter mediated by cotransfected wild-type or constitutively active PKCθ, as well as by endogenous PKC in ionomycin- and/or phorbol ester-stimulated cells. This did not represent a general inhibition of activation events, since PKC-independent (but Ca^{2+}-dependent) activation of an IL-4 promoter element was not inhibited by 14-3-3τ under similar conditions. Overexpression of wild-type 14-3-3τ also inhibited phorbol ester-induced PKCθ translocation from the cytosol to the membrane in Jurkat cells, while a membrane-targeted form of 14-3-3τ caused increased localization of PKCθ in the particulate fraction in unstimulated cells. Membrane-targeted 14-3-3τ was more effective than wild-type 14-3-3τ in suppressing PKCθ-dependent IL-2 promoter activity, suggesting that 14-3-3τ inhibits the function of PKCθ not only by preventing its translocation to the membrane but also by associating with it. The interaction between 14-3-3 and PKCθ may represent an important general mechanism for regulating PKC-dependent signals and, more specifically, PKCθ-mediated functions during T-cell activation.

Members of the protein kinase C (PKC) family of intracellular serine/threonine kinases play critical roles in the regulation of cellular differentiation and proliferation in many cell types and in response to diverse stimuli, including hormones, neurotransmitters, and growth factors (reviewed in references 36, 40, 41, and 55). Ten mammalian PKC genes have been isolated, and their protein products can be grouped into three subfamilies on the basis of their domain structure. Members of the Ca^{2+}-dependent subfamily (PKCα, -β, and -γ) contain three conserved domains, namely, the diacylglycerol- or phorbol ester-binding C1 domain, which contains two repeats of a cysteine-rich zinc finger, the phospholipid- and Ca^{2+}-binding C2 domain, and the catalytic C3 and C4 domains. The Ca^{2+}-independent enzymes (PKCδ, -ε, -η, -θ, and -μ) contain a C2-like N-terminal domain that binds acidic phospholipid but not Ca^{2+} (37). A third subfamily includes PKCζ and PKCι/λ with a single cysteine-rich domain which does not bind phorbol ester. PKC enzymes are regulated by phosphorylation and binding of defined cofactors, and enzyme activation is associated with its redistribution among different cellular compartments (32, 36). Receptors for activated C kinase (RACKs) play an important role in mediating PKC translocation and, by extension, in PKC-dependent functions (32). The differential expression of PKC isoforms in mammalian tissues and the differences in their intracellular localization and cofactor requirements suggest that distinct PKC isoforms may be independently regulated, respond to discrete ligands, and/or possess distinct substrate specificities (40, 55).

Members of the 14-3-3 protein family form a group of highly conserved 27- to 30-kDa acidic proteins expressed in a wide range of organisms and tissues. 14-3-3 proteins possess various biological activities, including activation of tyrosine and tryptophan hydroxylases, stimulation of Ca^{2+}-dependent exocytosis, obligatory cofactor activity for ADP ribosylation by *Pseudomonas aeruginosa* exoenzyme S (denoted FAS activity), and potential regulation of gene transcription and the cell cycle (1, 9, 35). Recently, 14-3-3 proteins were found to bind directly several oncogene or proto-oncogene products involved in signal transduction pathways, i.e., the Raf-1/B-Raf protein kinases (16, 17, 25, 26, 30, 62), the Bcr or Bcr-Abl kinases (45), polyomavirus middle tumor antigen (44), cdc25 phosphatases (12), the catalytic subunit (p110) of phosphatidylinositol 3-kinase (7), and Cbl (28). Activation of Raf-1 by 14-3-3 in intact cells or in vitro was also demonstrated in some studies (15, 16, 26, 62) but not in other (30, 56) studies. The crystal structures of two 14-3-3 proteins, which have recently been elucidated, reveal a dimeric structure whose ligand-binding pocket can accommodate two putative ligands (27, 61). In support of this model, it was recently shown that the 14-3-3 dimer mediates

* Corresponding author. Mailing address: Division of Cell Biology, La Jolla Institute for Allergy and Immunology, Science Center Dr., San Diego, CA 92121. Phone: (619) 558-3527. Fax: (619) 558-3525. Electronic mail address: amnon@liai.org.

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‡ Present address: INSERM U80, Hôpital E. Herriot, 69437 Lyon Cedex 03, France.

complex formation in vivo between the Raf-1 and Bcr kinases, two known 14-3-3 ligands that do not bind directly to each other (8).

14-3-3 proteins have also been implicated as potential regulators of PKC. Some 14-3-3 isoforms can be phosphorylated by PKC at a low stochiometry (2, 23), although the biological significance of this event is unclear. Studies which analyzed the effects of 14-3-3 on PKC activity in vitro reported conflicting results. While some described 14-3-3-mediated inhibition of PKC activity that can be overcome by high concentrations of diacylglycerol (46, 58, 59), others documented phosphatidylserine- or diacylglycerol-independent activation of PKC by 14-3-3 (20, 57). Still others failed to find direct effects of 14-3-3 on PKC (11, 34) and concluded that the effects of 14-3-3 on PKC-mediated exocytosis in adrenal permeabilized chromaffin cells are indirect and reflect an unrelated synergistic activity (34). These conflicting results may reflect the use of different assay conditions and/or 14-3-3 preparations. Despite all of these studies which suggest some functional interactions between 14-3-3 proteins and PKC, previous attempts to detect physical associations between 14-3-3 proteins and PKC were unsuccessful (8, 17).

In view of these contradictory findings, we sought to analyze potential interactions between PKC and 14-3-3 in intact T cells. We elected to use the 14-3-3τ isoform (39), which is expressed abundantly in T cells, and human PKCθ which we have recently isolated and characterized (3–5, 14). PKCθ is a Ca^{2+}-independent enzyme characterized by its unique profile of expression in skeletal muscle, lymphoid organs, and hematopoietic cell lines, in particular T cells (3, 4, 10, 31, 42, 60). We found recently that, among several PKC isoforms tested, PKCθ was the only one capable of significantly stimulating Ras-dependent transcription from an AP-1 element in EL4 leukemic T cells (5). These studies implicate PKCθ as a specific constituent of the signaling cascade that is involved in T-cell activation by the T-cell receptor–CD3 complex.

We report here that PKCθ and 14-3-3τ associate both in vitro and in intact T cells. Furthermore, overexpression of 14-3-3τ dramatically inhibited PKCθ-dependent activation of the interleukin 2 (IL-2) promoter and had a marked effect on the intracellular localization of PKCθ in resting or phorbol ester-stimulated Jurkat T cells. These results demonstrate that PKC and 14-3-3 can interact directly. This interaction may represent an important general mechanism for regulating PKC-dependent signaling events and, more specifically, PKCθ-mediated functions during T-cell activation.

MATERIALS AND METHODS

Cell culture, transient transfection, and activation. Human Jurkat leukemia T cells and Jurkat-TAg cells which stably express the simian virus 40-derived large T antigen (11a) were kept at logarithmic growth phase in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM minimum essential medium nonessential amino acids, 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.3), 50 μM β-mercaptoethanol, and antibiotics. For transfection, the cells were washed three times in supplement-free RPMI 1640 medium and suspended at 5×10^7 cells per ml in unsupplemented medium, and aliquots of 400 μl (2×10^7 cells) were dispensed into 0.4-cm-gap Gene Pulser cuvettes (Bio-Rad). Plasmid DNA was added, and the cells were electroporated with a Bio-Rad Gene Pulser (280 V, 960 μF). The cells were then cultured in 13 ml of complete RPMI 1640 medium. For chloramphenicol acetyltransferase (CAT) assays (see below), the cells were stimulated 24 h later in triplicate with the indicated concentrations of phorbol 12-myristate 13-acetate (PMA) and/or ionomycin (both from Calbiochem) dissolved in dimethyl sulfoxide (DMSO) and harvested after an additional 24-h period. In translocation experiments, the cells were collected 48 h after transfection, washed in phosphate-buffered saline (PBS) (pH 7.2), and stimulated with 50 ng of PMA per ml at 37°C for 20 min. The final concentration of DMSO was always ≤0.1%.

Expression vectors and reporter gene constructs. pEF (29)-based mammalian expression vectors encoding histidine-tagged wild-type (wt) human PKCθ (His_6-PKCθ), dominant-negative PKCθ in which a conserved ATP-binding lysine residue in the catalytic domain has been mutated to arginine (K409R), or constitutively active PKCθ in which a conserved alanine residue in the pseudosubstrate region has been mutated to glutamic acid (A148E), were generated as described previously (5). Generation of the mammalian 14-3-3τ expression vector (pEFneo-14-3-3τ) and a bacterial vector expressing wt human 14-3-3τ as a glutathione *S*-transferase (GST) fusion protein (pGEX-GST-14-3-3τ), as well as the expression of the fusion protein in *Escherichia coli*, has been described previously (7).

In order to generate a membrane-targeted version of 14-3-3τ (14-3-3τmm), a cDNA fragment encoding a CAAX membrane-targeting motif and an adjacent six-lysine polybasic membrane localization signal derived from K-*ras* (18), was amplified by standard PCR techniques. *Bgl*II and *Xba*I sites were added to the 5′ and 3′ ends of the cDNA fragment, respectively. Conversely, the corresponding *Bgl*II and *Eco*RI sites were created at the 3′ and 5′ ends, respectively, of the 14-3-3τ cDNA by PCR amplification. The sequences of the PCR products were confirmed by sequencing with a Sequenase T7 DNA polymerase kit (U.S. Biochemical Corp.). The K-*ras*-derived and 14-3-3τ cDNA fragments were ligated, and the chimeric cDNA was subcloned into pEFneo at the *Eco*RI and *Xba*I sites.

A reporter plasmid containing 298 bp of the human IL-2 promoter upstream of the CAT reporter gene, pIL-2/+1-CAT (53) was obtained from M. Karin (University of California at San Diego). A CAT reporter plasmid containing five tandem repeats of AP-1- and NF-AT-binding sites corresponding to nucleotides −88 to −61 in the IL-4 promoter, pSVO-5× AP-1/NF-AT-CAT (50), was a kind gift from L. Glimcher and M. Hodge (Harvard School of Public Health).

CAT assay. A mixed-phase assay for CAT activity was performed as described previously (38). Briefly, Jurkat cells were washed in PBS, suspended in buffer A (250 mM Tris-HCl [pH 7.8], 5 mM EDTA), and lysed by three cycles of freezing and thawing. The extract was centrifuged at 16,000 × *g* for 10 min, and 36 μl of the supernatant was incubated in a microtiter plate (5 min at 37°C) with 12 μl of 5 mM chloramphenicol in buffer A. Twelve microliters of [^{3}H]acetyl coenzyme A (20 μCi/ml; specific activity, 6.7 Ci/mmol) (ICN Biochemicals) and 150 μM acetyl coenzyme A in 75 μM HCl were added and incubated for 2 h. The reaction was terminated by adding 180 μl of 7 M urea (in water) to each well. Aliquots of 200 μl were transferred to scintillation vials containing an additional 300 μl of 7 M urea, and scintillation fluid (0.8% 2,5-diphenyloxazole in toluene) was added. The protein concentration of the cell extracts was determined by the Bradford protein assay (Bio-Rad), and specific CAT activity was expressed as counts per minute of acetyl coenzyme A incorporated into acetylated chloramphenicol per microgram of protein. The results represent the average CAT activity of triplicate samples, and the standard deviations are indicated in the figures by error bars.

Cell lysis, immunoprecipitation, and partial purification of recombinant His_6-PKCθ. Jurkat cells were lysed in TN1 buffer (20 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% Nonidet P-40 [NP-40], 10 μg [each] of leupeptin and aprotinin per ml) for 30 min on ice, the samples were centrifuged at 16,000 × *g* for 20 min, and the supernatant was collected and used for immunoprecipitations or for purification of His_6-PKCθ. Lysates were incubated (4 h, 4°C) with optimal dilutions of polyclonal rabbit anti-PKCθ (4) or anti-Raf-1 (C12; Santa Cruz Biotechnology) antibodies. Immune complexes were collected with protein A-Sepharose beads (Pharmacia) and washed five times in TN1 buffer. His_6-PKCθ from transfected cells was partially purified with Ni^{2+}-nitrilotriacetic acid (NTA) resin (4). Immunoprecipitates, partially purified PKCθ, or whole-cell extracts were boiled in sodium dodecyl sulfate (SDS) sample buffer, resolved by SDS–10% polyacrylamide gel electrophoresis (PAGE), and analyzed as described below.

Binding of PKCθ from cell lysates to recombinant 14-3-3τ. Jurkat-Tag cells were transfected with 5 μg of pEFneo-PKCθ (or control vector) plasmid DNA. Forty hours later, the cells were lysed in TN1 buffer, and lysates from 10^7 cells were incubated with 5 μg of GST or GST-14-3-3τ fusion protein and 30 μl of glutathione-Sepharose beads for 1 h at 4°C. The binding mixtures were washed five times in TN1 buffer and analyzed by immunoblotting with a mouse anti-PKCθ monoclonal antibody (MAb) (Transduction Laboratories).

Immunoblotting and Far-Western blotting. Electrophoresed proteins were transferred to nitrocellulose membranes which were blocked with 5% dry milk in TBS-T (30 mM Tris-HCl [pH 7.5], 125 mM NaCl, 0.1% Tween 20) for 1 h. The membranes were incubated (4 h at room temperature or overnight at 4°C) with anti-PKCθ (4), anti-PKCα and -β (19), or anti-Raf-1 polyclonal antibodies (each diluted 1/1,000) or with MAbs specific for 14-3-3τ (7), PKCθ (Transduction Laboratories), or actin (clone C4; ICN Biochemicals). Following washing, binding was detected with secondary horseradish peroxidase-conjugated sheep anti-mouse or donkey anti-rabbit immunoglobulins (ECL Western blotting detection system; Amersham).

To assess direct interactions between 14-3-3τ and electrophoresed, nitrocellulose-bound proteins, membranes containing Ni^{2+}-NTA-purified PKCθ or immunoprecipitated Raf-1 were prepared as described above, incubated at 4°C overnight with 10 μg of GST-14-3-3τ or GST (as a negative control) per ml, and washed. Bound GST proteins were detected by incubating the membrane with an anti-GST MAb (Santa Cruz) for 3 h, followed by a horseradish peroxidase-conjugated sheep anti-mouse antibody.

Cell fractionation. Cells were resuspended in buffer B [20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 10 mM β-mercaptoethanol, 10 μg (each) of leupeptin and aprotinin per ml], transferred to a 1-ml syringe, and sheared by being passed

30 times through a 25-gauge needle. The lysates were centrifuged at 280 × *g* for 7 min to precipitate nuclei, and the supernatant was collected. An aliquot of the whole-cell extract was saved, and the remainder was centrifuged at 16,000 × *g* for 20 min. The supernatant (cytosol) was collected, and the pellet was washed in buffer B, resuspended in buffer B containing 1% NP-40 for 30 min on ice, and centrifuged again at 16,000 × *g*. The supernatant representing the particulate (membrane) fraction was saved, and the detergent-insoluble fraction (cytoskeleton) was resuspended in 5× SDS sample buffer (5× SDS sample buffer is 0.3125 M Tris-HCl [pH 6.8], 10% SDS, 50% glycerol, 25% β-mercaptoethanol) and then diluted to 1×. Each fraction was resuspended in the same volume as that of the original extract. Samples containing 10^6 cell equivalents from each fraction were separated by SDS–10% PAGE and immunoblotted as indicated in the figure legends.

IL-2 quantitation. The amount of IL-2 secreted by Jurkat-TAg cells was determined by comparison with a human IL-2 standard using a commercially available enzyme-linked immunosorbent assay kit (Endogen), according to the manufacturer's protocol.

RESULTS

Association of 14-3-3τ with PKCθ in intact T cells. In order to test whether 14-3-3 can associate with PKC in intact cells, we cotransfected Jurkat-TAg cells with 14-3-3τ and histidine-tagged PKCθ expression vectors and precipitated the recombinant PKCθ with a Ni^{2+}-NTA resin. Washed precipitates were separated by SDS-PAGE, and nitrocellulose-bound proteins were blotted with anti-14-3-3τ or anti-PKCθ antibodies. The tagged PKCθ bound to the resin or in whole-cell extracts was detected by an anti-PKCθ serum (Fig. 1A, top blot). The results (Fig. 1A, bottom blot) indicate that 14-3-3τ coprecipitated with PKCθ from cells transfected with both cDNAs, but not from cells transfected with PKCθ alone. Our polyclonal anti-PKCθ serum is not sufficiently sensitive to recognize PKCθ at endogenous levels in whole-cell extracts (4); however, it readily detected PKCθ in extracts of the PKCθ-transfected cells (Fig. 1A, top blot). The lack of detectable endogenous 14-3-3 in Ni^{2+}-NTA precipitates from cells transfected with PKCθ alone (bottom blot) may reflect the low stoichiometry of this association. Indeed, when more cells and a higher concentration of the anti-14-3-3τ MAb were used, we could detect coimmunoprecipitation of endogenous 14-3-3τ and PKCθ from untransfected Jurkat cells (Fig. 1B).

To further evaluate the ability of 14-3-3τ to bind PKCθ, we incubated lysates from PKCθ-transfected cells with recombinant GST or a GST-14-3-3τ fusion protein. As shown in Fig. 1C, GST-14-3-3τ bound PKCθ from cells overexpressing the kinase. Binding of endogenous PKCθ (in control vector-transfected cells) was not detected under these conditions.

14-3-3τ binds PKCθ directly. To determine whether the association between PKCθ and 14-3-3τ is direct, we used the Far-Western technique to probe electrophoresed, Ni^{2+}-NTA-precipitated PKCθ with an *E. coli*-derived recombinant GST-14-3-3τ fusion protein and detected binding with an anti-GST MAb. For a positive control, we used in parallel an immunoprecipitate of Raf-1, a kinase known to associate directly with 14-3-3 proteins (16, 17, 25, 26, 30, 62). As shown in Fig. 2A, GST-14-3-3τ, but not the control GST protein, bound to a ~80-kDa protein precipitated from PKCθ-transfected cells. The same protein was not detected in cells transfected with empty pEF vector, and it comigrated with authentic PKCθ detected by anti-PKCθ immunoblotting (Fig. 2B). As expected, specific binding of 14-3-3τ to Raf-1 was also detected under the same conditions (Fig. 2). Binding to Raf-1 appeared to be more effective, since more 14-3-3τ bound to endogenous Raf-1 from 5×10^6 untransfected cells than to overexpressed PKCθ from 15×10^6 transfected cells. These results indicate that 14-3-3τ can bind PKCθ directly.

14-3-3τ inhibits PKCθ-mediated activation of the IL-2 promoter. In order to examine potential functional interactions between 14-3-3τ and PKCθ in intact T cells, we analyzed the effects of 14-3-3τ overexpression on a PKC-dependent event, namely, activation of the IL-2 promoter, in transiently transfected T cells. We reported previously that PKCθ can activate the complete IL-2 promoter as well as isolated elements of this promoter in murine EL4 leukemic T cells (3, 5). Similarly, PKCθ can activate a reporter plasmid containing the IL-2 proximal promoter attached to a CAT gene in Jurkat cells (Fig. 3). Thus, transiently transfected wt PKCθ stimulated reporter gene activity in Jurkat cells stimulated with PMA plus ionomycin to a level four- to fivefold higher than in cells transfected with the empty pEF vector. As demonstrated before for several PKC isoforms (5, 13), mutation of a conserved alanine residue in the pseudosubstrate region to glutamic acid (A148E) caused constitutive activation of PKCθ, which was manifested by the ability of this mutant protein to activate the reporter in the presence of ionomycin alone. For a control, we also assessed the effect of an inactive PKCθ mutant (K409R) which cannot bind ATP. As expected, this protein was incapable of stimulating the IL-2 promoter and, moreover, inhibited PMA-plus-ionomycin-stimulated activity by ~50% compared with cells transfected with the empty pEF vector (Fig. 3).

Next, we examined the effect of 14-3-3τ overexpression on

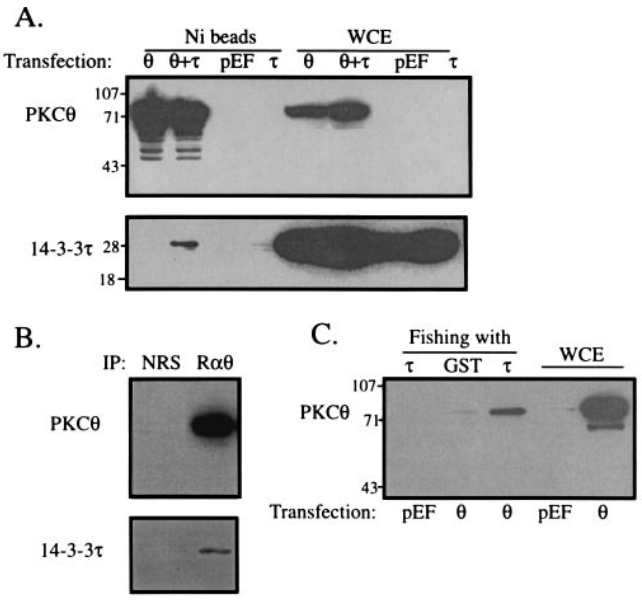

FIG. 1. Association of 14-3-3τ with PKCθ in T cells. (A) Jurkat-TAg cells (2×10^7) were transfected with pEFneo-PKCθ (θ), pEFneo-14-3-3τ (τ), a combination of pEFneo-PKCθ plus pEFneo-14-3-3τ (θ+τ) (9 and 1 μg of DNA, respectively), or control pEFneo alone (pEF). The total amount of plasmid DNA introduced into each group of cells was adjusted to 10 μg by supplementation with empty pEF DNA as necessary. The cells were lysed 48 h later in TN1 buffer. Lysates from 10^7 cell equivalents which were incubated with Ni^{2+}-NTA resin for 4 h and washed (Ni beads) or whole-cell extracts (WCE) from 10^6 cell equivalents were suspended in sample buffer, separated by SDS-PAGE, transferred to nitrocellulose, and analyzed by immunoblotting with rabbit anti-PKCθ (upper blot) or anti-14-3-3τ (lower blot) MAb. The results are representative of six similar experiments. (B) A similar experiment demonstrating the coimmunoprecipitation of endogenous PKCθ with 14-3-3τ from untransfected Jurkat cells. Lysates from 3×10^7 cells were immunoprecipitated (IP) with our polyclonal rabbit anti-PKCθ serum (Rαθ) or, as a negative control, normal rabbit serum (NRS). The membrane was immunoblotted with an anti-PKCθ MAb. A similar result was obtained in three of six experiments. (C) Lysates from PKCθ (θ) or control vector (pEF)-transfected cells were incubated with GST or GST-14-3-3τ, and bound PKCθ was detected by immunoblotting with the corresponding antibody. PKCθ immunoblots of whole-cell extracts (WCE) are shown for comparison. Similar results were obtained in three separate experiments. Molecular mass markers (in kilodaltons) are indicated.

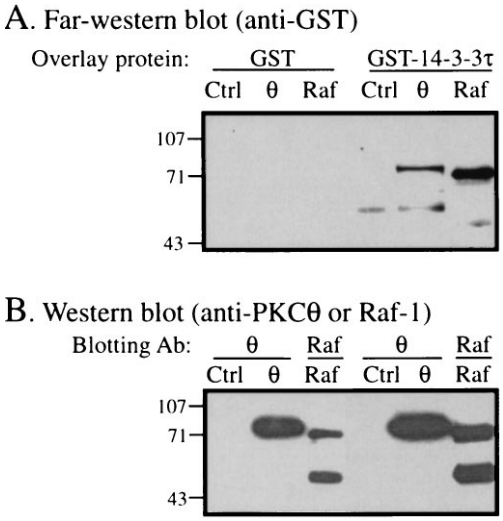

FIG. 2. Direct binding of recombinant 14-3-3τ to PKCθ. Jurkat-TAg cells were transfected with 5 μg of pEFneo-PKCθ (θ) or the control (Ctrl), empty pEFneo DNA. After 48 h, the cells were lysed and recombinant PKCθ was precipitated in duplicate from 15×10^6 cell equivalents with Ni^{2+}-NTA beads. As a positive control, Raf-1 (Raf) was immunoprecipitated in parallel from 5×10^6 cells. The precipitates were washed, separated by SDS-PAGE, and transferred to nitrocellulose membranes. (A) The membrane was overlaid with 10 μg of recombinant GST or GST-14-3-3τ fusion protein per ml, and binding was detected with an anti-GST MAb. (B) The membrane was stripped and reprobed with anti-PKCθ or anti-Raf-1 polyclonal antibodies to demonstrate the presence of the corresponding precipitated proteins. Similar results were obtained in two additional experiments. Ab, antibody. Molecular mass markers (in kilodaltons) are indicated.

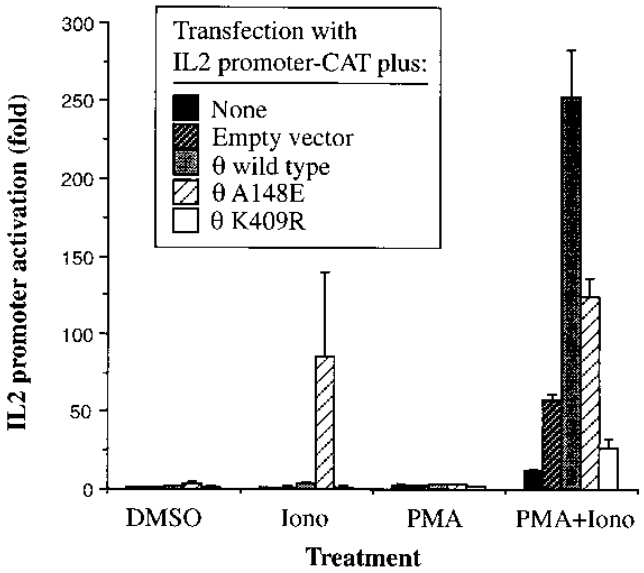

FIG. 3. Activation of the IL-2 promoter by PKCθ. Jurkat-TAg cells were transfected with 5 μg of IL-2 promoter-CAT reporter plasmid alone or in combination with 15 μg of empty pEF, wild-type PKCθ, constitutively active PKCθ (A148E), or inactive PKCθ (K409R). After 24 h, the cells were stimulated in triplicate with 1 μg of ionomycin (Iono) per ml, 5 ng of PMA per ml, or a combination of the two. The cells in the control groups received an equal volume of the solvent (DMSO). The cells were harvested after an additional 24 h, and CAT activity was measured. Activity is expressed as fold activation over the CAT activity in unstimulated cells transfected with the reporter gene alone. Similar results were obtained in another experiment.

the PKCθ-mediated activation of the IL-2 promoter by cotransfecting Jurkat-TAg cells with PKCθ plus increasing amounts of 14-3-3τ DNA. 14-3-3τ inhibited activation of the IL-2 promoter by both wt and constitutively active PKCθ in a dose-dependent manner (Fig. 4A). Maximal inhibition of ~95% (after subtracting background reporter activity in the presence of the empty pEF vector) was obtained with 5 to 10 μg of 14-3-3τ DNA, i.e., an approximately one- to twofold molar excess over the amount of the cotransfected PKCθ. Immunoblotting with anti-PKCθ antibodies demonstrated that transfection with 14-3-3τ did not affect the expression level of wt PKCθ (Fig. 4B) or A148E (which was expressed at a considerably lower level than the wt protein) (Fig. 4C), thus excluding the possibility that inhibition results from decreased PKCθ expression in the presence of 14-3-3τ. As shown later (see Fig. 9), inhibition was also observed when a similar experiment was performed with regular (non-TAg) Jurkat cells in which transfected 14-3-3τ is not overtly overexpressed. Thus, the inhibition of IL-2 promoter activation by 14-3-3τ is unlikely to be an artifact resulting from nonphysiological levels of 14-3-3τ overexpression.

In order to demonstrate the inhibitory activity of 14-3-3τ on activation of the IL-2 promoter in a more physiological system, we also assessed the effect of transient 14-3-3τ transfection on IL-2 promoter activation by endogenous PKC enzymes. Jurkat (non-TAg) cells were stimulated with PMA plus ionomycin in order to provide the requisite signals for IL-2 gene induction. Compared with a ~40-fold activation of the IL-2 promoter in PKCθ-transfected cells, CAT activity was stimulated to a lower extent (~8-fold) in cells transfected with the empty vector, a level which reflects the activation of endogenous PKC (Fig. 5A). When the cells were cotransfected with the 14-3-3τ expression vector, stimulation of the IL-2 promoter decreased from approximately eight- to twofold in a manner dependent on the dose of the 14-3-3τ vector DNA (Fig. 5B). This result is in agreement with the previous findings with PKCθ-transfected cells (Fig. 4A) and demonstrates that 14-3-3τ also inhibits IL-2 promoter activation mediated by endogenous PKC.

14-3-3τ does not inhibit Ca^{2+}-dependent promoter activation. In order to ascertain the specificity of the inhibitory effect of 14-3-3τ on IL-2 promoter activation and to exclude nonspecific suppressive effects, we examined the effects of 14-3-3τ overexpression on the activation of an unrelated promoter element. We used for this purpose a CAT reporter construct containing multimerized tandem NF-AT and AP-1 elements derived from the IL-4 promoter. The NF-AT element in this plasmid is activated in a PKC-independent manner by ionomycin alone (49, 50), unlike the NF-AT element in the IL-2 promoter which overlaps with an AP-1 site and, therefore, is both PKC and Ca^{2+} dependent (6, 21). Indeed, ionomycin, but not PMA, stimulated CAT activity three- to fourfold compared with unstimulated (DMSO-treated) cells in Jurkat cells transfected with this reporter construct (Fig. 6). Importantly, cotransfection with the 14-3-3τ expression vector did not inhibit this effect. Overexpression of 14-3-3τ inhibited the PMA-plus-ionomycin-driven activation of the IL-2 promoter by 50%, i.e., from ~20- to ~10-fold stimulation, in the same experiment (result not shown). These results indicate that 14-3-3τ overexpression does not lead to a general suppression of T-cell activation.

14-3-3τ inhibits IL-2 secretion mediated by PKCθ. To confirm the inhibitory effect of 14-3-3τ on the PKCθ-mediated activation of the IL-2 promoter by an independent and a physiologically more relevant assay, we studied the effect of 14-3-3τ overexpression on PKCθ-mediated IL-2 secretion. IL-2 production was quantified in PMA-plus-ionomycin-stimulated

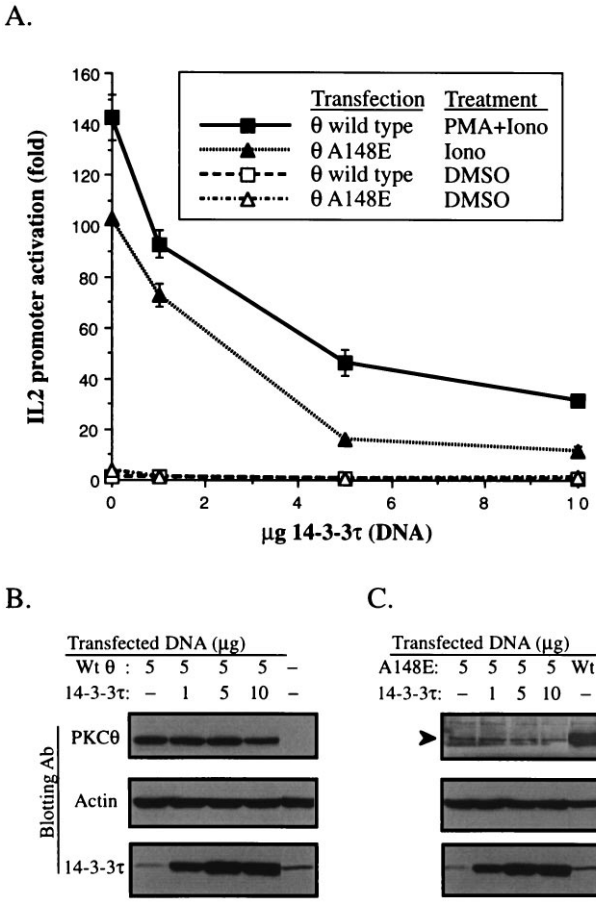

FIG. 4. 14-3-3τ inhibits PKCθ-mediated activation of the IL-2 promoter. Jurkat-TAg cells were triple transfected with 5 μg of IL-2 promoter-CAT reporter plasmid, 5 μg of wt or constitutively active (A148E) PKCθ plasmid, and 0 to 10 μg of pEFneo-14-3-3τ plasmid. After 24 h, the cells were treated in triplicate for an additional 24 h with ionomycin (Iono) (1 μg/ml), ionomycin plus PMA (5 ng/ml), or solvent (DMSO), as indicated. (A) CAT activity. Values are expressed as fold activation over the CAT activity in unstimulated cells cotransfected with 20 μg of empty vector plus 5 μg of IL-2 promoter-CAT reporter plasmid. The levels of background CAT activity in stimulated cells transfected with empty pEF (instead of PKCθ) were 25-fold (PMA plus ionomycin) and 3-fold (ionomycin). Similar results were obtained in three (wt) and two (A148E) additional experiments. (B) SDS-PAGE-separated lysates from wt-PKCθ-transfected cells (10^6 cell equivalents) were analyzed by immunoblotting with anti-PKCθ, anti-actin, or anti-14-3-3τ antibodies (Ab). The amounts of PKCθ and 14-3-3τ vector DNA are indicated above each lane. (C) Similar immunoblot analysis of lysates from PKCθ A148E-transfected cells. The lower expression level of A148E is evident from comparison of the A148E signal with the wt PKCθ signal.

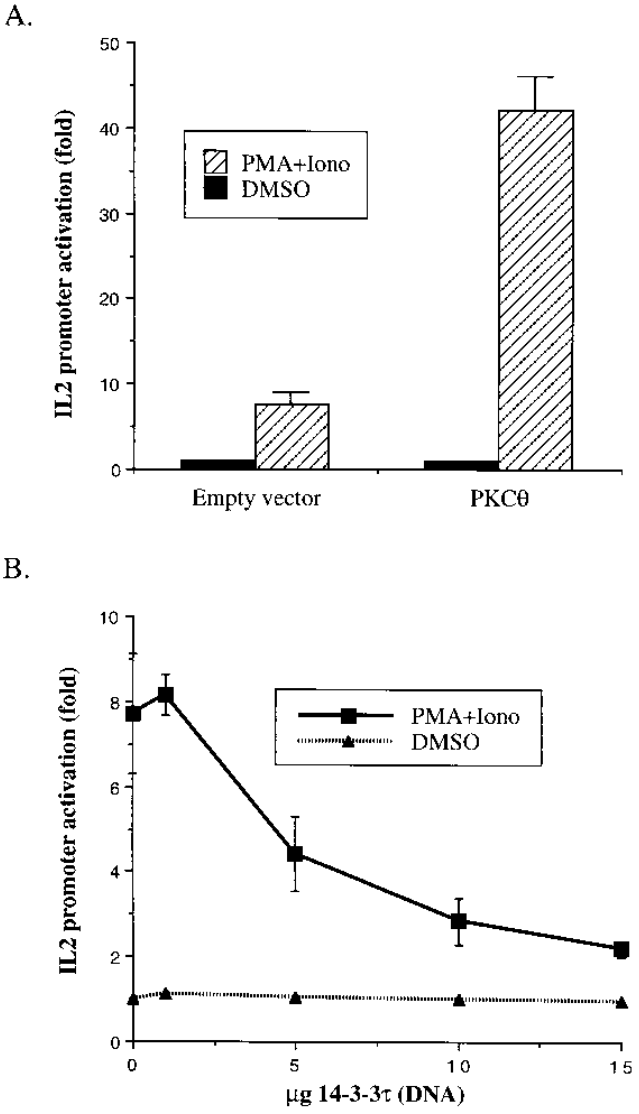

FIG. 5. 14-3-3τ inhibits IL-2 promoter activation by endogenous PKC. (A) IL-2 promoter activation in unstimulated (DMSO) (black bars) or PMA-plus-ionomycin (PMA+Iono)-stimulated (hatched bars) Jurkat cells cotransfected with 5 μg of IL-2 promoter-CAT reporter plasmid and 20 μg of empty pEFneo or pEFneo-PKCθ plasmid DNA. This result is representative of three experiments. (B) Jurkat cells were cotransfected with 5 μg of IL-2 promoter-CAT reporter plasmid plus 0 to 15 μg of pEFneo-14-3-3τ plasmid DNA. Each transfection was supplemented with the necessary amount of empty pEFneo plasmid DNA in order to adjust the total amount of plasmid DNA to 25 μg. After 24 h, the cells were treated in triplicate for an additional 24 h with solvent (DMSO) or with ionomycin (Iono) (1 μg/ml) plus PMA (5 ng/ml). The cells were harvested, and CAT activity was measured. Values are expressed as fold activation over the CAT activity in unstimulated cells cotransfected with 20 μg of empty vector plus 5 μg of IL-2 reporter.

Jurkat-TAg cells which were cotransfected with wt PKCθ plus 14-3-3τ. Consistent with the results shown in Fig. 5A, stimulated, empty vector-transfected cells produced IL-2 (~115 pg/ml), and this amount increased substantially (to ~300 pg/ml) in cells transfected with the PKCθ expression vector (Fig. 7). When the cells were cotransfected with pEF-14-3-3τ, the incremental IL-2 secretion stimulated by the transfected PKCθ was inhibited by 62%. Thus, the inhibitory effect of 14-3-3τ on IL-2 gene induction is also evident at the level of the endogenous IL-2 gene.

14-3-3τ overexpression inhibits PKCθ translocation from the cytosol to the membrane. IL-2 transcription depends on PKC activation, and translocation of PKC to the plasma membrane is one hallmark of its activation (32, 36, 41). Therefore, it is possible that 14-3-3τ suppresses PKCθ-dependent IL-2 promoter activation by tightly associating with PKCθ in the cytosol and inhibiting its translocation to the particulate fraction. We addressed this possibility by determining whether 14-3-3τ overexpression will affect PMA-induced translocation of PKCθ. Jurkat cells which were transfected with PKCθ alone or together with 14-3-3 cDNA were stimulated with PMA. Levels of PKCθ and 14-3-3τ in the cytosolic, particulate (membranes), or NP-40-insoluble (cytoskeleton) fractions from these or unstimulated cells were assessed by immunoblotting

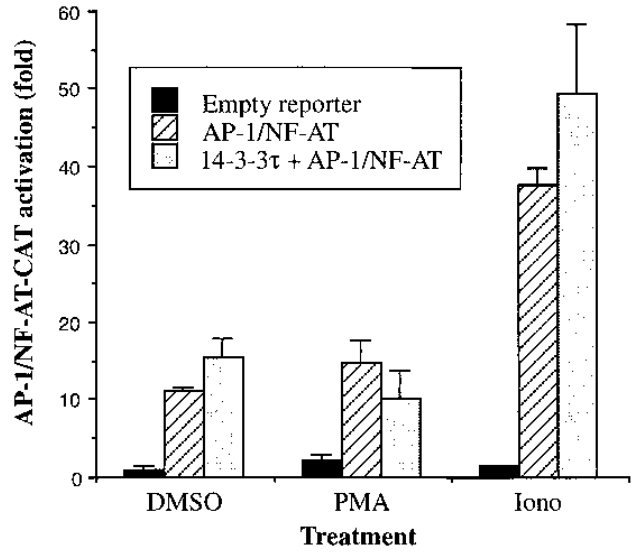

FIG. 6. 14-3-3τ does not inhibit activation of an IL-4 promoter-derived NF-AT element by ionomycin. Jurkat cells were cotransfected with 5 μg of the reporter plasmid pSVO-5× AP-1/NF-AT-CAT (50) plus either 20 μg of empty pEFneo (hatched bars) or 10 μg of pEF-neo plus 10 μg of pEFneo-14-3-3τ (gray bars). After 24 h, the cells were treated with 5 ng of PMA per ml, 1 μg of ionomycin (Iono) per ml, or solvent (DMSO) for an additional 24 h. The cells were harvested and lysed, and CAT activity was determined. Values are expressed as fold activation over the basal activity (onefold) obtained in unstimulated cells cotransfected with the empty reporter vector pSVO-CAT plus 20 μg of pEFneo (black bars).

with the corresponding antibodies (Fig. 8). As a control for the quality of the cellular fractionation, we also assessed the expression of endogenous PKCα and -β using a cross-reactive antiserum that recognizes these two enzymes (19). We have shown previously (3) and confirm here (Fig. 8A) that these two

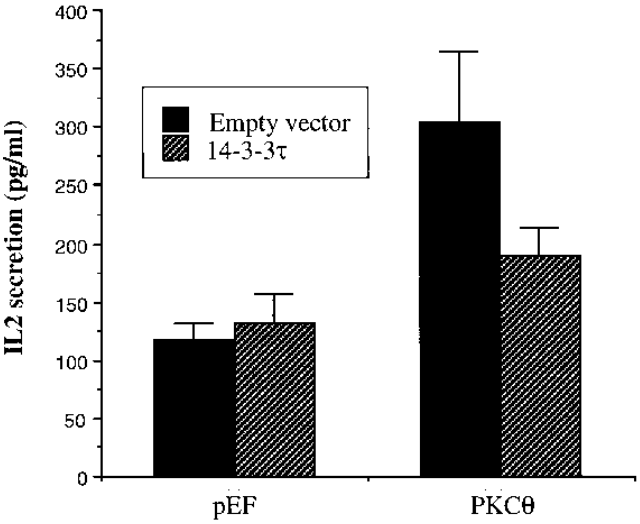

FIG. 7. 14-3-3τ inhibits PKCθ-mediated IL-2 secretion. Jurkat-TAg cells were transfected with 5 μg of empty pEF or pEFneo-PKCθ (wt) plasmid DNA plus 15 μg of pEFneo (black bars) or pEFneo-14-3-3τ (hatched bars). After 24 h, aliquots of the transfected cells (5×10^5/ml) were stimulated in triplicate with 5 ng of PMA per ml plus 1 μg of ionomycin per ml and supernatants were collected after an additional 24-h culture period for determination of IL-2 titers as described in Materials and Methods. The concentration of IL-2 in parallel samples of unstimulated (DMSO-treated) cells was below the detection level of the assay. This experiment was repeated twice with similar results.

isoforms are expressed exclusively in the cytosol of resting Jurkat cells and undergo a near-complete translocation to the particulate fraction upon PMA treatment. Expression of actin as an indirect measure of protein loading was also determined.

In cells transfected with PKCθ alone, a large fraction (~40%) of the enzyme was present in the cytosol before stimulation; ~10 and ~50% were localized in the particulate and detergent-insoluble fractions, respectively. PMA treatment caused a nearly complete translocation of cytosolic PKCθ to the particulate fraction (Fig. 8A, top blot). Overexpression of wt 14-3-3τ (14-3-3wt) did not have a significant effect on PKC distribution in unstimulated cells but inhibited PMA-induced translocation such that only ~40% of the cytosolic PKCθ redistributed to the membrane after stimulation (Fig. 8B, top blot).

In order to further address the association between PKCθ and 14-3-3τ in intact cells, we generated a modified form of 14-3-3τ to which the membrane-targeting sequence CAAX (C = cysteine, A = aliphatic amino acid, X = any amino acid) from K-Ras (18) was added at its N terminus. We then examined whether this membrane-targeted 14-3-3τ (14-3-3mm) would induce increased translocation of PKCθ to the particulate fraction. Indeed, in 14-3-3mm-cotransfected cells, a large fraction of PKCθ was present in the particulate fraction in the absence of any stimulation (compare the top blot of Fig. 8C with the top blots of Fig. 8A and B); PMA did not increase this effect. Under all transfection conditions, a sizeable proportion of PKCθ (unlike PKCα and -β) was present in the NP-40-insoluble fraction of the unstimulated cells, and PMA stimulation did not affect this level. Endogenous 14-3-3τ was localized exclusively in the cytosol (Fig. 8A, top blot), and a small fraction of 14-3-3τ was localized in the particulate and detergent-insoluble fractions in both 14-3-3wt or 14-3-3mm-transfected cells either before or after PMA stimulation (Fig. 8B and C, top blots). It is difficult to interpret potential effects of transient 14-3-3τ overexpression on PKCα and -β localization in this experiment, since endogenous PKCα and -β are expressed in the entire cell population, whereas the transfected 14-3-3τ protein is expressed in only a fraction of cells.

The stimulus-independent PKCθ translocation to the particulate fraction in 14-3-3mm-expressing cells is consistent with the data demonstrating an in vitro association between 14-3-3τ and PKCθ and suggests that the membrane-localized 14-3-3 is associated with PKCθ in situ. Nevertheless, immunoblotting with anti-14-3-3τ did not reveal an increased level of expression of 14-3-3mm in the particulate or detergent-insoluble fractions by comparison with 14-3-3wt (compare the top blots in Fig. 8B and C). This may reflect the fact that only a minority of 14-3-3mm actually becomes farnesylated in the transfected cells, and/or that membrane-binding sites for farnesylated 14-3-3mm are largely saturated in the face of overwhelming 14-3-3 overexpression. If so, the marked PKCθ translocation under these conditions suggests a high stoichiometry of a putative PKCθ–14-3-3τ complex in situ.

Effect of membrane-targeted 14-3-3τ on IL-2 promoter activation. The partial inhibition of PMA-induced PKCθ translocation to the membrane by 14-3-3τ may be the mechanism by which 14-3-3 inhibits PKC-mediated IL-2 promoter activation. Alternatively, or in addition, the association between PKCθ and 14-3-3τ may in itself inhibit PKC-dependent functions. Thus, we examined whether the membrane-targeted form of 14-3-3τ (which does not inhibit PKCθ translocation [Fig. 8C]) also inhibits the PKCθ-mediated IL-2 promoter activation. The effects of transfected 14-3-3wt and 14-3-3mm proteins on promoter activation were compared in transient cotransfection assays. 14-3-3mm inhibited the PKCθ-mediated activation of

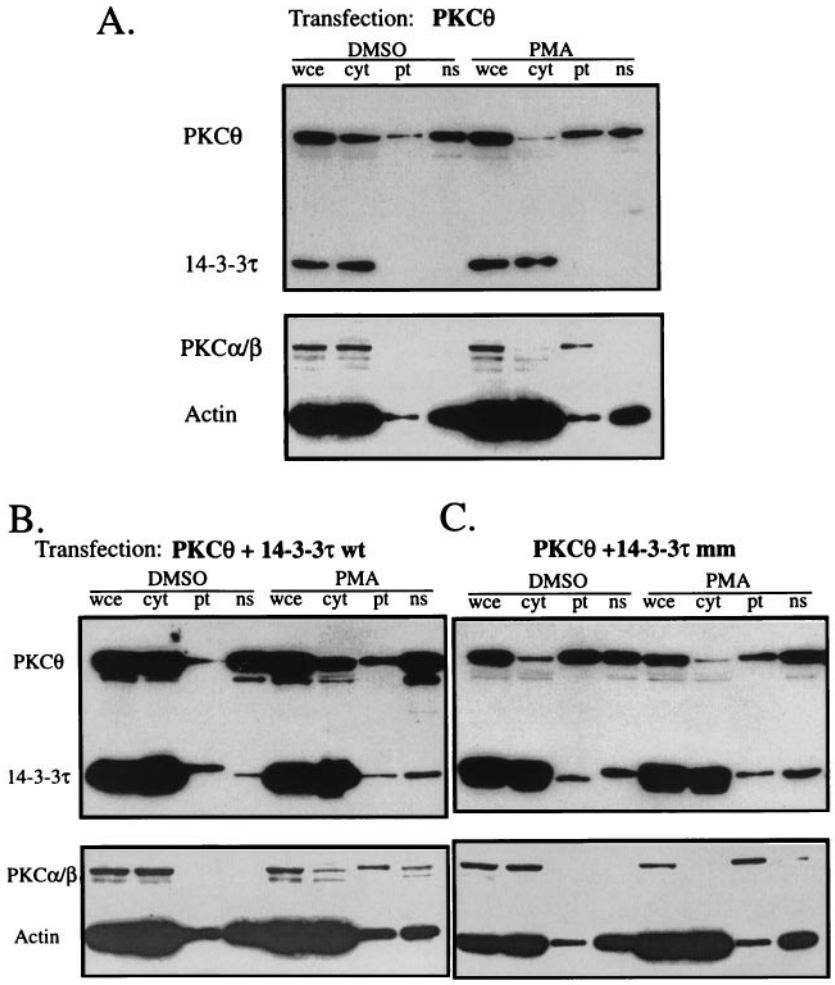

FIG. 8. Effects of wild-type or membrane-targeted 14-3-3τ overexpression on the intracellular localization of PKCθ. Jurkat-TAg cells were cotransfected with 10 μg of pEFneo-PKCθ plus 10 μg of empty pEFneo (A), 10 μg of pEFneo-PKCθ plus 10 μg of pEFneo-wild-type (wt)-14-3-3τ (B), or 10 μg of pEFneo-PKCθ plus 10 μg of pEFneo-membrane-targeted (mm)-14-3-3τ (C). After 48 h, the cells were treated for 20 min with PMA (50 ng/ml) or with solvent (DMSO). The cells were lysed, and whole-cell extracts (wce) were prepared. A sample of each extract was fractionated into cytosolic (cyt), particulate (pt), and NP-40-nonsoluble (ns) fractions, which were separated by SDS-PAGE, transferred to nitrocellulose in duplicate, and immunoblotted with anti-PKCθ and anti-14-3-3τ antibodies (upper blots) or with an anti-actin MAb and a polyclonal PKCα- and -β-specific antiserum (lower blots) as indicated. Similar results were obtained in another experiment.

the IL-2 promoter to a markedly higher degree than 14-3-3wt. Thus, maximal inhibition was higher, and 50% inhibition was obtained with ~1 μg of 14-3-3mm compared with ~8 μg of 14-3-3wt DNA, respectively (Fig. 9A). This effect was seen despite the fact that 14-3-3mm was expressed at a level similar to, or even lower than, 14-3-3wt (Fig. 9B). To maximize the difference between the two 14-3-3τ forms, we used in this experiment regular Jurkat cells in which the expression levels of transfected DNAs are lower than in Jurkat-TAg cells. Indeed, maximal inhibition of IL-2 promoter activity in these cells required ~10 μg of 14-3-3τ (wt) DNA (Fig. 9A) compared with only ~5 μg in Jurkat-TAg cells (Fig. 4A). These results suggest that 14-3-3τ inhibits the function of PKCθ not only by preventing its translocation to the membrane but also by merely associating with it.

DISCUSSION

14-3-3 proteins were recently found to associate with and, in some cases, modulate the activity of, several proto-oncogene and oncogene products. One of the earliest activities attributed to 14-3-3 proteins was modulation of PKC activity, based on in vitro studies which yielded conflicting results (11, 20, 34, 46, 57–59). In order to address the potential interaction between these two protein families in a more rigorous manner and to explore its physiological significance, we have used recombinant 14-3-3τ in conjunction with transfection assays and analysis of physical and functional interactions in intact T cells. The present work demonstrates that PKCθ and 14-3-3τ associate in vitro and in intact cells, and this association is direct (at least in vitro). In Jurkat cells, 14-3-3τ overexpression induces marked effects which include inhibition of PMA-induced PKCθ translocation from the cytosol to the membrane, increased stimulus-independent localization of PKCθ to the membrane by membrane-targeted 14-3-3τ, and profound inhibition of IL-2 gene transcription.

The association between PKCθ and 14-3-3τ was demonstrated by the following: (i) coprecipitation of the endogenous or transfected proteins, (ii) the ability of a GST-14-3-3τ fusion protein to bind PKCθ from cell lysates and (iii) direct binding of recombinant 14-3-3τ to PKCθ in overlay assays. Previous attempts to demonstrate an association between 14-3-3 and

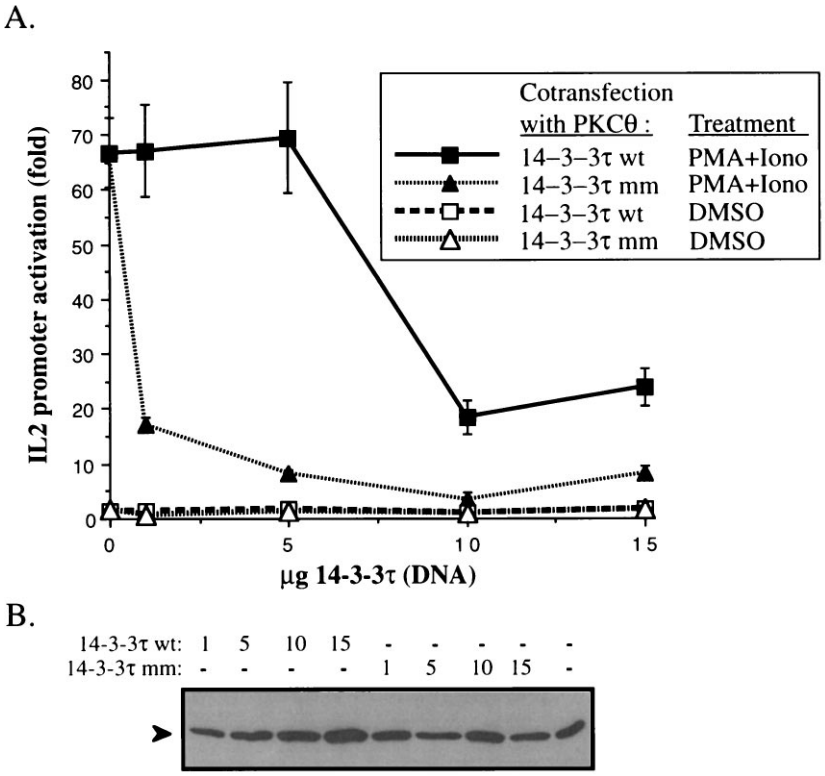

FIG. 9. Inhibition of PKCθ-mediated activation of the IL-2 promoter by wild-type and membrane-targeted 14-3-3τ. (A) Jurkat cells were triple transfected with 5 μg of IL-2 promoter-CAT reporter plasmid, 5 μg of pEFneo-PKCθ DNA, and 0 to 15 μg of pEFneo-14-3-3wt or pEFneo-14-3-3mm. The total amount of the pEFneo DNA was adjusted to 20 μg in each case by adding the necessary amount of empty pEFneo. After 24 h, the cells were treated in triplicate with ionomycin (Iono) (1 μg/ml) plus PMA (5 ng/ml) or with an equal volume of solvent (DMSO) for an additional 24 h. The cells were harvested and lysed, and CAT activity was measured. The values are expressed as fold activation over the CAT activity in unstimulated cells cotransfected with 20 μg of empty vector plus 5 μg of the IL-2 reporter. (B) Expression of 14-3-3τ. Extracts (10^6 cell equivalents) from the different transfection groups were analyzed by immunoblotting with an anti-14-3-3τ MAb. The position of the immunoreactive 14-3-3τ band is indicated by the arrowhead. These results are representative of two experiments.

PKC have failed (8, 17). This could be due to one of several reasons. First, the low stoichiometry of the association between 14-3-3 and PKC in vitro may confound its detection. In our hands, detection of this association required more-sensitive blotting conditions (in the absence of overexpression of both proteins) and was not observed consistently. It is possible, however, that in intact cells the stoichiometry of this association may in fact be considerably higher. Lysis conditions, or denaturation of the membrane-bound PKCθ used in the overlay assay, may be unfavorable for maintaining an optimal PKCθ–14-3-3 association. Second, it is also possible that optimal interaction between PKCθ and 14-3-3τ requires an additional factor (e.g., a protein or lipid), consistent with the findings that, in the cell, 14-3-3 proteins form dimers (22, 23) that are potentially capable of simultaneously binding two ligands (8, 27, 61). Finally, members of the 14-3-3 protein family may interact specifically with distinct PKC isoforms (as well as with other 14-3-3 ligands). Thus, PKCα, which has previously been screened as a potential 14-3-3 ligand (8, 17), may not interact with the 14-3-3 isoforms used in those studies, i.e., β and ζ.

At present, the molecular basis of the direct association between 14-3-3 and PKC is unknown. However, distinct regions in both proteins can be considered as likely candidates. Several 14-3-3 ligands, i.e., Raf-1, Bcr, and polyomavirus middle tumor antigen, contain a cysteine-rich domain and, in the case of the first two, this domain was found to be essential for 14-3-3 binding (8, 16, 17, 30). More specifically, Cys-165 and Cys-168 in Raf-1 are important for this interaction since mutation of these residues, which have corresponding homologs in PKC (i.e., Cys-190 and Cys-193 in PKCθ), abolished the ability of Raf-1 to associate with 14-3-3 (30). This implies that the cysteine-rich domain of PKCθ might be involved in the association with 14-3-3. However, PKC does not display a serine-rich domain which, in the case of Raf-1 and Bcr, was also found to contribute to 14-3-3 binding (8, 30). This effect may be mediated by phosphorylated serine residues (30).

With regard to 14-3-3, residues 122 to 137 of 14-3-3τ and similar, highly conserved sequences in other 14-3-3 isoforms, show a strong homology to a conserved sequence at the C-terminal region of annexins. Members of the annexin family were found to bind PKC (33) and inhibit its activity (52), and RACK1, a recently isolated cellular receptor for activated PKC, also shows homology to the same region of annexin (47). Moreover, a synthetic peptide based on this region of annexin I was found to bind PKC, inhibit the binding of RACKs to PKC (33), and inhibit translocation of PKC to the particulate fraction of *Xenopus* oocytes (48). Similarly, a homologous annexin II-derived peptide inhibited 14-3-3-mediated exocytosis (51). Thus, the corresponding sequence of 14-3-3 may mediate binding to PKC and other 14-3-3 ligands. Interestingly, this sequence is located in a region that defines one corner of the ligand-binding pocket in the crystal structure of 14-3-3τ (61).

The apparently low stoichiometry of the association between PKCθ and 14-3-3τ raises a question about its biological importance. The ability of wild-type 14-3-3τ to inhibit by >50% the PMA-induced translocation of PKCθ to the membrane, and

the predominant membrane expression of PKCθ in unstimulated cells expressing membrane-targeted 14-3-3τ, albeit falling short of proving direct association in vivo, is consistent with such an association, and implies a potent and functionally meaningful interaction between the two proteins in the cell. This is also supported by the profound inhibition of IL-2 promoter activation mediated by 14-3-3τ overexpression.

IL-2 promoter activation represents a distal event in a complex signaling cascade which involves discrete steps occurring both upstream and downstream of PKC. Thus, it is not clear whether 14-3-3τ acts directly on PKC itself or indirectly via interaction with a PKC-coupled signaling element. For example, Raf-1, which binds 14-3-3 (16, 17, 25, 26, 30, 62), is an important element in the pathway leading to IL-2 gene induction (43), and Raf-1 can be activated by PKC (24, 54). The possibility that 14-3-3 interferes with an upstream signaling event essential for PKC activation is unlikely since 14-3-3 overexpression inhibited IL-2 promoter activation by constitutively active PKC. However, several lines of evidence do suggest that the target for 14-3-3-mediated inhibition is PKC itself. First, we have demonstrated an association between PKCθ and 14-3-3τ in vitro and in intact cells, and this association appears to be direct in the overlay assay. Second, overexpression of wild-type or membrane-targeted 14-3-3τ affected the intracellular localization of PKCθ. Third, the reported effects of purified 14-3-3 proteins on PKC activity in vitro, while conflicting (11, 20, 34, 46, 57–59), also support a direct interaction. However, these arguments do not rule out the possibility that 14-3-3 also interferes with other discrete steps in the pathway leading to IL-2 production. The ability of 14-3-3 to bind several signaling proteins does suggest, in fact, that other regulatory interactions with 14-3-3 are likely to affect T-cell activation as well.

If 14-3-3τ functions to maintain PKCθ in the cytosol and prevent its activation and translocation to the particulate fraction, this effect may account for the inhibition of PKCθ-mediated IL-2 gene induction by overexpressed 14-3-3τ. However, our finding that 14-3-3τ overexpression also inhibits activation of the IL-2 promoter by constitutively active (i.e., cofactor-independent) PKCθ suggests that, in addition to preventing activation and membrane translocation of resting PKC, 14-3-3 can also inhibit the enzymatic activity of preactivated PKC, at least when expressed at sufficiently high levels. This inhibition may result from blocking the access of substrates or ATP to the catalytic site of PKC via complex formation with 14-3-3. The first possibility seems more likely since it was reported that 14-3-3-mediated inhibition of PKC in vitro does not occur by competition with ATP (58).

A major question raised by our findings is how 14-3-3 proteins, when bound to PKC, inhibit its enzymatic activity. Mochly-Rosen et al. recently characterized RACK proteins that act as cellular receptors for activated PKC and suggested that the affinity of PKC enzymes for their RACKs increases upon cofactor (e.g., diacylglycerol) binding (32). Thus, RACKs may function as important determinants of the substrate specificity of distinct PKC isoforms by acting as anchoring proteins that target activated PKC enzymes to particular cellular compartments in which their respective substrates are localized. On the basis of our results and the homology between 14-3-3 and RACK proteins, it is tempting to propose that 14-3-3τ functions as a PKCθ receptor which associates with PKCθ in its resting state in the cytosol. Upon activation, the affinity of PKCθ for 14-3-3τ may decrease. This will cause PKCθ to dissociate from 14-3-3τ and translocate to the membrane where it would become activated and bind to its specific RACK.

Clearly, additional studies, which are in progress, are needed in order to establish the validity of the model proposed by our findings. Furthermore, it will be important to determine whether this model can be generalized to other PKC and/or 14-3-3 isoforms. Since distinct PKC and 14-3-3 isoforms (as well as RACK proteins) are localized in different cellular compartments both before and after cellular activation, coordinate and isoform-specific interactions among distinct PKC and 14-3-3 proteins may provide a considerable level of flexibility for regulating multiple PKC-dependent functions. The findings reported herein open the way to additional studies that will establish the role of 14-3-3 proteins in regulating the activation and function of PKC in general and, more specifically, during T-cell activation.

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