The protein kinase C (PKC) family has been clearly implicated in T-cell activation as have several nonreceptor protein-tyrosine kinases associated with the T-cell receptor, including p59fyn. This report demonstrates that PKC and p59fyn specifically interact in vitro, in the yeast two-hybrid system, and in T-cells. Further indications of direct interaction are that p59fyn potentiates PKC catalytic activity and that PKC is a substrate for tyrosine phosphorylation by p59fyn. This interaction may account for the localization of PKC following T-cell activation, pharmacological disruption of which results in specific cell-signaling defects. The demonstration of a physical interaction between a PKC and a protein-tyrosine kinase expands the class of PKC-anchoring proteins (receptors for activated C kinases (RACKs)) and demonstrates a direct connection between these two major T-cell-signaling pathways.

Signal transduction networks comprise parallel pathways that achieve integrated responses at multiple levels including second messenger accumulation and activation of kinases and transcription factors. Two major pathways involve nonreceptor protein-tyrosine kinases (1) and the protein kinase C (PKC) family of serine/threonine kinases (2). Direct interaction between the ε (3) and ζ (4) members of the PKC family and particular protein-tyrosine kinase signaling proteins have recently been described. These reports are of interest because precedents for direct integration of serine/threonine and tyrosine kinase pathways by protein-protein interactions are rare, with perhaps the most prominent being identification of ras as a mediator between receptor tyrosine kinases and the MAPK pathway of serine/threonine kinases (5).

T-cell receptor (TCR) stimulation leads to hydrolysis of phosphatidylinositol-4,5-bisphosphate to produce diacylglycerol (DAG) and inositol-3,4,5-trisphosphate (6). These results, respectively, in direct activation of PKC and in increased intracellular calcium, which can then influence PKC activation among its other effects. Use of activators and inhibitors has established that PKC activation in T-cells is necessary but not sufficient for proliferation and production of characteristic cytokines (7). Furthermore, in the murine thymoma line EL-4, transiently overexpressing PKC, the PKC activator phorbol-12-myristate-13-acetate (PMA) increased transcription of a reporter gene regulated by the IL-2 promoter (8). Although T-cells express 10 of the 11 PKC isozymes (9), PKC is of special interest because it is the most nearly T-cell-restricted isozyme in its expression (10). In addition, activation of T-cells results in PKC translocation to the zone of TCR clustering present at the contact between the T-cell and an antigen-presenting cell (APC) (11).

PKC-mediated events can operate in a manner largely independent of other signaling pathways (12). A prominent parallel pathway following stimulation of the TCR involves activation of a tyrosine kinase signaling cascade (6). Among the protein-tyrosine kinases that have been described as reversibly associated with the TCR complex are src family members p59fyn and p56lck and syk family member ZAP-70 (6). p59fyn is notable for its low fractional stoichiometry of association with the TCR CD3 complex, which suggests a transient modulatory role, and for its associations with a wide range of other proteins implicated in T-cell signaling including ZAP-70 (13), c-cbl (14), fas (15), shc (16), IL-7 receptor (17), CD43 (18), α-tubulin (19), inositol-3,4,5-trisphosphate kinase (20) and the inositol-3,4,5-trisphosphate receptor (21). These interactions, mediated primarily through SH2 and SH3 domains, can be short lived. In the case of ZAP-70, for example, the association with p59fyn in a T-cell hybridoma was found as early as 10 s after activation, peaked at 5 min, and was gone by 10 min (13). Although 10 min is much longer than the interaction between a typical enzyme and substrate, it is short compared with the time course for expression of mitogenic cytokines, implying that a variety of other interactions are also likely to be involved in the regulation of cytokine secretion.

T-cells express one of the two splicing variants of p59fyn, the other being found primarily in brain (22). The enzyme is posttranslationally modified by N-terminal myristylation or palmitoylation, which is thought to facilitate membrane localization (23). Although clearly implicated in T-cell signaling by biochemical and genetic experiments using transgenic mice (24), the precise physiological functions of p59fyn remain unclear. It is believed to be involved in TCR-induced calcium release from intracellular stores mediated by the inositol-3,4,5-trisphosphate receptor (25), a process thought to be regulated in part by PKC as well. In other hematopoietic cells, p59fyn is associated with Btk (26) and with WASp (27); mutations in each have been implicated in human immune disorders.

Recent progress in the understanding of PKC function has focused on the fact that individual isozymes translocate from one cell compartment to other subcellular sites following physiological stimulation, often manifested as a shift in distribution.
from the soluble fraction to the particulate fraction. This phenomenon is believed to reflect a conformational change in PKC leading to specific interaction with particular anchoring proteins, designated receptors for activated C kinase (RACKs) (28). Restricting localization to defined sites within the cell represents a general strategy (29) that serves to limit the substrates exposed to the catalytic site of each PKC isozyme, providing physiological specificity for a family of enzymes that can phosphorylate a wide range of proteins in vitro. Two such anchoring proteins have been described in detail, RACK1, specific for βPKC (30), and RACK2, specific for εPKC (31). Each is found in the particulate fraction and shows saturable binding to its cognate PKC following stimulation. In addition to promoting localization, anchoring proteins can also potentiate PKC catalytic activity, presumably by stabilizing the active conformation (29). Both RACK1 and RACK2 have sequence homology to the WD-40 family of proteins previously implicated in signaling (32), and in both cases, the interaction is centered on the regulatory domain of PKC, which has the most extensive sequence variation within the PKC family. Notably, neither RACK serves as a substrate for PKC itself (30, 31). However, other PKC-binding proteins, several of which are implicated in regulating membrane-lysosome interactions, have been shown to be substrates (33). Of particular relevance to the present work, the pleckstrin homology domains of Tec family protein-tyrosine kinases Btk and Emt have been suggested as possible anchors (3). The more distantly related atypical class εPKC has been shown to both bind and phosphorylate ZIP, a protein thought to provide a scaffold-linking εPKC to cytokine receptor tyrosine kinases (4).

Given the pharmacologically established significance of PKC in general for the T-cell response (2) and the intracellular localization of εPKC in close proximity to the T-cell receptor following activation (11), specific functions for endogenous anchoring proteins of εPKC are likely. Support for this view comes from disruption of activation-induced translocation and anchoring of εPKC in Jurkat T-cells by overexpression of either the human immunodeficiency virus protein Nef (34) or the signaling protein 14-3-3 (35). In the latter case, PKC-dependent activation of the IL-2 promoter was shown to be inhibited. Recent work using electroporated antibodies to disrupt PKC function in peripheral blood lymphocytes has further implicated εPKC in early responses to T-cell activation, specifically up-regulation of the IL-2 receptor (36). In the present work, identification of p59fyn as an endogenous anchoring protein for εPKC has provided a basis for experiments indicating a role for this interaction in the regulation of IL-4 in nontransformed T-cell lines, using both electroporated antibodies and a small organic compound as pharmacological probes.

**EXPERIMENTAL PROCEDURES**

**Reagents**—Phorbol-12-myristate-13-acetate (PMA) was from LC Laboratories. Phosphatidylserine (PS) and dacylglycerol (DAG) were from Avanti. Ionomycin and tetanus toxoid were from Calbiochem. Lectin from Phaseolus vulgaris phytohemagglutinin, piceatannol, enolase, and histone type IIIs were from Sigma. TER14867 (c reverted-N,N-dimethylaminomethyl)-lindane HC1) was obtained from Sigma Aldrich (catalog number S793558). OKT3 antibody to CD3 (ATCC) was purchased from mouse ascites on a mouse A sorbent kit (Pierce).

**Rericombinant Proteins**—Human εPKC was PCR-amplified from Jurkat cell cDNA, cloned into the baculovirus transfer vector pBlueBacHisB (Invitrogen), and confirmed by sequencing. The recombinant N-terminal His-tagged εPKC was isolated from the Sf-9 insect cell on a nickel chelation resin (Qiagen) according to the manufacturer’s protocol. The resulting protein was 85% pure by silver-stained SDS-PAGE gel and was catalytically active for autophosphorylation and using histone IIIs as substrate.

εPKC-V1 domain (amino acids 1–140) and the V1 domain of εPKC (amino acids 1–140) were PCR-amplified from Jurkat cell cDNA and expressed in XLI-Blue strain of Escherichia coli (Strategen) using pQE-30 vector (Qiagen) with an N-terminal His tag for nickel chelation purification and modified to encode a C-terminal c-myc epitope for immunological detection.

Human p59fyn cDNA was PCR-amplified from human T-cell cDNA, cloned in-frame into pMAL-c expression vector to produce a fusion protein with maltose-binding protein (MBP), and the sequence was verified using an ABI373 sequencer (Applied Biosystems). Protein was purified on an amionos addy column following the manufacturer’s protocols (New England Biolabs). The resulting MBP-p59fyn fusion protein was 90% pure and was catalytically active as measured by autophosphorylation and by using enolase as substrate. Similarly, RACK1 and RACK2 were cloned into the pGEX-2X expression vector (American Pharmacia Biotech) to generate GST fusion proteins and purified using the manufacturer’s protocols. The resulting GST-p59fyn fusion protein was 90% pure and was also catalytically active.

**In Vitro Kinase Assays**—Recombinant MBP-p59fyn or MBP (2.5 μg) was incubated with or without substrates, 2.5 μg of εPKC-V1, and/or 2 μg of acid-denatured enolase, in 50 μl of assay buffer (10 mM MnCl2, 40 mM Hepes buffer, pH 7.6, and 5 μCi of [γ-32P]ATP) for 15 min at room temperature (37). Reactions were stopped by adding 12.5 μl of 5× SDS-PAGE sample buffer and boiling for 5 min. Samples were resolved by SDS-PAGE, transferred onto nitrocellulose, and exposed to x-ray film using an intensifying screen at ~80 °C. Parallel manipulations were used for εPKC autophosphorylation or phosphorylation of histone IIIs by εPKC in the absence or presence of 40 μM myocardial type IIIs (Sigma) with or without activating lipids (DAG and phosphatidylserine, 0.8 and 50 μg/ml, respectively) and 10 μg of GST-p59fyn, all in 20 mM MgCl2, 20 mM Tris-HCl, pH 7.5, 12 mM 2-mercaptoethanol, 20 μM ATP, and 5 μCi [γ-32P]ATP.

**Affinity Sorbent Binding**—100 μg of recombinant His-tagged εPKC-V1 was immobilized on 0.5 ml of agarose-nickel resin (Qiagen) and incubated for 30 min at room temperature with 0.5 μg Triton X-100-soluble Jurkat extract cell, prepared in solubilization buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium orthovanadate, 0.2 mM phenylmethylsulfonyl fluoride, and 20 μg/ml each of leupeptin, aprotinin, and soybean trypsin inhibitor). The resin was washed three times with 50 mM Tris-HCl, pH 7.5, 12 mM 2-mercaptoethanol, 0.2 mM NaCl, 0.1% polyethylene glycol, and eluted with standard SDS-PAGE sample buffer: 1% SDS, 1% 2-mercaptoethanol, 0.3 mM Tris-HCl, pH 6.8, 10% glycerol, 0.05% bromophenol blue. The sample was directly loaded on a 10% SDS-PAGE gel followed by Western blotting; after transfer to nitrocellulose membrane, the resolved and immobilized proteins were probed with anti-phosphotyrosine antibodies (Transduction Labs, 1:1000) or anti-p59fyn antibodies (Transduction Labs, 1:1000) or anti-p59fyn antibodies (Transduction Labs, 1:1000) or anti-p59fyn antibodies (Transduction Labs, 1:1000).

**Microplate Binding Assay**—Polyethylene 96-well plates (Corning) were coated with indicated amounts of purified MBP-p59fyn or MBP in PBS for 1 h at 37 °C. The plates were washed with TBST buffer (20 mM Tris, pH 7.4, 150 mM NaCl, and 0.05% Tween 20), blocked with 1% BSA in PBS at room temperature for 1 h, and washed again with TBST. Recombinant εPKC or V1 fragments of εPKC or εPKC were added in a binding containing 10 mM MnCl2, 150 mM NaCl, 40 mM Hepes, pH 7.6, 1 mM ATP, and 0.1% BSA and incubated 30 min with shaking at room temperature. The plates were then washed 5 times with TBST buffer followed by incubation with peroxidase-conjugated anti-εPKC antibody in 1% BSA/PBS (Transduction Labs) for 1 h at room temperature. The wells were washed 5 times with TBST buffer and developed using the 3,3',5,5'-tetramethylbenzidine peroxidase substrate system according to the manufacturer’s directions (Kirkegaard and Perry Laboratories), followed by reading at OD 650 nm. Background binding of PKC constructs to MBP alone, <0.1 OD, was subtracted to yield the specific binding to MBP-p59fyn. No signal was seen in the absence of PKC.
Interaction of θPKC and p59fyn in T-cells

T-cell cDNA and cloned in-frame downstream of VP16 transcription activation domain in pVP16 vector to form the prey construct. The full-length human p59fyn in which Lys-296 was mutated to Ala (K296A) was PCR-amplified from the wild type p59fyn cDNA. Full-length, regulatory (amino acids 1–248), and kinase (amino acids 249–505) p59fyn cDNAs were added to the LexA fusions, which were ligated into pLexA (Oncogene) was added, and the mixture was incubated for an additional 20 min at 4 °C. Triton-soluble protein was resolubilized and lysed on the PKC-activating phospholipids, DAG and phosphatidylserine, followed by overnight incubation at 4 °C with primary antibodies, anti-θPKC monoclonal antibodies (Transduction Laboratories), or anti-p59fyn polyclonal antibodies (Santa Cruz Biotechnology) diluted 1:500 in 1% BSA in PBS. Membranes were washed 3 times for 10 min in PBS, 0.05% Tween 20. Secondary antibody conjugated to horseradish peroxidase at a 1:10,000 in 1% BSA PBS. The immunoblots were incubated for 1 h at room temperature. The membranes were washed as above and developed for electrochemiluminescence detection as described by the manufacturer (Amer sham Pharmacia Biotech).

Protein Expression Analyses—T-cells grown and stimulated as described above were incubated for 24 h in culture media, followed by secretion of cytokines. In particular, cytokines were measured using enzyme-linked immunosorbent assay reagents from R&D Systems or BIO SOURCE, following the manufacturer’s protocols for detection of bound biotinylated antibodies with peroxidase-streptavidin affinity reagents. Human peripheral blood lymphocytes isolated from 19005

Cells were generally cultured overnight in modified Yssel’s media containing 10 ng/ml IL-2 and 10 ng/ml IL-4 (R&D Systems). Cells were cultured for 3–5 days, then expanded in media containing 10 ng/ml IL-2 and 10 ng/ml IL-4 (R&D Systems). The lines were maintained by anti-CD3 stimulation every 21 days, followed by expansion with IL-2 + IL-4. Cells were maintained in modified Yssel’s media. Isovolemic media supplementation with 2% heat-inactivated human AB serum, 2.5 mg/ml AlbuMAX II, 1× insulin-transferrin-selenium, and 1% penicillin, streptomycin (all from Life Technologies, Inc.) in the presence of 10 ng/ml IL-2 and 10 ng/ml IL-4 (R&D Systems). Jurkat cells (ATCC) were maintained in RPMI 1640 (Life Technologies, Inc.)) and 2.5% penicillin/streptomycin (Life Technologies, Inc.), supplemented with 10% fetal bovine serum (Hy Clone, and lysine to assay for protein-protein interaction. Colonies from the UTL plates were picked and plated on THULL medium grids to retest the interaction observed on primary THULL medium plates. About 25 independent transformants were analyzed for each bait-prey pair.

In Vitro Binding—The first variable region of novel class PKC isozymes had previously been shown to contain anchoring protein binding sites (41). Accordingly, initial experiments were designed using that domain as a biochemical bait to identify θPKC-binding proteins. Sorbents were prepared from θPKC-V1, comprising residues 1–140. Adsorbed and eluted proteins from Jurkat lymphoma cell extracts were characterized using antibodies to phosphotyrosine (Tyr(P)) and to known T-cell-signaling proteins. These results led to the preliminary identification of p59fyn as the most prominent adsorbed Tyr(P)-containing protein (Fig. 1). The other bands in lane 3 stained with anti-Tyr(P) antibody are attributable to leached-off protein contaminants present in the θPKC preparation used to make the sorbent (see lane 2) or to p59fyn degradation products (see lane 4). In control experiments using sorbents constructed from the V1 regions of θPKC and ePKC, no band at the molecular weight of p59fyn was observed (not shown).

RESULTS

Cells were generally cultured overnight in modified Yssel’s media without IL-2 and IL-4 before stimulation. Typically, 5 × 105 cells were washed with PBS, pelleted, and resuspended in 2 × 5 ml warmed modified Yssel’s media. Direct PKC stimulation was achieved by treatment with 10 mM PMA and the calcium ionophore ionomycin at 25 ng/ml or by a combination of 20 nM PMA plus 1 µg/ml phytobezgin for 10–15 min at 37 °C followed by washing in 10 ml of PBS. Alternatively, cells were stimulated for 1 h using OKT3 (1 µg/ml), an antibody to the CD3 complex of the TCR.

Immunoprecipitation Analyses—Cells were lysed by resolubilizing cell pellets in solubilization buffer + 1% Triton X-100, followed by a 10-min incubation in an ice bath. Lysates were clarified by centrifugation at 12,000 × g for 10 min at 4 °C. Triton-soluble protein was quantified by BCA protein assay (Pierce). A minimum of 350 µg of lysate protein was used for each immunoprecipitation, to which was added 2.5–5 µg of monoclonal antibody to θPKC (Transduction Laboratories), polyclonal antibody to p59fyn (Upstate Biotechnology), or control antibodies. After incubation overnight (16 h) at 4 °C with end-over-end rotation on a rotating platform, protein PLUS G/A-agarose (Oncor) was added to the mixture and the mixture was incubated for an additional 20 min at 4 °C with rotation. The agarose was then washed four times with immunoprecipitation buffer.

SDS-PAGE sample buffer was added to the washed immune complexes and boiled for 5 min. Samples were resolved on 8 or 10% SDS-PAGE gels and transferred to nitrocellulose. The membranes were incubated in blocking buffer (5% nonfat dry milk in PBS) for 1 h at room temperature, followed by overnight incubation at 4 °C with primary antibodies, anti-θPKC monoclonal antibodies (Transduction Laboratories), or anti-p59fyn polyclonal antibodies (Santa Cruz Biotechnology) diluted 1:500 in 1% BSA in PBS. Membranes were washed 3 times for 10 min in PBS, 0.05% Tween 20. Secondary antibody conjugated to horseradish peroxidase at a 1:10,000 in 1% BSA PBS. The immunoblots were incubated for 1 h at room temperature. The membranes were washed as above and developed for electrochemiluminescence detection as described by the manufacturer (Amersham Pharmacia Biotech).
Fig. 1. Binding of p59fyn to θPKC-V1 affinity sorbent. Recombinant θPKC-V1 was immobilized via a histidine tag on a nickel-agarose resin and incubated with Jurkat cell extracts. Lane 1, total Jurkat phosphotyrosine (Tyr(P))-containing proteins; lane 2, background Tyr(P) immunoreactivity present in the θPKC-V1 preparation before the addition of Jurkat cell extracts; lanes 3 and 4, proteins eluted from the resin with SDS-PAGE sample buffer following washing. Proteins were visualized by Western blotting; after separation on a 10% gel, the proteins were transferred to nitrocellulose and probed with anti-Tyr(P) antibody (lanes 1–3) or anti-p59fyn antibody (lane 4).

Fig. 2. Binding of recombinant θPKC to p59fyn in a microplate assay. A, titration of MBP-p59fyn immobilized on a microplate is shown for the indicated amounts of θPKC in solution. B, the V1 variable domains of θPKC and θPKC were compared for ability to bind MBP-p59fyn. Detection of bound protein was accomplished using an enzyme-conjugated antibody. The average of duplicate determinations for a representative replicate of at least four different protein preparations is plotted, with background signal (~0.1 optical density) found with MBP alone subtracted.

Fig. 3. Potentiation of θPKC kinase activity by p59fyn. θPKC autophosphorylation (lanes 1–4) and phosphorylation of histone IIIs (lanes 5–8) were assayed by incorporation of radiolabeled phosphate in the absence or the presence of GST-p59fyn, and in the absence or presence of DAG/phosphatidylserine (PS), as indicated. Equal amount of material was loaded in each SDS gel lane.
The strongest binding was to the kinase domain, although some binding to the regulatory domain was observed. When transfected into the same parental bait construct yeast strain, approximately equal amounts of the fusion proteins were produced for full-length p59fyn and for both the kinase and regulatory domains, as measured by Western blotting with anti-p59fyn antibodies. Thus, the reporter gene signal is attributable to variations in the affinity of the interacting bait and prey proteins.

Specificity of the interaction was explored in two respects. First, the V1 region of ΔPKC, which is the isozyme most closely related to ΔPKC by sequence, showed no binding to p59fyn or its major fragments. Second, p56ck, which is closely related to p59fyn by sequence (39) and is also critical for the TCR-mediated signal transduction, showed reduced binding. The quantititative comparison of ΔPKC-V1 binding to p59fyn and p56ck is shown in Fig. 6A. Unlike p59fyn, for which the kinase domain was as effective as the intact protein, the two major domains of p56ck were not nearly as effective as the whole protein. Furthermore, when p59fyn kinase and regulatory domain prey constructs were mixed into a murine T-cell cDNA library and screened using ΔPKC-V1 as bait, the positive clones recovered were predominantly from p59fyn, consistent with the degree to which they had been spiked into the library. Likewise, screening a human cDNA library yielded several p59fyn clones but no p56ck clones.

Also shown in Fig. 6A is evidence that the catalytic function of p59fyn is necessary for binding to ΔPKC-V1; the K296A p59fyn mutant, which has no kinase activity (42), did not bind to ΔPKC-V1. Both wild type full-length p59fyn and the kinase domain constructs were catalytically active in the yeast cells as determined by anti-Tyr(P) Western blot analysis of total yeast proteins. In contrast, yeast cell extracts containing ΔPKC-V1 bait alone or in combination with the K296A mutant had no detectable Tyr(P) by the same analysis.

The yeast two-hybrid system was further used to characterize TER14687, the (−)-2-(N,N-dimethylaminomethyl)-1-indanone HCl, a compound identified by preliminary screening of an empirically diverse set of compounds (43) for activity in a Jurkat cell IL-2 production assay (35). In the yeast reporter assay, the compound was found to block the association between ΔPKC-V1 and p59fyn (Fig. 6B). Because this compound undergoes a spontaneous elimination at neutral pH to form a more reactive compound, the active species probably results from reaction with a media component; the final active structure has not yet been identified. Whatever the ultimate structure of TER14687, however, its effects are apparently specific to the interaction of interest because it had no effect on a Tal1/E2A two-hybrid construct. In this control, an active transcription factor is reconstituted by noncovalent association of domains from two separate transcription factors. Moreover, when added to media at the 60 μM concentration used in the two-hybrid experiments, TER14687 did not affect growth of normal yeast cells.

Physiological Role—Prior work had shown that disrupting ΔPKC translocation by overexpressing a binding protein suppressed IL-2 production in Jurkats (35) and that ΔPKC was one of the first PKC isozymes to be activated following T-cell stimulation (36). Under the conditions used here, PMA stimulation caused all isozymes studied to translocate from the soluble to particulate fraction in both Jurkat lymphoma cells and normal T-cell lines. These include closely related ΔPKC and εPKC as well as the more distantly related βPKC. However, ΔPKC was the only isozyme that translocated following physiological stimulation using the OKT3 antibody to CD3 (Fig. 7A). ΔPKC translocation in nontransformed T-cells following OKT3 activation was blocked by TER14687 (Fig. 7B). No effect of the compound on other isozymes was observed in Jurkats or normal T-cells. Thus, a compound that had been found to specifically inhibit ΔPKC binding to p59fyn was also found to cause a selective effect on ΔPKC translocation. TER14687 therefore provides a useful tool for exploring the physiological consequences of ΔPKC translocation.

OKT3 stimulation of normal human T-cells in the presence of 5 μM TER14687 resulted in substantial suppression of the up-regulation of surface antigens normally associated with T-cell activation. The results shown in Fig. 8A for CD69 are representative of effects observed in parallel experiments examining CD25 and CD40L. Expression of the cytokines IL-4 and γ-IFN was also measured for several cell lines that express these cytokines but not IL-2 upon stimulation. Culture supernatants collected 24 h after OKT3 stimulation were analyzed by enzyme-linked immunosorbent assay assays; tritiated thymidine uptake was used to assess the proliferation response. The representative example in Fig. 8 (panels B–D) shows that TER14687 caused a more pronounced suppression of IL-4 as compared with γ-IFN. Considering all experiments together, there was a trend to reduced proliferation at higher doses of TER14687, possibly as a secondary consequence of the reduc-
tion in secretion of IL-4, which acts as an autocrine growth factor. Reductions in γ-IFN were correlated with reduced proliferation, whereas the more extreme reductions of IL-4 secretion were independent of effects on proliferation.

Specificity of the TER14687 results was enhanced by several negative results. At 5 μM in the cell media, TER14687 had no effect on calcium flux, an early sequel to OKT3 activation that was blocked by 25 μM piceatannol, a tyrosine kinase inhibitor. Cytotoxic effects of TER14687 were modest, below 5 μM; the higher tolerance of yeast cells to TER14687 may be because of active export pumps, which are known to be readily inducible in yeast (44). At 50 μM in vitro, the compound did not inhibit the kinase activity of either PKC or p59fyn.

Additional indications that the cytokine effects arose from the direct interaction between θPKC and p59fyn were provided by exploratory experiments using antibodies to either θPKC or p59fyn as pharmacological probes, introduced into the cells by electroporation. For these experiments, the cells were stimulated for 30 min using PMA and ionomycin, conditions that produced results comparable with OKT3 activation with regard to θPKC translocation and cytokine secretion. Suppression of IL-4, with little effect on γ-IFN, was seen with antibodies to either protein but not with a control antibody against choline acetyltransferase (CAT) (Fig. 9). As shown in the figure, these results were significantly more variable in replicate experiments than was the case with TER14687, possibly because of variable degradation of the antibody. Consistent with a progressive degradation of the antibody, it was found that doubling the length of time the cells were stimulated eliminated the IL-4 suppression effect of the antibodies electroporated into the cells before stimulation. OKT3 activation, which required 60 min of stimulation to increase cytokine secretion, was thus not feasible using antibodies as pharmacological probes.

DISCUSSION

The key experimental result that has emerged from the present study is that a specific protein-tyrosine kinase, p59fyn, interacts with a specific PKC isozyme, θPKC, in a manner that goes well beyond the typical interaction of an enzyme with its substrate. Rather, the interaction is quite analogous to that previously described between other PKC isozymes and their anchoring RACKs (28). This result, together with other recent evidence expanding the range of direct PKC interactions with proteins involved in signaling pathways (3, 4), thus has ramifications beyond T-cell biology.

Signal transduction networks are inherently nonlinear, replete with feedback loops, and convergent in several aspects. This complexity makes definitive proof for the role of any single
ever. The issue could not be resolved from in vitro nase activity is required for p59fyn binding to ZAP-70 (13), activity increasing stability of a binding event. Specifically, ex-
tivity increasing stability of a binding event. Specifically, ex-
co-immunoprecipitation is unusual for enzyme interactions
from T-cells by immunoprecipitation also contained phospho-
tion.

Second, the two proteins are associated in vivo, as detected by co-immunoprecipitation from cell extracts (Fig. 4). The use of nontransformed human T-cell lines, rather than immortal-
ized tumor lines or genetically engineered overexpressing lines, enhances the credibility of this result. One possible divergence from the previous PKC/RACK literature concerns the depend-
ence of the interaction on activating phospholipids. Lipid-de-

PKC binds, Olszowy, M. W., and Bleackley, R. C. (1996) J. Biol. Chem.

On DAG for binding because removing cytokines from the cell
media overnight consistently reduced the amount of particu-
lar fraction of PKC to some degree, suggesting that the cyto-
kines needed to promote growth of the cells also caused a
variable level of activation.

Third, the yeast two-hybrid system established that the in-
teraction encompasses an extended region of p59fyn, with
strong binding via the kinase domain of p59fyn (Fig. 6). This
result is consistent with evidence that p59fyn is a substrate for
tyrosine phosphorylation by p59fyn (Fig. 5). PKC obtained from T-cells by immunoprecipitation also contained phospho-

Fig. 9. Electroporated antibody effects on cytokine expres-
sion. Antibodies were introduced into T-cells under electroporation
conditions in which >50% of the cells took up antibody with minimal
loss in cell viability. The effects of antibodies to PKC and to p59fyn on
IL-4 expression induced by PMA + ionomycin are compared with a
control anti-CAT antibody. Filled bars are with antibody; open bars are
electroporated mock experiment without antibody; all are normalized
gains against cells that were not electroporated.

component difficult to achieve and even more difficult for the
interaction of two components. Accordingly, the credibility of
p59fyn as an anchoring protein for PKC is fortified by being
the common conclusion from three independent lines of exper-
imentation. First, the two proteins interact in vitro, as measured by using affinity sorbents on cell extracts (Fig. 1) as well as by direct binding of recombinant proteins (Fig. 2). Fur-

PKC is a substrate for PKC obtained

References—We thank Daria Moehly-Rosen for inspiration and

guidance throughout this work, Stan Hollenberg for providing protocols, vectors, and yeast strains for the two-hybrid experiments,
Mike Csukai for sharing information regarding RACK2, and Amnon
Altman and Avi Kupfer for helpful discussions. Michael Julius, Steve
Hedrick, and Robert Messing offered valuable critical review of the
work on several lively occasions. Our colleagues at Telik (previously Terrapin Technologies) provided helpful assistance in many aspects of
the work, with special thanks to Deb Higgins, Don Schmidt, Rich
Sportsman, Marvin Siegel, Wes Zmolek, Åsa Engqvist-Goldstein, Ami
McElroy, Scott Mayfield, Karin Bauer, Renee Holloman, Nick Boet-
ticher, Chris O’Hara, Steve Tauchida, Francis Dy, Cheri Harkrader,
David Stockett, and Ehud Droi.

Acknowledgments—

REFERENCES

1. Bolen, J. B., and Brugge, J. S. (1997) Annu. Rev. Immunol. 15, 371–404
2. Moehly-Rosen, D., and Kauvar, L. M. (1998) Adv. Pharmacol. 44, 91–145
3. Yao, L., Suzuki, H., Ozawa, K., Deng, J., Lehel, C., Fukamachi, H., Anderson, W. B., Kawakami, Y., and Kawakami, T. (1997) J. Biol. Chem. 272, 10033–10039
4. Puls, A., Schmidt, S., Grawe, F., and Stabel, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 6191–6196
5. Katz, M. E., and McCormick, F. (1997)Curr. Opin. Genet. Dev. 9701, 75–79
6. Cantrell, D. (1996) Annu. Rev. Immunol. 14, 259–274
7. Szamel, M., and Resch, K. (1995) Eur. J. Biochem. 229, 1–15
8. Baier-Bitterlich, G., Uerball, F., Bauer, B., Fresser, F., Wachter, H., Grunicke, H., Utermann, G., Altman, A., and Baier, G. (1996) Mol. Cell. Biol. 16, 1842–1850
9. Genot, E. M., Parker, P. J., and Cantrell, D. A. (1995) J. Biol. Chem. 270, 9835–9839
10. Meller, N., Altman A., and Isakov, N. (1998) Stem Cells 16, 178–192
11. Monks, C. R., Kupfer, H., Tamir, I., Barlow, A., and Kupfer, A. (1997) Nature 385, 83–86
12. Entschladen, F., Niggemann, B., Zanker, K. S., and Friedl, P. (1997) J. Im-
munol. 159, 3203–3210
13. Fusaki, N., Matsuda, S., Nishizumi, H., Umemori, H., and Yamamoto, T. (1999) J. Immunol. 163, 1369–1377
14. Tsuyugak, A. Y., Mahajan, S., Fincke, J. E., and Bolen, J. B. (1996) J. Biol.

Chem. 271, 27130–27137
15. Alkinkson, R. A., Osterberg, H., Kane, K., Pinkoski, M. J., Caputo, A., Olzowy, M. W., and Bleackley, R. C. (1996) J. Biol. Chem. 271, 5968–5971
16. Li, B., Subleski, M., Fusaki, N., Yamamoto, T., Copeland, T., Princler, G. L., Jung, H., and Katama, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1001–1005
17. Page, T. H., Lali, F. V., and Foxwell, B. M. (1995) Eur. J. Immunol. 25, 2956–2960
18. Pedraza-Alva G., Merida, L. B., and Burakoff, S. J., Rosenstein, Y. (1996) J. Biol. Chem. 271, 27564–27568
19. Marie-Caroline, A., Kirchgesner, H., Eckerskorn, C., Meuer, S. C., and Schraven, B. (1995) Eur. J. Immunol. 25, 3290–3297
Interaction of θPKC and p59fyn in T-cells

371, 297–300
33. Jaken, S. (1996) Curr. Opin. Cell Biol. 8, 168–173
34. Smith, B., Krushelnicky, B. W., Mochly-Rosen, D., and Berg, P. (1996) J. Biol. Chem. 271, 16753–16757
35. Meller, N., Liu, Y.-C., Collins, T. L., Bonnefoy-Berard, N., Bai, G., Isakov, N., and Altman, A. (1996) Mol. Cell. Biol. 16, 5782–5791
36. Szamel, M., Appel, A., Schwinzer, R., and Resch, K. (1996) J. Immunol. 157, 2207–2214
37. Huang, T. R., Luo, K., and Sefton, B. M. (1996) Science 245, 497–499
38. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 251–258
39. Perlmutter, R. M., Math, J. D., Lewis, D. B., Poet, R., and Wilson, C. B. (1988) J. Cell. Biochem. 38, 117–126
40. Ausubel, F. M., Brent, R., Kriegstein, E. R., Moore, D. D., Seidman, J. G., Struhl, K., and Chanda, V. B. (eds) (1998) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
41. Johnson, J. A., Gray, M. O., Chen, C. H., and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966
42. Cooke, M. P., Abraham, K. M., Forbush, K. A., and Perlmutter, R. M. (1991) Cell 65, 281–291
43. Kauvar, L. M., Villar, H. O., Sportsman, J. R., Higgins, D. L., and Schmidt, D. E., Jr. (1998) J. Chromatogr. B 715, 93–102
44. Reed, R. J., Kauf, E. A., and Bjornst, M. A. (1997) J. Biol. Chem. 272, 12091–12099
45. Ryan, J. J. (1997) J. Allergy Clin. Immunol. 99, 1–5

20. Renzoni, D. A., Pugh, D. J., Siligardi, G., Das, P., Morton, C. J., Rossi, C., Waterfield, M. D., Campbell, I. D., and Ladbury, J. E. (1996) Biochemistry 35, 15646–15653
21. Jayaraman, T., Ondrias, K., Ondriasova, E., and Marks, A. R. (1996) Science 272, 1492–1494
22. Rigley, K., Slocombe, P., Proudfoot, K., Wahid, S., Mandair, K., and Bebbington, C. (1995) J. Immunol. 154, 1136–1145
23. Koegl, M., Zlatkine, P., Ley, S. C., Courtneidge, S. A., and Magee, A. I. (1994) Biochem. J. 303, 749–753
24. Perlmutter, R. M., Levin, S. D., Appleby, M. W., Anderson, S. J., and Alberola-Ila, J. (1993) Annu. Rev. Immunol. 11, 451–459
25. Davidson, D., Viallet, J., and Veillette, A. (1994) Mol. Cell. Biol. 14, 4554–4564
26. Yang, W., Malek, S. N., and Desiderio, S. (1995) J. Biol. Chem. 270, 20832–20840
27. Banin, S., Truong, O., Katz, D. R., Waterfield, M. D., Brickell, P. M., and Gout, I. (1996) Curr. Biol. 6, 961–968
28. Mochly-Rosen, D., and Gorden, A. S. (1998) FASEB J. 12, 35–42
29. Montixi, C., Langel, C., Bernard, A. M., Thimonier, J., Dubois, C., Wurbel, M. A., Chauvin, J. P., Piers, M., and He, H. T. (1998) EMBO J. 17, 5334–5348
30. Ron, D., Chen, C. H., Caldwell, J., Jamieson, L., Orr, E., and Mochly-Rosen, D. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 839–843
31. Csekai, M., Chen, C. H., De Matteis, M. A., and Mochly-Rosen, D. (1997) J. Biol. Chem. 272, 29200–29206
32. Neer, E. J., Schmidt, C. J., Nambudripad, R., and Smith, T. P. (1994) Nature 371, 297–300
33. Jaken, S. (1996) Curr. Opin. Cell Biol. 8, 168–173
34. Smith, B., Krushelnicky, B. W., Mochly-Rosen, D., and Berg, P. (1996) J. Biol. Chem. 271, 16753–16757
35. Meller, N., Liu, Y.-C., Collins, T. L., Bonnefoy-Berard, N., Bai, G., Isakov, N., and Altman, A. (1996) Mol. Cell. Biol. 16, 5782–5791
36. Szamel, M., Appel, A., Schwinzer, R., and Resch, K. (1998) J. Immunol. 157, 2207–2214
37. Huang, T. R., Luo, K., and Sefton, B. M. (1996) Science 245, 497–499
38. Vojtek, A. B., Hollenberg, S. M., and Cooper, J. A. (1993) Cell 74, 251–258
39. Perlmutter, R. M., Math, J. D., Lewis, D. B., Poet, R., and Wilson, C. B. (1988) J. Cell. Biochem. 38, 117–126
40. Ausubel, F. M., Brent, R., Kriegstein, E. R., Moore, D. D., Seidman, J. G., Struhl, K., and Chanda, V. B. (eds) (1998) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York
41. Johnson, J. A., Gray, M. O., Chen, C. H., and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966
42. Cooke, M. P., Abraham, K. M., Forbush, K. A., and Perlmutter, R. M. (1991) Cell 65, 281–291
43. Kauvar, L. M., Villar, H. O., Sportsman, J. R., Higgins, D. L., and Schmidt, D. E., Jr. (1998) J. Chromatogr. B 715, 93–102
44. Reed, R. J., Kauf, E. A., and Bjornst, M. A. (1997) J. Biol. Chem. 272, 12091–12099
45. Ryan, J. J. (1997) J. Allergy Clin. Immunol. 99, 1–5