Inhibition of the c-Jun N-terminal Kinase/AP-1 and NF-κB Pathways by PICOT, a Novel Protein Kinase C-interacting Protein with a Thioredoxin Homology Domain*

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Protein kinase C-θ (PKCθ) is a Ca²⁺-independent PKC isoform that is selectively expressed in T lymphocytes (and muscle), and is thought to play an important role in T cell receptor-induced activation. To gain a better understanding of the function and regulation of PKCθ, we have employed the yeast two-hybrid system to identify PKCθ-interacting proteins. We report the isolation and characterization of a cDNA encoding a novel 355-amino acid (37.5-kDa) PKCθ-interacting protein termed PICOT (for PKC-interacting cousin of thioredoxin). PICOT is expressed in various tissues, including in T cells, where it colocalizes with PKCθ. PICOT displays an N-terminal thioredoxin homology domain, which is required for the interaction with PKC. Comparison of the unique C-terminal region of PICOT with expressed sequence tag data bases revealed two tandem repeats of a novel domain in T cells that is highly conserved from plants to mammals. Transient overexpression of full-length PICOT (but not its N- or C-terminal fragments) in T cells inhibited the activation of c-Jun N-terminal kinase (but not extracellular signal-regulated kinase), and the transcription factors AP-1 or NF-κB. These findings suggest that PICOT and its evolutionary conserved homologues may interact with PKC-related kinases in multiple organisms and, second, that it plays a role in regulating the function of the thioredoxin system.

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 (1–3). The PKC family consists of 11 known mammalian members that are expressed in a wide variety of tissues and cell types. Based on sequence similarities, domain structures, and cofactor requirements, PKC isoenzymes can be grouped into three subfamilies. 1) The Ca²⁺-dependent conventional enzymes, consisting of PKC-α, -βI, -βII, and -γ, contain three conserved domains, namely the diacylglycerol/phorbol ester binding C1 domain, which contains two repeats of a cysteine-rich zinc finger, the phospholipid- and calcium-binding C2 domain, and the catalytic C3 and C4 domains. 2) The Ca²⁺-independent enzymes (PKC-δ, -ε, -η, -θ and -μ) are termed novel PKCs. The C2-like N-terminal domain of these enzymes can bind acidic phospholipids but not Ca²⁺. 3) A third PKC subfamily, termed atypical PKCs, includes PKC-ζ and -λ that possess a single cysteine-rich domain, lacking the ability to bind phospholipids or phorbol esters. PKC activity is regulated by defined cofactors that interact with specific regions of the regulatory domain as well as transphosphorylation by serine/threonine kinases and autophosphorylation. The activation is accompanied by a conformational change that releases the basic pseudosubstrate region from the catalytic cleft of the kinase domain. In addition, interaction with specific proteins, termed receptors for activated PKC, that function as selective scaffolds for activated PKCs at discrete subcellular compartments, play a role in activation of PKC (4).

In the course of isolating novel PKC isoforms that play a role in T cell antigen receptor (TCR) signaling, we isolated PKCθ, a Ca²⁺-independent PKC isoform characterized by a unique tissue distribution, i.e. in skeletal muscle, lymphoid organs, and hematopoietic cell lines, particularly in T cells (5). PKCθ plays a selective role in the activation of the c-Jun N-terminal kinase (JNK)/AP-1 pathway and the interleukin-2 gene in T cells (6–8), and to colocalize with the TCR complex to the contact site between antigen-specific T cells and antigen-presenting cells (9), where it participates in the formation of a supramolecular activation cluster (10). In order to gain a better understanding of the function and regulation of PKCθ in TCR signaling, we have employed the yeast two-hybrid system to identify PKCθ-interacting proteins. Here we describe the isolation of a novel PKCθ-interacting protein having a unique domain structure, which consists of an N-terminal thioredoxin (Trx)-homologous domain followed by two tandem repeats of a novel, evolutionary conserved protein domain. We have termed this protein PICOT (for PKC-interacting cousin of thioredoxin). Transient overexpression of PICOT inhibited the activation of JNK and two transcription factors i.e. AP-1 and NF-κB, induced by PKCθ or by combinations of T cell-activating stimuli, suggesting that this novel protein plays an important role in regulating T cell activation and the function of PKCθ.
**RT-PCR and Northern Blotting—** Multi-Tissue cDNA Panels (CLONTECH) were screened by RT-PCR with a pair of PICOT-specific primers according to the instructions of the manufacturer. For amplification of a 1-kilobase pair PICOT fragment, 35 cycles were used, and the glyceraldehyde-3-phosphate dehydrogenase control was amplified using 25 cycles. After 200 ng of total RNA from Jurkat cells was prepared using standard procedures. The PICOT plasmid isolated from the yeast two-hybrid screen or a full-length glyceraldehyde-3-phosphate dehydrogenase cDNA were used for generation of 32P-labeled probes using a commercial kit (Rediprime II, Amersham Pharmacia Biotech).

**Subcellular Fractionation—** Jurkat cells were lysed and separated into surface membrane, cytosol, and detergent-insoluble fractions as described (12).

**Immunofluorescence—** Transfected or nontransfected Jurkat-TAg cells were left unstimulated, or stimulated with 100 ng/ml PMA for 10 min at 37 °C. Cells were then spun down, washed with cold phosphate-buffered saline (PBS), fixed with 3.7% paraformaldehyde, and permeabilized in 0.05% saponin. Transfected cells were then stained with a polyclonal rabbit anti-PICOT antibody and an anti-EGFP mAb. Nontransfected cells were stained with a polyclonal anti-PKCδ antibody and Alexa 488-conjugated anti-PICOT antibody. Samples were then incubated with fluorescein isothiocyanate isocyanate-conjugated secondary antibodies (Pierce) or Alexa 594 (Molecular Probes, Eugene, OR), respectively, and were subsequently washed four times with 1% bovine serum albumin in PBS. After the final wash, samples were mounted on glass slides using a drop of Aqua-Poly/ mount (Polysciences, Inc., Warrington, PA). Samples were viewed with a Plan-Apochromat 63×/1.4 objective on a Nikon microscope. Images were taken using a Bio-Rad MRC 1024 laser scanning confocal imaging system.

**Immunoprecipitation and Immunoblotting—** Lysates (1–2 × 10^7 cells) were mixed with antibodies (1–2 μg) for 2 h, followed by addition of 40 μl of protein A/G Plus- sepharose beads (Santa Cruz Biotechnology) for an additional h at 4 °C. Immunoprecipitates were washed twice with 1× Nonidet P-40 lysis buffer and twice with PBS (pH 7.2). After boiling in 20 μl 2x Laemmli sample buffer, samples were subjected to SDS-PAGE and electrotransferred to nitrocellulose membranes (Bio- Rad). Membranes were immunoblotted with the indicated primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. Bands were visualized by chemiluminescence (Amersham Pharmacia Biotech). When necessary, membranes were stripped by incubation in 62.5 mM Tris-HCl, pH 6.7, 100 μM 2-mercaptoethanol, 2% SDS for 1 h at 65 °C, washed, and then reprobed with other antibodies as indicated.

**In Vitro Kinase Assays—** In vitro kinase assays of immunoprecipitated JNK or ERK2 were conducted as described. Briefly, washed JNK1 or ERK2 immunoprecipitates were assayed using 2 μg of GST-c-Jun fusion protein or myelin basic protein as substrates, respectively, in 20 μl of JNK or ERK2 kinase buffers containing 3 μCi of [γ-32P]ATP (30 Ci/mmol, Amersham Pharmacia Biotech). Kinase reactions were incubated for 20 min at 30 °C with gentle shaking, and were stopped by the addition of 20 μl of 2× Laemmli buffer. Proteins were resolved by SDS-13% PAGE, transferred to nitrocellulose, and subjected to autoradiography. Substrate phosphorylation was quantified by PhosphoImager (Storm 860; Molecular Dynamics, Sunnyvale, CA) analysis. The nitrocellulose membranes were routinely reprobed with anti-JNK or -ERK2 antibodies to confirm equal expression levels of the immunoprecipitated kinases.

**Reporter Assays—** Transfected Jurkat-TAg cells were harvested, washed twice with PBS, and lysed in 100 μl of lysis buffer (100 mM KPi, pH 7.8, 1 mM dithiothreitol, 0.5% Triton X-100) for 10 min at room temperature. The lysates were then centrifuged (15,000 × g, 5 min at 4 °C). Fifty μl of the supernatant were mixed with 100 μl of assay buffer (17.5 mM glycyglycine, pH 7.8, 10 mM MgCl₂, 5 mM ATP, 0.135 mM coenzyme A, 0.235 mM luciferin), and the luciferase activity determined in a luminometer (Monolight 2010; Analytical Luminescence Laboratory, Sparks, MD). The protein content was determined using the Bio-Rad protein assay. The final results were expressed as arbitrary relative luciferase units per microgram of protein.

**RESULTS**

**Isolation of a cDNA Encoding a Novel PKCδ-interacting Protein—** To identify proteins that interact with PKCδ in vitro, we performed three independent yeast two-hybrid screens using the full-length PKCδ, its regulatory domain, or its catalytic domain as baits to screen a Jurkat T lymphoma cDNA library fused to a cDNA encoding a transcription activation domain (14). Since expression of catalytically active forms of PKCδ as
LexA fusion proteins was toxic to yeast (data not shown), a point-mutated cDNA encoding a catalytically inactive PKC\(\alpha\) (K409R) was used for the yeast two-hybrid screening. Approximately 5 × 10^7 independent clones were screened with each bait. No clones growing on selective medium were obtained with the isolated regulatory or catalytic domains of PKC\(\alpha\), respectively (Table I). Screening with the catalytically inactive, full-length PKC\(\alpha\) bait resulted in 17 primary positive colonies, which grew on selective medium and were \(\beta\)-galactosidase-positive when tested in a filter lift assay (Table I). Thus, the observed interaction in yeast requires full-length PKC\(\alpha\), but does not depend on its intact catalytic activity.

Ten of the positive, PKC\(\alpha\)-interacting clones were found to contain cDNAs that specifically interacted with the bait. Partial sequence analysis revealed that these clones encoded identical cDNAs. The two longest cDNAs (−1,250 base pairs in length) were sequenced, and found to contain an open reading frame (ORF) encoding a putative protein of 335 amino acids (Fig. 1a). Since the putative protein product contained a \(\text{Trx}\)-homologous domain (see below), it was named PICOT (for PKC-interacting cousin of thioredoxin) (see below). Northern blot analysis using one of the yeast two-hybrid clones as a probe indicated that a hybridizing mRNA of 1.5 kilobase pairs is exclusively localized in the cytosol, with additional, very low abundance in subcellular fractions. Membrane, detergent-insoluble, and cytosolic fractions of Jurkat cells were immunoblotted with a PICOT-specific rabbit antiserum. d, expression of PICOT in different human tissues analyzed by RT-PCR (top panel). The same human cDNA panel was also screened for the expression of PKC\(\alpha\) (middle panel) and, as a loading control, glyceraldehyde-3-phosphate dehydrogenase (bottom panel).

**Table I**

| DNA-binding domain hybrid \(\text{"bait"}\) | Activation domain hybrid \(\text{"prey"}\) | Growth on Ura His-Trp-Leu medium | Colony color |
|------------------------------------------|---------------------------------|----------------------------------|-------------|
| PKC\(\alpha\) K409R                     | –                               | –                                | White       |
| PKC\(\alpha\) reg. dom. (1–378)         | –                               | –                                | White       |
| PKC\(\alpha\) cat. dom. (379–706)       | –                               | –                                | White       |
| PKC\(\alpha\) K409R                     | PICOT                           | ++                               | Blue        |
| PKC\(\alpha\) K409R                     | PICOT-N (1–146)                 | –                                | White       |
| PKC\(\alpha\) K409R                     | PICOT-C (133–335)               | –                                | White       |
| PKC\(\alpha\) regulatory dom. (1–378)   | PICOT                           | –                                | White       |
| PKC\(\alpha\) regulatory dom. (1–378)   | PICOT-N (1–146)                 | –                                | White       |
| PKC\(\alpha\) catalytic dom. (379–706)  | PICOT-C (133–335)               | –                                | White       |
| PKC\(\alpha\) catalytic dom. (379–706)  | PICOT-N (1–146)                 | –                                | White       |
| PKC\(\alpha\) catalytic dom. (379–706)  | PICOT-C (133–335)               | –                                | White       |

**Fig. 1.** Primary sequence and mRNA expression of PICOT. a, the primary sequence of PICOT deduced from the cDNA. The two repeats of the evolutionary conserved novel domain (see Fig. 2C) are underlined. b, Northern blot analysis of the expression of PICOT mRNA in Jurkat T cells. The PICOT plasmid isolated from the yeast two-hybrid screen was used as a probe. c, expression of PKC\(\alpha\) protein in subcellular fractions. Membrane, detergent-insoluble, and cytosolic fractions of Jurkat cells were immunoblotted with a PKC\(\alpha\)-specific rabbit antiserum. d, expression of PICOT in different human tissues analyzed by RT-PCR (top panel). The same human cDNA panel was also screened for the expression of PKC\(\alpha\) (middle panel) and, as a loading control, glyceraldehyde-3-phosphate dehydrogenase (bottom panel).
expression in the membrane fraction, but no detectable expression in the detergent-insoluble cellular fraction.

The tissue distribution of PICOT was analyzed by RT-PCR using a commercial cDNA panel derived from different human tissues. The expression of PICOT mRNA was more ubiquitous than that of PKC8; nevertheless, it was not expressed in all tissues. It was abundant in heart, spleen, and testis, with low expression in the membrane fraction, but no detectable expression in the membrane fraction.
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![Image](50x557 to 297x729)

**Fig. 4. Localization of PICOT and PKCα in T cells.** Jurkat-TAg cells were cotransfected with PICOT and PKCα expression vectors for 48 h (two top rows) or were left untransfected (bottom row). The transfected cells were either unstimulated or stimulated for the final 10 min of culture with PMA (100 ng/ml). The cells were fixed, permeabilized, and stained as described under "Materials and Methods." Localization of the relevant proteins was analyzed by confocal microscopy. The right column represents an overlay of the PKCα (left column) and PICOT (middle column) images. The images shown are representative of ~50 cells observed in each group.

Table: Localization of PICOT and PKCα in T cells

| Transfection | PKCα | PICOT | Overlay | PMA |
|--------------|------|-------|---------|-----|
| Yes          | ![Image](a) | ![Image](b) | ![Image](c) | - |
| Yes          | ![Image](d) | ![Image](e) | ![Image](f) | + |
| No           | ![Image](g) | ![Image](h) | ![Image](i) | - |

same conditions, no interaction with PKCα was detected, suggesting that PICOT displays some selectivity with regard to its ability to associate with PKC isoforms.

The Trx-like Domain of PICOT Is Sufficient for Interaction with PKCα—In order to further define the structural basis for the interaction of PICOT with PKCα, we expressed the PICOT N-terminal region corresponding to its Trx-like domain (residues 1–146) and its C-terminal fragment representing the two tandem PICOT homology domains (residues 133–335) as GST fusion proteins, and used these recombinant proteins to precipitate cell lysates from PKCα-transfected Jurkat cells. As shown in Fig. 3c, the N-terminal, Trx-like domain of PICOT was sufficient for PKCα binding, although it was less effective than the full-length protein. In contrast, the C-terminal region of PICOT did not display any detectable binding to PKCα. In yeast, only full-length PICOT associated with PKCα (Table I), suggesting that the affinity of the interaction is lower in yeast. This difference may reflect a favorable conformation of PICOT for this interaction in vitro (where it was expressed as a GST fusion protein) compared with yeast (where PICOT was expressed as a fusion protein with a transcription activation domain).

Intracellular Localization of PICOT and PKCα—In order to determine the relative localization of PICOT versus PKCα in situ, transfected or untransfected Jurkat cells were stained with PICOT- and PKCα-specific antibodies, and analyzed by confocal microscopy. In unstimulated cells, which were transfected with both PICOT and PKCα, both proteins colocalized to a distinct cytoplasmic area under the plasma membrane (Fig. 4a and b), and their colocalization was evident when the two individual images were overlaid (Fig. 4c). PMA stimulation caused translocation of both PICOT and PKCα to a more extended membrane (or submembrane) area, with colocalization of the two proteins still evident (Fig. 4d–f).

We also analyzed the localization of the relevant endogenous protein in untransfected cells. The quality of this analysis was lower due to the lower expression of the proteins and the fact that it was necessary to use a polyclonal anti-PKCα antibody, which produces a less satisfactory staining than that obtained with the monoclonal antibody used in the transfected cells. Nevertheless, endogenous PICOT and PKCα also appeared to colocalize in the same area of the cell, with an overall staining pattern similar to the one observed in the transfected cells (Fig. 4, g–i).

Selective Inhibition of JNK Activation by PICOT—As PICOT was identified on the basis of its interaction with PKCα, we examined the effect of transient PICOT overexpression on the activation of the stress-activated protein kinase JNK, since it represents a selective target of PKCα in the TCR/CD28 signaling pathway (7, 8). Jurkat-TAg cells were cotransfected with different combinations of expression plasmids encoding PICOT, PKCα and/or constitutively active calcineurin (CnA&CaM-AI). The latter plasmid was used since calcineurin cooperates with PKCα in the activation of JNK and the interleukin-2 promoter (7, 8).

As predicted, transient overexpression PKCα caused activation of the cotransfected epitope-tagged JNK reporter, and this effect was somewhat augmented by CnA coexpression (Fig. 5a, lanes 3 and 5 versus lane 1 in the two upper panels). When the cells were additionally cotransfected with a PICOT expression vector, the PKCα- or PKCα/CnA-induced JNK activation was significantly reduced (Fig. 5a, lanes 4 and 6). Overexpression of PICOT alone also seemed to reduce the basal JNK activity (lane 2). Densitometric analysis of the phospho-c-Jun bands revealed that PICOT reduced the PKCα- or PKCα/CnA-induced JNK activation by 70% and 55%, respectively. Immunoblotting of immunoprecipitated JNK with a specific antibody confirmed that all groups expressed a similar level of transfected JNK (Fig. 5a, third lane from the top). Similarly, immunoblotting of cell lysates from the same cells with antibodies specific for PKCα, PICOT, or HA-CnA confirmed the expected overexpression of the corresponding proteins in the cells (Fig. 5a, three bottom panels).

In order to examine the selectivity of this inhibitory effect of PICOT, we also assessed the effect of transient PICOT overexpression on the activation of another MAP kinase, i.e. ERK2, using similar in vitro immune complex kinase assays. As reported before (8), ERK2 can be non-selectively activated by both PKCα and PKCα and PKCα (Fig. 5b, lanes 3 and 5 versus lane 1 in the upper panel). Coexpression of PICOT alone did not reduce ERK2 activity and, in some experiments, even enhanced it (data not shown). Similarly, coexpression of PICOT with PKCα (lane 4) or PKCα (lane 6) did not inhibit the PKCα-induced ERK2 activity. Immunoblotting with an ERK2-specific antibody confirmed the equivalent expression levels of ERK2 in most groups, with the exception of the two PKCα-transfected groups, which displayed lower ERK2 expression, thereby making the PKCα-induced ERK2 activation even more pronounced than the apparent level. PKCα and/or PICOT were properly overexpressed in the transfected cells. Thus, the PICOT-mediated inhibition of PKC-induced MAP kinase activation is specific for JNK and, furthermore, PICOT alone can induce ERK, but not JNK, activation.

Next, we determined the effect of PICOT overexpression on JNK activation induced by several stimuli, including a physiological stimulus provided by anti-CD3/CD28 antibodies. As reported previously (8, 18), stimulation of Jurkat cells with this antibody combination, a combination of PMA plus ionomycin, or by UV irradiation, all induced marked activation of the cotransfected JNK1 reporter when compared with the unstimulated cells (Fig. 5c, lane 1). Coexpression of PICOT in the same cells reduced the basal or anti-CD3/CD28-induced JNK activity by ~80%, but had a much smaller effect on the PMA/ionomycin- or UV-induced kinase activity (lane 2). Furthermore, the inhibitory effect of PICOT overexpression was very similar to that caused by overexpressing a dominant-negative PKCα (Δ-K/R) mutant (lane 3), supporting the notion that PI-
PKC isoforms tested, PKC Cells—Our earlier studies demonstrated that, among several four lower panels are shown in the PKC activation of NF-κB coexpression, this combination induced a combination of expression vectors plus an HA-tagged JNK1 plasmid. JNK1 activity was determined in in vitro immune complex (anti-HA) kinase assays (two top panels). The same immunoprecipitates were immunoblotted with an anti-JNK antibody (third panel from top), and aliquots of cell lysates were immunoblotted with anti-PKCα, -PKCβ, or -HA antibodies to reveal the proper overexpression of the transfected proteins. b, the activation of ERK2 was assessed in a similar way in anti-c-Myc (9E10 mAb) immunoprecipitates from cells transfected with the indicated plasmid combinations plus a c-Myc epitope-tagged ERK2 expression vector. Control immunoblots of the immunoprecipitates (with anti-ERK2 antibodies) or cell lysates (with anti-PKCα, -PKCβ, or -PICOT antibodies) are shown in the four lower panels. c, cells were transfected with empty vector, PICOT, or dominant-negative (K/R) PKCβ expression vectors and were left unstimulated or stimulated with the indicated stimuli for the final 5 min (anti-CD3/CD28 or PMA plus ionomycin) or 1 min (UV) of culture. JNK1 was immunoprecipitated and tested for in vitro kinase activity as in a.

PICOT inhibits PKCβ-induced JNK activation. Jurkat-Tag cells were transfected with the indicated combinations of expression vectors plus an HA-tagged JNK1 plasmid. JNK1 activity was determined in in vitro immune complex (anti-HA) kinase assays (two top panels). The same immunoprecipitates were immunoblotted with an anti-JNK antibody (third panel from top), and aliquots of cell lysates were immunoblotted with anti-PKCα, -PKCβ, or -HA antibodies to reveal the proper overexpression of the transfected proteins. b, the activation of ERK2 was assessed in a similar way in anti-c-Myc (9E10 mAb) immunoprecipitates from cells transfected with the indicated plasmid combinations plus a c-Myc epitope-tagged ERK2 expression vector. Control immunoblots of the immunoprecipitates (with anti-ERK2 antibodies) or cell lysates (with anti-PKCα, -PKCβ, or -PICOT antibodies) are shown in the four lower panels. c, cells were transfected with empty vector, PICOT, or dominant-negative (K/R) PKCβ expression vectors and were left unstimulated or stimulated with the indicated stimuli for the final 5 min (anti-CD3/CD28 or PMA plus ionomycin) or 1 min (UV) of culture. JNK1 was immunoprecipitated and tested for in vitro kinase activity as in a.

COT exerts its inhibitory activity by interfering with the cellular function of PKCβ.

PICOT Inhibits the Activation of AP-1 and NF-κB in T Cells—Our earlier studies demonstrated that, among several PKC isoforms tested, PKCα functions as a selective AP-1 activator via a Ras-dependent pathway (6). Therefore, we assessed the effect of transient PICOT overexpression on the activation of an AP-1 reporter plasmid. As expected, PMA stimulation or transient overexpression of PKCβ caused a marked increase of AP-1 activity, and the constitutively active plasmid (PKCβ-A/E) was more active than the wild-type kinase in that regard (Fig. 6a). When the cells were cotransfected with increasing amounts of the PICOT expression plasmid, a dose-dependent inhibition of the basal or PKCβ-induced AP-1 activity was observed. Five μg of the PICOT plasmid reduced the basal activity of AP-1 by ~90%, and the activities induced by wild-type or constitutively active PKCβ (in the absence of PMA stimulation) by ~95 and ~60%, respectively. The failure of PICOT to inhibit AP-1 activity in PMA-stimulated cells may reflect activation of AP-1 by other, endogenous PKC isoforms that are not sensitive to the inhibitory effect of PICOT, e.g. PKCα (see Fig. 3b).

Further analysis of the structural requirements for the inhibition of AP-1 activation by PICOT revealed that the full-length protein was necessary. Thus, while native PICOT was capable of inhibiting AP-1 activation induced by constitutively active PKCβ by 60%, the isolated N- or C-terminal fragments of PICOT displayed minimal inhibitory activity (Fig. 6b).

In order to determine the effect of transient PICOT overexpression on the activity of another transcription factor that is known to be induced by stress signals, we assessed the activation of the transcription factor NF-κB by the combined stimulation of anti-CD3 plus anti-CD28 antibodies. In the absence of PICOT coexpression, this combination induced a ~5-fold activation of NF-κB. Transient expression of a lower dose of PICOT (5 μg) caused a minimal reduction of activity, but at the higher dose (10 μg plasmid DNA), NF-κB activity was reduced by 63% (Fig. 6c). As in the case of AP-1, this inhibition required the full-length PICOT protein (data not shown). The selectivity of this effect is indicated by the fact that the PMA-induced activation of NF-κB was not inhibited under the same conditions.

DISCUSSION

Several recent studies have relied on the yeast two-hybrid system to isolate PKC-interacting proteins, which were found to represent either substrates or regulators of distinct PKC isoforms (19–21). Here, we have used a similar strategy to isolate proteins that interact with PKCβ, a Ca2+-independent PKC isoform that is expressed selectively in T cells (5) and has been implicated in T cell-specific functions (6–10, 22). This study reports the isolation and partial characterization of a novel PKCβ-interacting protein termed PICOT. Our results establish the expression of the corresponding mRNA and protein in T and other cells, and the association of PICOT with PKCβ in intact T cells and in vitro which requires the N-terminal, Trx-homologous domain of PICOT. Since PICOT interacts with kinase-inactive PKCβ, and our preliminary results indicate that PICOT is not phosphorylated by PKCβ in vitro (data not shown), PICOT most likely does not represent a PKCβ substrate. Furthermore, PICOT associated in vitro not only with PKCβ, but also with PKCζ. Nonetheless, its interaction with PKC is not promiscuous since it did not associate with another PKC isoform, i.e. PKCε. The degree of specificity of the interaction between PICOT and distinct PKC isoforms, particularly in intact cells, remains to be established.

The stoichiometry of the association between PICOT and PKC appears to be low. Although the two proteins could be coimmunoprecipitated from transfected, overexpressing cells, we could not reproducibly demonstrate coimmunoprecipitation of the relevant endogenous proteins. This may reflect the use of lysis conditions that are unfavorable for the maintenance of
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**Fig. 6. PICOT overexpression inhibits AP-1 and NF-κB activation.** a. Jurkat-TAg cells were cotransfected with an AP-1:luciferase reporter plasmid plus the indicated expression vectors. One group was additionally stimulated with PMA (100 ng/ml) for the final 6 h of culture. Luciferase activity in cell extracts was determined as described under "Materials and Methods." The overexpression of PICOT was verified by immunoblotting (data not shown). b. cells were transfected with the indicated combinations of empty vector, constitutively active (A/E) PKCθ, and/or full-length (wt), N-terminal (N), or C-terminal (C) fragments of PICOT. The activity of a cotransfected AP-1 reporter was determined as in a. c. PICOT inhibits NF-κB activation. Jurkat-TAg cells were cotransfected with an NF-κB:luciferase reporter plasmid plus the indicated amounts of a PICOT expression vector. The cells were either left unstimulated, or stimulated with a combination of anti-CD3 plus anti-CD28 antibodies, or with PMA (100 ng/ml), for the final 6 h of culture. The inset show the expression level of transfected PICOT determined by anti-HA immunoblotting.

this association, or the requirement of other cellular factors (e.g., lipids or adaptor proteins) for optimal interaction between these two proteins. In addition, the association may take place in specific sites within the cell, and only under specific conditions. However, analysis by confocal microscopy revealed overlap, albeit incomplete, between the intracellular localization of PICOT and PKCθ, even in untransfected cells. Nevertheless, our data indicating that PICOT regulates cellular functions mediated by PKCθ or physiological stimuli in T cells suggest that the association between PICOT and PKC is physiologically relevant. Thus, the activation of two important elements in the TCR/CD28 signaling cascade leading to interleukin-2 production, i.e. JNK and AP-1, both of which are selectively activated by PKCθ (6–8), was inhibited by coexpressed PICOT. This effect was selective and did not reflect a general inhibition of cellular functions, since the activation of another MAP kinase, ERK2, was not inhibited by PICOT. Since JNK positively regulates AP-1 activity by phosphorylating two regulatory serine residues in the activation domain of c-Jun (18), it is not surprising that the PKCθ-mediated activation of AP-1 was also inhibited by PICOT.

The JNK/AP-1 pathway is not the only target for inhibition by PICOT, since this novel protein also inhibited the anti-CD3 plus anti-CD28-induced activation of another transcription factor, i.e. NF-κB. Since both of these pathways are commonly activated in response to stress signals and inflammatory stimuli (23–28), our findings suggest that PICOT may regulate stress-induced signaling pathways in other cell types and organisms. Although the N-terminal, Trx-homologous region of PICOT was sufficient to mediate PKC binding, inhibition of AP-1 or NF-κB activation required the intact protein. This raises the possibility that The N-terminal domain of PICOT binds regulators and/or effectors, whereas the C-terminal region mediates the biological functions of this protein. At present, we do not know whether PICOT homologues expressed in lower organisms also interact with, and potentially regulate, protein kinases. Studies to examine the effects of PICOT on stress responses triggered by different stimuli are currently in progress.

Of particular interest is the novel domain that appears as two tandem repeats at the C-terminal region of PICOT. We have provisionally termed this domain PICOT homology (PIH) domain (Fig. 2c). This domain, which hitherto has not been recognized, is highly conserved in evolution from plants to mammals. However, in contrast to PICOT, only one repeat of this domain is found in lower organisms, where it constitutes most of the putative protein encoded by the corresponding EST sequences. The high degree of conservation of this domain suggests that it plays an important, yet to be identified, role in cellular functions. Studies are in progress to isolate PIH domain-interacting proteins.

Although the exact mechanism by which PICOT inhibits the activation of the JNK/AP-1 pathway or NF-κB remains to be elucidated, the homology of PICOT to Trx is of particular interest. The evolutionary conserved Trx system has evolved to protect cells from damage mediated by reactive oxygen species, which are generated as part of a cellular defense mechanism against invading pathogens (17, 29, 30). Various cellular insults, i.e. mitogens, inflammatory stimuli, UV or ionizing radiation, ischemia, phorbol ester, and hydrogen peroxide up-regulate the expression of Trx and induce its translocation to the nucleus. Trx exerts both extracellular and intracellular functions, including its extracellular ability to protect cells from tumor necrosis factor- or Fas-mediated apoptosis (17). Trx is known to promote the DNA binding and transcriptional activ-
NF-κB-mediated in part by regulation of the transcription factors both cell viability and proliferation (17, 29, 30), actions that are highly conserved throughout evolution, plays an important role in regulating possibility that PICOT functions as an endogenous antagonist of Trx or its activating enzyme, Trx reductase, via its ability to compete for substrate binding. Since the Trx system, which is highly conserved throughout evolution, plays an important role in regulating the intracellular redox state, which is critical for both cell viability and proliferation (17, 29, 30), actions that are mediated in part by regulation of the transcription factors NF-κB and AP-1 (30), it is possible that PICOT has a highly conserved role in regulating the Trx system. Our findings, that transient PICOT overexpression inhibits the activation of AP-1 and one of its upstream activators (JNK), as well as NF-κB, are consistent with this putative function.

Finally, since the production of reactive oxygen species (27) and the concomitant induction of genetic programs that mediate defense mechanisms against pathogenic agents (36, 37) are highly conserved in evolution, including in plants (38), an intriguing possibility is that PICOT and its evolutionary conserved homologues regulate innate immunity defense mechanisms. Furthermore, the conservation of Trx system (17, 29, 30) and the PKC superfamily (39, 40) during evolution, and the findings that PKC homologues play a role in plant defense mechanisms against viral pathogens (13, 41), raise the intriguing possibility that an axis consisting of PICOT/PKC/Trx homologues plays a general and well conserved important regulatory role in cellular functions. Thus, the biological significance of PICOT and its putative homologues may extend well beyond its role in T cell activation. Additional studies aimed at elucidating the physiological role and regulation of PICOT are likely to shed light on these notions.

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REFERENCES
1. Newton, A. (1995). J. Biol. Chem. 270, 28495–28498
2. Nishizuka, Y. (1995). FASEB J. 9, 484–496
3. Jaken, S. (1996). Curr. Opin. Cell Biol. 8, 168–173
4. Mochly-Rose, D. (1995). Science 268, 247–251
5. Baier, G., Telford, D., Giampa, L., Coggeshall, K. M., Baier-Bitterlich, G., Isakov, N., and Altman, A. (1993). J. Biol. Chem. 268, 4997–5004
6. Baier-Bitterlich, G., Ueberall, F., Bauer, B., Fresser, P., Wachtler, H., Grunnicke, H., Utermann, G., Altman, A., and Baier, G. (1996). Mol. Cell. Biol. 16, 1842–1850
7. Ghaefian-Talabrizi, N., Bauer, B., Utermann, G., Ueberall, F., and Baier, G. (1999). Eur. J. Immunol. 29, 132–142
8. Werlen, G., Jacinto, E., and Karin, M. (1998). EMBO J. 17, 3101–3111
9. Monks, C. R., Fupfer, H., Tamir, I., Barlow, A., and Kupfer, A. (1997). Nature 385, 83–86
10. Monks, C. R., Freiberg, B. A., Kupfer, H., Siami, N., and Kupfer, A. (1998). Nature 395, 82–86
11. Liu, T.-C., Elly, C., Langdon, W. Y., and Altman, A. (1997). J. Biol. Chem. 272, 168–173
12. Meller, N., Liu, Y. C., Collins, T. L., N.-B., Baier, G., Isakov, N., and Altman, A. (1996). Mol. Cell. Biol. 16, 5782–5793
13. Subramaniam, R., Despres, C., and Ericsson, N. (1997). Plant Cell 9, 653–664
14. Golemis, E. A., Serebreikina, I., Gurtat, J., and Brent, R. (1997) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K., eds) Vol. 3, pp. 20.1.1–20.1.14, Wiley Interscience, New York
15. Witte, S., and Krawinkel, U. (1997). J. Biol. Chem. 272, 22243–22247
16. Liu, Y.-C., Elly, C., Yoshida, H., Bennefey-Berard, N., and Altman, A. (1996). J. Biol. Chem. 271, 14593–14595
17. Nakamura, H., Nakamura, K., and Yodoi, J. (1997). Annu. Rev. Immunol. 15, 351–369
18. Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M., and Ben Neriah, Y. (1994). Cell 77, 727–736
19. Staudinger, J., Zhou, J., Burgess, R., Eledge, S. J., and Olson, E. N. (1995). J. Cell Biol. 126, 263–271
20. Diaz-Meco, M. T., Municio, M. M., Sanchez, P., Lozano, J., and Moscat, J. (1996). Mol. Cell. Biol. 16, 105–114
21. Puls, A., Schmidt, S., Grawe, F., and Stabel, S. (1997). Proc. Natl. Acad. Sci. U. S. A. 94, 6191–6196
22. Meller, N., Altman, A., and Isakov, N. (1996). Stem Cells 15, 178–192
23. Verma, I. M., Stevenson, J. K., Schwartz, E. M., Van Antwerp, D., and Miyamoto, S. (1995). Genes Dev. 9, 2723–2735
24. Baeuerle, P. A., and Baltimore, D. (1996). Cell 87, 13–20
25. Karin, M. (1997). Nature 386, 1066–1071
26. Karin, M., Liu, Z., and Zandi, E. (1997). Curr. Opin. Cell Biol. 9, 240–246
27. Muller, J. M., Roper, R. A., and Baeuerle, P. A. (1997). Methods 11, 301–312
28. O’Neill, L. A. J., and Greene, C. (1997). J. Leukocyte Biol. 63, 550–567
29. Powis, G., Oblong, J. E., Gasdaska, P. Y., Berggren, M., Hill, S. R., Kirkpatrick, D. L., Chantler, E. L., Gallegos, A., Chen, T., Marshall, N., and Cotgreave, I. A. (1994). Oncol. Res. 6, 539–544
30. Holmgren, A., and Bjornstedt, M. (1995). Methods Enzymol. 252, 199–208
31. Hayashi, S., Haji-Kanakishii, K., Makino, Y., Eguchi, H., Yodoi, J., and Tanaka, H. (1997). Nucleic Acids Res. 25, 4035–4040
32. Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., and Yodoi, J. (1997). Proc. Natl. Acad. Sci. U. S. A. 94, 6336–6348
33. Holmgren, A. (1985). Annu. Rev. Biochem. 54, 237–271
34. Oblong, J. E., Berggren, M., Gasdaska, P. Y., and Powis, G. (1994). J. Biol. Chem. 269, 11714–11720
35. Kang, S. W., Baines, I. C., and Rhee, S. G. (1998). J. Biol. Chem. 273, 6303–6311
36. Fearon, D. T., and Lockshay, R. H. (1996). Science 272, 50–53
37. Medzhitov, R., and Janeway, C. A. J. (1997). Cell 91, 295–298
38. Whitham, S., Dinesh-Kumar, S. P., Choi, D., Hehl, R., Corr, C., and Baker, B. (1994). Cell 78, 1101–1115
39. Kruse, M., Gamulin, V., Cekovic, H., Panic, Z., Muller, I. M., and Muller, W. E. (1996). J. Mol. Evol. 43, 374–383
40. Meller, H., and Parker, P. J. (1998). Biochem. J. 332, 281–292
41. Sokolova, M., Pruner, D., Tacke, E., and Rohde, W. (1997). FEBS Lett. 400, 201–205

PICOT, a Novel PKC-interacting Protein

eties of AP-1 and NF-κB as well as the activity of the estrogen receptor (17, 31, 32). It mediates these effects by reducing cysteine residues in the p50 subunit of NF-κB, the two components of AP-1, i.e. c-Jun and c-Fos, and Rel-F, an endonuclease that participates in AP-1 activation (17, 31, 32). These modifications are necessary for the binding of these transcription factors to their cognate DNA sequences in the promoter regions of various genes (17).

Since PICOT does not possess the essential catalytic motif of Trx (Cys-Gly-Pro-Cys) and, in fact, lacks the first of the two cysteine residues in the catalytic center of Trx abolishes its enzymatic (33) and mitogenic (34) activities, and converts the mutated proteins into competitive inhibitors of Trx reductase (34). Another type of peroxiredoxins, the peroxiredoxins, contain a single conserved cysteine residue, but do not share homology with Trx (35). This structural feature of PICOT raises the interesting