Selective Inhibition of Protein Kinase C Isozymes by Fas Ligation*

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Activation of protein kinase C (PKC) can protect cells from apoptosis induced by various agents, including Fas ligation. To elucidate a possible interaction between Fas-mediated apoptotic signals and activation-related protective signals, we investigated the impact of Fas ligation on PKC activity. We demonstrate that engagement of Fas on human lymphoid Jurkat cells triggered apoptosis, and Fas ligation resulted in partial blockade of cellular PKC activity. The phorbol 12-myristate 13-acetate-mediated translocation of PKCα from the cytoplasm to the membrane was inhibited by treatment with anti-Fas antibody, whereas the translocation of PKCε or δ was not affected. In vitro kinase assay of PKCα or ε phosphotransferase activity demonstrated that Fas ligation inhibited the ability of PKCα to phosphorylate histone H1 as substrate but did not inhibit ε isozyme activity. This inhibition of PKCα activity mediated by Fas ligation was reversed by okadaic acid, a phosphatase inhibitor, suggesting the involvement of a member of the protein phosphatase 2A subfamily in this component of Fas signaling. Identical patterns of PKC isozyme inhibition were obtained using mouse thymoma cells overexpressing the fas gene (L5+/+). These results suggest that the selective inhibition of a potentially protective, PKC-mediated pathway by Fas activation may, to some extent, contribute to Fas-induced apoptotic signaling.

The Fas/APO-1 antigen, a member of the tumor necrosis factor receptor family, is a transmembrane molecule and is expressed by a variety of cells, including transformed cell lines and activated T lymphocytes (1–3). The function of Fas/APO-1 appears to be the induction of apoptosis, and a growing number of Fas-associated molecules and signal pathways have been discovered (1, 4–6). Fas mutation or disruption of its function in lymphoproliferation (Lpr) mice leads to a progressive lymphadenopathy and autoimmune syndrome resembling human systemic lupus erythematosus (7–9). The cytotoxic activity of T lymphocytes is also dependent upon normal expression of Fas (10). Thus, Fas/APO-1-induced apoptosis is required for the maintenance of at least two immunological processes in vivo: the normal elimination of potentially autoreactive peripheral T cells and calcium-independent T cell cytotoxicity (6, 11, 12). The mechanisms of Fas-induced apoptosis have been studied extensively (13, 14). The molecules that bind to the intracellular domain of Fas, the death domain, have been identified as MORT1/FADD, TRADD, and RIP (15–18). The association of MORT1/FADD in turn recruits caspase-8/FLICE/MACH-1 to the death complex (19, 20). Caspase-8/FLICE/MACH-1 then transmits the activation signal to ICE and CPP32 and executes the death program (19, 20). Another signal transduction event leading to apoptosis after Fas ligation appears to be mediated by ceramide and involves the activation of p21ras (21–24). Studies have shown that a serine/threonine phosphatase is involved in ceramide signaling pathways (25–27). This ceramide-activated protein phosphatase (CAPP) is a cytosolic enzyme and has been implicated as a specific mediator of ceramide action. CAPP is very sensitive to low concentrations of okadaic acid and therefore belongs to the heterotrimeric subfamily of the protein phosphatase 2A group. Although CAPP has been demonstrated to mediate some of the cellular actions of ceramide, the link between ceramide-induced phosphatase activation and subsequent intracellular events is still not clear.

Protein kinase C (PKC) has been implicated as one of the critical components of multiple signaling pathways, including T cell activation processes (28–30). At least 11 isotypes of PKC have been discovered, and these isotypes can be classified according to their structure and cofactor requirements for activation (31–34). PKCα, β1, β2, and γ are dependent upon calcium for activity, whereas PKCs δ, η, and θ are not. PKCε and λ/ι cannot be activated by phorbol esters or diacylglycerols, although they belong to the PKC family by structure (33). The differential expression of PKC isotypes in mammalian tissues and the different substrate specificities of different PKCs indicate that distinct PKC isotypes may have precise cellular localizations and substrate preferences. T cells express at least seven different isotypes of PKC: α, β1, δ, ε, η, θ, and ζ. PKC isotypes are regulated by phosphorylation and binding of various cofactors. Upon activation, the enzyme is redistributed among different cellular compartments (35–37). The roles of PKCα, ε, and θ have been implicated in T cell activation by transfection experiments (28–30, 38, 39). These three isotypes of PKC regulate T cell activation via control of transcriptional factors such as AP-1, NFAT, and nuclear factor-κB, which, in turn, modulate the activity of the interleukin-2 gene promoter/enhancer (28–30, 38, 39). PKCθ translocates from the cytoplasm to the membrane in response to antigen-specific activation (30, 39). The θ isoform of PKC interacts with 14-3-3, and overexpression of 14-3-3 blocks the activation-mediated translocation of PKCθ (39). Other studies have demonstrated that the human immunodeficiency virus Nef protein inhibits the translocation of PKCθ after phorbol ester stimulation (40). PKCα activity is inactivated by ceramide, perhaps through the action of CAPP (27).

A protective effect of PKC activity in apoptosis has also been

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1 The abbreviations used are: CAPP, ceramide-activated protein phosphatase; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; Ab, antibody.
PKC inhibitors have been shown to block phosphorylation of Bcl-2 and lead to apoptosis, whereas activation of PKC induced phosphorylation of Bcl-2 and abolished the apoptotic process (44–46). Individual isoforms of PKC may play specific roles in the induction of apoptosis. In human U-937 myeloid leukemia cells, PKCζ is cleaved in the third variable region by caspase-3 during apoptosis induced by various agents, and overexpression of cleaved PKCζ fragment resulted in cell death (47). Furthermore, cleavage of another isoform, PKCδ, by caspase-3-cysteine protease caused cells to undergo apoptosis, whereas overexpression of the anti-apoptotic factors Bcl-2 or Bcl-XL blocked the cleavage of PKCδ (44–46).

Cell surface expression levels of Fas on resting T lymphocytes can be induced rapidly in response to T cell activation (1, 48, 49). Activation-induced T cell death has been implicated in the termination of immune response (6, 11, 12). Ligation of Fas significantly suppresses TCR/CD3 complex-mediated early signal transduction events, including inhibition of TCR/CD3-triggered tyrosine phosphorylation of cellular proteins (50). Thus, Fas engagement may attenuate T cell activation events, whereas T cell activation-related events, such as activation of PKC, may reciprocally protect cells against apoptosis. This opportunity for cross-talk between signaling pathways led us to examine the potential interrelationship between these two seemingly opposing processes: apoptosis and activation. Here, we demonstrate that engagement of Fas triggers the apoptotic process in human Jurkat and mouse thymoma cells stably expressing the fas gene (LF(+)) but not in mouse thymoma cells stably transfected with antisense fas gene (LF(−)). Fas ligation selectively inhibits the activation of different isoforms of PKC in both Jurkat and LF(−) cells. The translocation of PKCζ in response to phorbol 12-myristate 13-acetate (PMA) stimulation is inhibited by prior Fas ligation. PKCζ-mediated phosphorylation of histone H1 is blocked by prior Fas activation. This inhibition of PKC activity by Fas activation could be prevented by pretreatment with okadaic acid, indicating an involvement of a protein phosphatase in Fas signaling. The activity of PKCζ was not affected by Fas stimulation. Therefore, these data suggest that the integration of multiple pro- and anti-apoptotic signals resulting from Fas activation may be required to execute the apoptotic program successfully.

**Experimental Procedures**

**Cell Lines**—The human lymphoblastoid cell line Jurkat (American Type Culture Collection, Rockville, MD) was maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated newborn calf serum (Hazeltan Research Products, Inc., Lenoxa, KA), 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. LF1210 mouse thymoma cells were transfected with the fas gene (LF(+) or an antisense fas gene (LF(−)) (generous gifts from Dr. S. T. Ju, Boston University) and cultured in RPMI 1640 medium containing 10% heat-inactivated newborn calf serum plus 0.7 mg/ml Genetin.

**Cell Viability Assays**—Jurkat, LF(+), or LF(−) cells (0.5 × 10⁶ cells/ml) were cultured in six-well plates with 5 ml of medium containing 10% newborn calf serum plus 1.5 μg/ml anti-human Fas Ab (Pan Vera Corp., Madison, WI) for Jurkat and PH1 cells or 1.5 μg/ml IgM Ab as control. The mouse thymoma cells (LF) were cultured under the same conditions with the addition of 5 μg/ml mouse anti-mouse Fas Ab (Pharmingen, San Diego). Cells were collected at the time points indicated and enumerated using trypan blue dye exclusion to assess viability. Error bars represent the S.D. over five independent experiments.

**DNA Fragmentation Assay**—The cells (1 × 10⁷/ml) were cultured with 1.5 μg/ml anti-human Fas Ab and resuspended in 1 ml of 1% sodium citrate, 0.1% Triton X-100, 50 μg of propridium iodide, and 10 μl of RNase (1 mg/ml). The stained samples were kept in the dark at 4 °C overnight before DNA fragmentation analysis by FACScan (Becton Dickinson, Mountain View, CA).

**PKC Enzymatic Assay**—Human and mouse cells (1 × 10⁶ cells/ml) were cultured in five replicate wells of a six-well plate with 10 ml of medium containing 1.5 μg/ml anti-human Fas Ab or 5 μg/ml anti-mouse Fas Ab for 60 min and subsequently treated with 100 nM PMA for 15 min. For single stimulation experiments, the cells were exposed to either anti-Fas Ab or PMA alone for 60 or 15 min, respectively. For PKC enzyme activity inhibitor experiments, cells were exposed to PMA for 15 min after a 30-min preincubation for 15 min. After washing in 25 mM Tris-HCl (pH 7.5), 1% Triton X-100, 200 mM MgCl₂, 150 mM NaCl (9), the lysates were normalized for protein concentration, and 150-μg aliquots of protein were analyzed for PKC activity using a PKC assay kit containing a specific substrate peptide for PKC and an inhibitor mixture that blocks the activity of PKCα and calmodulin kinase (Upstate Biotechnology Inc., Lake Placid, NY). Subsequently, the 32P-incorporating substrate from each treatment was separated from the residual [32P]ATP using p81 phosphocellulose paper, and the radioactivity incorporated into the substrate was measured by scintillation counting.

**Immunoblotting and Cell Fractionation**—Cells (50 × 10⁶), following the different treatments described above, were washed twice with 1 × phosphate-buffered saline and resuspended in 1 ml of buffer B (20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 5 mM EGTA, 10 μM β-mercaptoethanol, 10 μg/ml leupeptin, 10 μg/ml aprotinin) (39). The cell suspensions were transferred to a 1-ml syringe and sheared by being passed 40 times through 25-gauge needle. The lysates were centrifuged at 280 × g for 10 min to precipitate nuclei, and the supernatants were collected. One-third of the whole cell extract was saved, and the remainder was centrifuged at 16,000 × g for 30 min. The supernatant was collected, and the pellet was washed in buffer B containing 1% Nonidet P-40 for 1 h on ice and centrifuged again at 16,000 × g. The supernatant representing the membrane fraction was saved. Each fraction (whole cell lysate, cytosol fraction, and membrane fraction) was normalized and separated on 5% acrylamide gel. Subsequently, the gel was immunoblotted with the anti-mouse (Santa Cruz Biotechnology, Santa Cruz, CA) or human (Transduction Laboratories, Lexington, KY) PKC Abs, which recognize the isoforms of PKCα, α, and ε. The blot was developed with an anti-mouse Ig alkaline phosphatase reagent (Onogene Science, Uniondale, NY).

**In Vitro Kinase Assay**—The cells (20 × 10⁶) were incubated with PMA at 100 nM for 15 min prior to 60 min of exposure to anti-Fas Abs. Fas-mediated phosphatase inhibitor experiments, okadaic acid was added to a final concentration of 50 μM 15 min before the next treatments. The cell lysates were then prepared with the lysis buffer (150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, 5 mM EGTA, 10 mM NaF, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride) (27). The lysates were normalized for protein concentration, and each sample (containing approximately 500 μg of protein) was immunoprecipitated with the corresponding anti-PKC Ab and collected by absorption to protein A-Sepharose. The immunocomplexes bound to protein A-Sepharose were washed with lysis buffer twice and kinase buffer twice (50 mM Tris-HCl (pH 7.4), 10 mM NaF, 1 mM Na₃VO₄, 0.5 mM EDTA, 0.5 mM EGTA, 2 mM MgCl₂, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride). Subsequently, the immunocomplexes bound to the beads were resuspended in reaction buffer (20 mM Tris-HCl (pH 7.4), 10 mM MgCl₂, 10 μM cold ATP, 0.4 mM [γ-32P]ATP, 0.25 μg/ml histone H1, 2.25 μCi of [γ-32P]ATP (6,000 Ci/mnmol) and incubated at 30 °C for 10 min. The reactions were terminated by the addition of protein loading buffer. Proteins were separated on 10% SDS-polyacrylamide gel, and the gel was subjected to autoradiography.

**Results**

**Fas-mediated Apoptotic Signals Inhibit PKC Activation**—Jurkat cells express a significant amount of Fas antigen on their surfaces (51). The sensitivity of these cells to treatment with anti-Fas Ab was examined in medium containing 10% serum (Fig. 1a). The number of viable Jurkat cells did not begin to decline until 5 h after exposure to the Ab, and the death proportion of dead cells increased steadily from that time on. By 24 h, approximately 65% of the Jurkat cells lost viability. Exposure to an unrelated, isotype-matched (IgM) antibody did not induce cell death in Jurkat cells; rather, the numbers of cells increased to approximately 6 h after exposure to the mouse IgM Abs (Fig. 1b), reflecting normal cell growth. The cell death observed was caused by apoptosis, as confirmed by cytofluorometric analysis of nuclear DNA fragmentation, as described previously (Fig. 1b). The percentage of cells with fragmented DNA 15 h after the addition of anti-Fas Ab was more than 20%, and by 24 h more than 30% of the cells contained fragmented DNA. This...
Fig. 1. Cell growth kinetics and DNA fragmentation in response to Fas ligation. Panel a, growth curves of Jurkat cells in the presence of either anti-Fas Ab or an irrelevant IgM Ab. Viable cells were enumerated at the time points indicated. Error bars represent the S.D. over five independent experiments. Panel b, percent of Jurkat cells with DNA fragmentation at different time points after the addition of anti-Fas Ab. Error bars represent the S.D. over five independent experiments.

We demonstrated previously that down-regulation or inhibition of PKC could induce Jurkat cells expressing oncogenic ras to undergo apoptosis and that Fas-mediated signals may be involved in this apoptotic process (51, 52). Several other groups have documented that PKC can regulate or modulate both proliferative and anti-apoptotic pathways in diverse cell types (28–30, 38, 39, 41–44, 53). The involvement of these common messengers in signaling pathways with disparate outcomes, cell activation or cell death, suggested that these opposing pathways may interfere with each other or utilize cross-talk to facilitate execution of their respective end points. T lymphocytes express several isotypes of PKC, and the functions of the isotypes \( \theta \), \( \alpha \), and \( \epsilon \) have been studied extensively in T cell activation (28–30). PMA mobilizes PKC, and together with increasing cytoplasmic free calcium, mimics some aspects of T cell activation. To examine the interrelationships described above, PKC enzymatic activity was measured. A PKC-specific enzymatic assay for detecting total PKC activity was used to measure the phosphotransferase activity of PKC in Jurkat cells after different combinations of Fas ligation or PMA treatment (Fig. 2a). Adding anti-Fas Ab alone for 60 min did not elicit PKC activation in Jurkat cells. The phosphotransferase activity of PKC was increased 15 min after treatment with PMA alone, at 100 nM, however, induction of total cellular PKC activity by PMA was attenuated substantially (by 60%). In reversing the sequence of the treatments, it was found that ligation of Fas after prior stimulation of the cells with PMA for 15 min did not suppress or interfere with PKC activation. Staurosporine, a PKC inhibitor, dramatically inhibited PKC activity in response to PMA, as expected. Because Fas-generated signals blocked PMA-mediated PKC activation, we next determined whether this blockade of PKC activation by Fas signaling was sustained at later time points after Fas ligation. PKC enzymatic activity was examined at various time points after exposure to anti-Fas antibody (Fig. 2b). Up to 6 h after Fas ligation, PMA treatment still could not elicit PKC activity. Because of the very large fraction of the cells undergoing apoptosis after that time point, it was impossible to collect enough cells to assay PKC enzymatic activity. The levels of PKC protein, measured by immunoblotting, at different time points after Fas engagement were similar, excluding the possibility that the effects of Fas ligation on PKC activity were the result of changes in the expression PKC proteins (Fig. 2b, inset).

Blockade of PKC\( \theta \), \( \epsilon \) Translocation from Cytoplasm to Membrane by Fas Ligation—One of the phenomena associated with PKC activation is the redistribution of the enzyme among different cellular compartments (35–37). It has been proposed that receptors for activated protein kinase C (or 14-3-3 \( \tau \)) or Nef oncoprotein may mediate PKC translocation (35, 39, 40). The major isozymes of PKC (\( \theta \), \( \alpha \), and \( \epsilon \)) undergo translocation from the cytoplasm to the cellular membrane in T cells stimulated with PMA (30, 35, 39, 40). To examine whether the inhibitory effect of Fas ligation on PKC activation might affect enzyme or isozyme translocation, cells were treated with PMA, anti-Fas Ab, or a combination of them (Fig. 3a). The levels of PKC\( \theta \), \( \alpha \), and \( \epsilon \) proteins in the nuclear-free whole cell, membrane, or cytoplasmic fractions from stimulated or unstimulated cells were assessed by immunoblotting with the corresponding antibodies. (Because the Fas-mediated cell death program is executed at the cytoplasmic level (10, 21, 51), isozyme data from the nuclear fraction were not included in this study.) Comparable amounts of the three isozymes were expressed in the cytoplasm of unstimulated Jurkat cells and underwent translocation to the membrane fraction upon PMA stimulation. The addition of anti-Fas Ab alone did not mobilize the enzyme isoforms to redistribute. After the addition of the anti-Fas antibody for 1 h, PKC\( \theta \) no longer translocated from the cytoplasm to the membrane in response to PMA stimulation, whereas PKC\( \alpha \) and PKC\( \epsilon \) translocated normally. The translocation of PKC\( \epsilon \), a PMA-insensitive isoform, was also examined under the same stimulatory conditions. Neither treatment with PMA nor anti-Fas Ab mobilized this isozyme from the cytoplasm to the cellular membrane.

Inhibition of PKC\( \alpha \) Activity by Fas-Mediated CAPP—It has been reported that ceramide generated intracellularly can activate CAPP, a member of the protein phosphatase 2A subfamily, which may then play an important role in the regulation of
ceramide-induced apoptosis in various types of cell lines, or of ceramide-mediated down-regulation of e-Myc in myeloid leukemia cells (25–27). Activation of CAPP in Molt-4, a human leukemia cell line, and in Jurkat cells blocked the activity of PKCα at the downstream level of enzyme translocation (27). Because ceramide has also been reported to play a role in Fas-induced apoptosis (21–24), we tried to determine the potential involvement of CAPP in Fas-mediated signaling and whether this phosphatase may interfere with PKC activity (Fig. 3b, two upper rows). The cells were treated with PMA, anti-Fas Ab, or a combination of the two, with or without exposure to okadaic acid or staurosporine (a serine/threonine protein kinase inhibitor), and the lysates were subsequently prepared for in vitro PKC kinase assay. After immunoprecipitation with either anti-PKCα or e Ab, the immunoprecipitates were collected on protein-A-Sepharose beads for in vitro kinase assay of phosphotransferase activity. Nuclear free whole cell lysates were also separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with either anti-PKC or e Ab to determine isoyzme protein levels (bottom two rows). The results presented are representative of three independent experiments.

Fig. 2. Inhibition of PKC activity after Fas ligation. Panel a, relative [32P] incorporation into a PKC-specific substrate peptide after Fas ligation, with or without subsequent PMA stimulation, in lysates of Jurkat cells. Error bars represent the S.D. over five independent experiments. Control, untreated cells; PMA + IONO, cells treated with 100 nM PMA plus 2 μM calcium ionophore for 15 min; PMA, cells treated with 100 nM PMA for 15 min; anti-Fas Ab, cells treated with 1.5 μg/ml anti-human Fas Ab alone; anti-Fas Ab + PMA, cells treated with 1.5 μg/ml anti-Fas Ab for 60 min before exposure to 100 nM PMA for 15 min; stauro. + PMA, cells treated with 0.1 μM staurosporine for 15 min before exposure to 100 nM PMA for 15 min; PMA + anti-Fas Ab, cells treated with 100 nM PMA for 15 min before anti-Fas Ab treatment (1.5 μg/ml). Panel b, PMA-induced relative [32P] incorporation into a PKC-specific substrate peptide at different time points after preincubation with anti-Fas Ab (1.5 μg/ml). Cells were harvested 15 min after the addition of PMA and lysates used for PKC activity assay. Error bars represent the S.D. over five independent experiments. Control, untreated cells; PMA, cells treated with 100 nM PMA for 15 min; anti-Fas 2 h, 4 h, or 6 h + PMA, cells treated with 1.5 μg/ml anti-Fas Ab for 2, 4, or 6 h, respectively, before exposure to 100 nM PMA. The levels of total cellular PKC protein in response to Fas ligation at the various time points indicated above were also determined (inset). Proteins in lysates from the cells were separated on a SDS-polyacrylamide gel and immunoblotted with an anti-PKC Ab that recognizes a region common to all isotypes of PKC.

Fig. 3. Selective inhibition of different PKC isoforms by Fas ligation. Panel a, effect of Fas ligation on the intracellular localization of PKC isoforms. Jurkat cells were treated with PMA, anti-Fas Ab, or a combination of the two. Nuclear-free whole cell lysates (WC), membrane fractions (M), and cytoplasmic fractions (C), were prepared. After immunoprecipitation with isotype-specific antibodies (α, ε, β, γ), immunoblotting was performed with the corresponding Abs. Control, untreated cells; PMA, cells treated with 100 nM PMA for 15 min; anti-Fas Ab, cells treated with 1.5 μg/ml anti-Fas Ab for 60 min; anti-Fas Ab + PMA, cells treated with 1.5 μg/ml anti-Fas Ab for 60 min before exposure to 100 nM PMA for 15 min. Panel b, effect of Fas ligation on the kinase activity of PKC isoforms. Jurkat cells were treated with PMA, anti-Fas Ab, or a combination of the two in the absence or presence of 10 nM okadaic acid (OA) or 0.1 μM staurosporine (Stau.). The lysates were immunoprecipitated with either anti-PKCα or e Ab, and the immunoprecipitates were collected on protein-A-Sepharose beads for in vitro kinase assay of phosphotransferase activity. Nuclear free whole cell lysates were also separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose, and immunoblotted with either anti-PKCα or e Ab to determine isoyzme protein levels (bottom two rows). The results presented are representative of three independent experiments.
Inhibition of PKCζ, ε by Fas Ligation

ulated with PMA could no longer phosphorylate histone H1. PMA-stimulated enzymatic activity of PKCζ immunocomplexes was similarly suppressed after Fas Ab treatment (not shown), consistent with the suppression of translocation of this isotype to the cell membrane (see panel a). In the presence of okadaic acid, a phosphatase inhibitor, at 10 nM, however, histone H1 was again phosphorylated by PKCs from PMA-activated, anti-Fas Ab-treated cells. PMA-stimulated PKCe isozyme activity was unaffected by Fas ligation or by okadaic acid. These results indicated that a CAPP-like phosphatase might be involved in the Fas-mediated signals that inactivate the PKCζ isofrom. In addition, the lack of an effect of Fas ligation on PMA-mediated PKCe activity may define discrete regulatory pathways for the different isotypes of PKC and may also explain the incomplete inhibition of PKC activity by Fas ligation (as demonstrated in Fig. 2a). Together, these data suggest that the signals generated by Fas engagement which interfere with the activity of certain PKC isotypes may function selectively at different steps along PKC pathways. The protein levels of the PKC isoforms under the same conditions were also determined, to exclude the possibility that changes in protein levels could account for the observed differences in enzymatic activities (Fig. 3b, two lower rows). The levels of PKCζ and ε proteins did not change after the different treatments.

Fas Ligation Results in Blockade of PKC Activity in Mouse Thymocytes Overexpressing the fas Gene—To confirm and generalize the results obtained in human Jurkat cells, mouse thymoma cells stably transfected with either fas (LF(1) or antisense fas (LF(-)) genes were studied (Figs. 4 and 5). The level of cell surface expression of Fas on LF(1) cells was comparable to that on Jurkat cells (51). After the addition of anti-mouse Fas Ab, the numbers of viable LF(1) cells started to decline at 12 h, and about 80% of the cells had died by 36 h (Fig. 4a). In comparison, the viability of LF(-) cells was not affected by Fas engagement; rather, by 12 h after the addition of anti-Fas Ab, the numbers of LF(-) cells were seen to increase. The occurrence of apoptosis in LF(1) cells after Fas ligation was confirmed by an assay of DNA fragmentation (Fig. 4b). By 15 h after the addition of anti-Fas Ab, more than 15% of LF(-) cells had fragmented DNA, and the percentage of DNA fragmentation was increased further at 24 h after Fas ligation (to more than 25%), which is in good agreement with the cell viability assay results seen in Fig. 4a. The percentage of DNA fragmentation in LF(-) cells did not increase at any time point after Fas engagement and remained comparable to that of the control (untreated) cells. Cell viability assays and DNA fragmentation assays were also performed in the parental LF1210 cells. Because there was only a very low level of Fas antigen expressed on the surface of LF1210 cells, apoptosis in response to Fas ligation was minor (data not shown). The effect of Fas ligation on PKC activity in these cell lines was then defined. A PKC enzymatic assay was performed in LF(1) and LF(-) cells after treatment with anti-Fas and/or PMA (Fig. 5a). PKC activity was not elicited by exposure to anti-Fas Ab for 60 min in either LF(1) or LF(-) cell lines, whereas 15 minutes of treatment with PMA activated PKC in both cells. 1 h after the addition of anti-Fas Ab, PKC activation in LF(1) cells was inhibited significantly (by 60%) but not in LF(-) cells. The levels of PKC protein were assayed in LF(+) and LF(-) cells by immunoblotting following the same treatments, and no changes in the protein levels were induced by PMA or anti-Fas Ab. To define further how the signals generated through Fas ligation may block the PMA-elicited PKC activation, the translocation status of PKCζ, α, and ε in response to different treatments was examined by immunoblotting protein lysates from the LF(+) cells (Fig. 5b). After different combinations of stimuliants, cytoplasmic and membrane fractions and the whole cell lysates of LF(+) cells were prepared for the detection of PKCζ, α, and ε. All three PKC isoforms were expressed in the cytoplasmic fraction of unstimulated LF(+) cells but not in the membrane fraction. In response to treatment with PMA, the majority of the PKCζ and α proteins translocated from cytoplasm to membrane, whereas PKCζ did not translocate. Fas engagement by itself did not cause redistribution of any of the three enzymes. After exposure of the cells to anti-Fas Ab for 1 h, PMA treatment no longer induced the translocation of PKCζ to membrane. However, the translocation of PKCα in response to PMA treatment was not affected by prior Fas ligation. Analysis of PKCε subcellular location by immunoblotting was also conducted; and as found in Jurkat cells, Fas-
initiated signals did not block the translocation of the enzyme (data not shown).

Involvement of a CAPP-like phosphatase in this Fas-activated signaling pathway was suggested in Jurkat cells. To determine if this observation was generalizable, in vitro PKC kinase assays were performed in LF(1) cells (Fig. 5c, two upper rows). Cell lysates were prepared after different treatments, and immunoprecipitations were carried out with anti-PKCα or ε Ab, and a PKC kinase assay was performed by mixing the immunocomplexes with histone H1 to serve as a substrate. Histone H1 was not phosphorylated by PKC isoyme immunocomplexes from control samples or from cells treated with anti-Fas Ab. However, histone H1 protein phosphorylation activity was present in PKCα and ε immunocomplexes from cells treated with anti-Fas Ab and was suppressed in the presence of staurosporine. 1 h after the addition of anti-Fas Ab, PKCα could not be activated by exposure to PMA, as assayed by histone H1 kinase activity, but PKCε isoyme activity was intact. Treatment with okadaic acid prevented the blockade of PKCα activity by Fas ligation. The protein levels of the PKC isoforms under the same conditions were also determined, to exclude the possibility that these different treatments may affect the expression of the isoforms (Fig. 5c, two lower rows). The different treatments did not alter the levels of PKCα or ε. These data were in good agreement with the findings generated in Jurkat cells and suggested again that Fas-generated signals may interfere selectively with the activities of different isotypes of PKC.

Preactivation of PKC Partially Protects from, or Delays, Fas-induced Apoptosis—PKC is an important component of signaling pathways regulating cell activation and proliferation (28–30, 53). Activation of PKC elicits a variety of cellular responses, mediated by phosphorylation of its downstream effector proteins on serine/threonine residues. It has been demonstrated that activation of PKC can protect cells from apoptosis mediated by various apoptotic stimuli, such as ceramide-induced
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Fas-mediated apoptosis, initiated by lymphocyte activation pathways, plays an important role in the elimination of immature autoreactive lymphocytes during thymic selection, as well as in the regulation of the peripheral lymphocyte pool, through the engagement of Fas antigen on the surface of lymphocytes by either anti-Fas Ab or Fas ligand (11, 12). The PKC family consists of more than 10 isoforms, and they are selectively engaged in various biological processes, including critical roles in mediating lymphocyte activation signals (31–34). Although the exact role of these different isozymes in cellular functions remains enigmatic, it has been documented that the activation of PKC by diacylglycerols or phorbol esters can protect cells from apoptosis induced by various biological or chemical agents, especially ceramide-induced apoptosis (31–34). Fas ligation, neutral sphingomyelinase, and tumor necrosis factor-\(\alpha\) can activate intracellular ceramide, which can then mediate apoptosis (21–24). This study examined the possible interactions between apoptotic signals elicited by Fas activation and protective events generated by PKC, both of which can be the direct results of T cell activation stimuli. We demonstrate herein that Fas engagement in human Jurkat cells and in mouse thymoma cells overexpressing Fas antigen (LF(+) cells) partially blocked phorbol ester-induced PKC activation, and this inhibition persisted for at least 6 h after Fas ligation. Fas ligation was not able to suppress PKC activity when the PMA stimulus was delivered before Fas ligation, however. The suppression of PKC activity by Fas ligation involved certain T cell activation-related isotypes of PKC (\(\theta\) and \(\epsilon\)) and blocked them at different steps of the enzyme activation pathway. The translocation of PKC\(\theta\) in response to PMA was blocked by ligation of Fas antigen. PKC\(\alpha\)-mediated translocation was not blocked, but its activity, as assayed by phosphorylation of histone H1, was inhibited after Fas ligation. This inhibition may be mediated by a member of the protein phosphatase 2A subfamily. Overall, these data suggest that blockade of certain isoforms of PKC, at different levels of the enzyme activation depending on the specific isozyme, may be a part of Fas signaling, PKC\(\alpha\) and \(\theta\) activation may be suppressed by Fas signals or Fas-related inhibitory proteins so as to ensure rapid execution of the apoptotic process. In addition, loss of PKC\(\alpha\) activity may itself directly promote apoptosis, as shown recently in an experimental system where a dominant negative PKC\(\alpha\) transgene induced programed cell death in COS cells (54).

This observed difference in the mechanism by which Fas ligation inhibits PKC\(\alpha\) activation compared with \(\theta\) is of interest. The process of regulation of PKC by lipids such as phosphatidylserine and diacylglycerol includes several discrete

![Fig. 6. Protective effect of PKC activation on the Fas-induced apoptotic process. Panel a. Jurkat cells, with or without pretreatment with PMA for 15 min, were cultured in medium containing anti-Fas Ab, and the numbers of viable cells were enumerated at the time points as indicated in the figure. The error bars represent the S.D. of five independent experiments. Panel b, DNA fragmentation in Jurkat cells was analyzed after Fas ligation in response to pretreatment with PMA. The error bars represent the S.D. of five independent experiments. Control, untreated cells; 100 nm PMA + anti-Fas Ab Th, 9h, 15h, 24h, cells treated with 100 nm PMA for 15 min before exposure to anti-Fas Ab for 7, 9, 15, or 24 h, respectively.](image-url)
events, such as membrane translocation and phosphorylation of these enzymes (28–30, 35–37). PKC participates in signal transduction pathways through mobilization from the cytoplasm to the cellular membrane, where it is then activated by membrane-associated stimulatory molecules. All isozymes of PKC contain the conserved regulatory regions, C1 and C2 (31–34, 55). Cleavage of certain isozymes, like PKCθ, in the third variable (V3) region deletes the two regulatory regions and results in a catalytically active fragment that appears to be associated with the induction of apoptosis. It has been demonstrated that PKCθ can be cleaved by caspase-3. One explanation for the blockade of PKCθ translocation by Fas ligation may be that the enzyme is a dual function molecule. Activation of the caspase protease family by Fas ligation may result in the generation of the pro-apoptotic fragment of PKCθ, and therefore the enzyme could function as a regulator in the death program. Alternatively, it is possible that PKCθ activation (in the absence of cleavage) would normally serve an anti-apoptotic, protective function, like PKCα. Experiments employing ectopic or overexpression of this enzyme isoform will be necessary to determine the role of PKCθ in pro-versus anti-apoptotic processes.

Several studies have demonstrated that sphingosine or lysosphingolipids can inhibit PKC activity, induce growth arrest at the G1 phase of the cell cycle, and down-regulate c-myc expression (26, 56). The mechanism by which C2-ceramide or sphingomyelinas inactivates the PKCs isoform, in particular, remains controversial (27, 57). In mouse epidermal cells (HEL-37) or human skin fibroblasts (SF3155), the phorbol ester-induced translocation of PKCa, but not of PKCε, was inhibited by ceramide treatment (57). Inhibition of translocation did not occur, however, in MOLT-4 human leukemia cells and Jurkat cells (27). Instead, the inactivation of PKCα by ceramide MOLT-4 and Jurkat cells appears to be through activation of CAPP. In our studies, we demonstrated that exposure to PMA could still induce the translocation of PKCa, even after engagement of Fas by anti-Fas Ab. Yet, the activity of the enzyme, as measured by the phosphorylation of histone H1 substrate, was suppressed dramatically, and this inhibition could be reversed by okadaic acid at concentrations in the range required for inhibition of protein phosphatase 2A in vitro. Because activation of the ceramide/Ras pathway is one component of Fas-induced signaling, the possible existence and activation of CAPP in Fas-mediated pathways may account for the subsequent intracellular events we observe, including the inhibition of PKCa activity. The activity of PKCs in the cells remained inhibited after Fas ligation even after the enzyme was immunoprecipitated, suggesting post-translational modification of the enzyme as a result of Fas ligation or, less likely, the association of an inhibitory protein that coprecipitates in an inhibitory complex with the enzyme. Activation of PKC by phorbol esters is associated with new phosphorylation of the PKC molecule itself. New phosphorylation of PKCa, but not of the ε isoform, by PMA treatment was partially inhibited in the presence of anti-Fas Ab. The finding that Fas ligation subsequent to prior PMA treatment does not inhibit PKC activity suggests that inhibitory process activated by the Fas signal may prevent activation of PKCa rather than inhibiting its enzymatic activity directly. Although a CAPP-like phosphatase may play a role in the inhibition of PKCa activity by Fas, there are no data to support a direct interaction between PKCa and a phosphatase. Rather, the data cumulatively suggest that activation of CAPP-like phosphatase by Fas ligation may indirectly regulate this isozyme of PKC. It is possible that CAPP may activate a PKCa-specific, inhibitory protein. In preliminary studies, we have found that cytoplasmic extracts from cells in which Fas-initiated signals had been induced contain an activity that inhibits PKC activation in vitro. The lack of responsiveness of PKCε to Fas ligation demonstrates the discrete and specific nature of Fas-generated signals and lends support to the general concept that there are distinct functions for the different isozymes of PKC and that these isoforms can themselves be regulated differentially by networks of kinases and phosphatases. A study has shown that down-regulation of c-myc expression in human myelogenous leukemia HL-60 cells could be regulated by an okadaic acid-insensitive, PMA-mediated pathway or an okadaic acid-sensitive, dephosphorylation mechanism (26).

Our findings suggest a model for the integration of death-inducing signals and survival signals. The apoptotic signals generated by Fas ligation initiate a cascade of reactions and drive the cells to undergo apoptosis. To ensure such a process, the signals mediated by Fas engagement may also suppress potential protective mechanisms, such as activation of the PKC pathway. If these protective pathways are activated prior to Fas ligation, the process of Fas-mediated apoptosis is delayed or is less inefficient. When the PKC pathway is activated, subsequent Fas ligation can no longer block the phosphotransferase activity of PKC, and the cells become more resistant to Fas-mediated apoptotic signals. Although preactivation of the PKC pathway did, to some extent, interfere with the apoptotic signal, it did not engender full protection. The initial Fas signal triggers multiple diverging pro-apoptotic pathways, with the major Fas-mediated apoptotic signal being through the ICE protease cascade. Although the ceramide/Ras pathway has been suggested as another result of Fas signaling which can induce apoptosis, its ability to act independently of the ICE pathway is unclear (21–24). PKCε activation protects cells from various apoptotic processes, including ceramide-mediated apoptosis (28–30). The protection against apoptosis provided by PKC activity may thus only extend to a subset of Fas-initiated signals, perhaps ceramide/Ras-related signals. Cross-linking of cell surface Fas antigen is known to suppress some of the TcR/CD3-mediated signal transduction events in human T lymphocytes (50). After pretreatment with anti-Fas Ab, CD3-stimulated production of inositol trisphosphate and tyrosine phosphorylation of multiple cell proteins was inhibited. The inhibition of PKC activity and translocation we observe to result from Fas ligation may account, in part, for this suppression, and this hypothesis is under investigation.

The data presented herein, together with the reports from other laboratories, clearly demonstrate that anti-apoptotic mechanisms or molecules may be induced by PKC activation. It is noteworthy that activation-induced cell death in T lymphocytes involves the initial activation of mitogenic (and protective) pathways (57) and subsequent desensitization to external stimuli (58). At later times following T cell activation, when Fas expression is up-regulated, susceptibility to Fas-mediated apoptosis increases, and protective molecules such as PKCε are repressed, perhaps facilitating cell death. The reciprocal interactions between protective and pro-apoptotic signals as shown here may account for the protective (anti-apoptotic) mechanisms proposed to be present at early times after activation (50, 59). The general concept that activation signals in the cell might actively inhibit or oppose apoptotic programs has been proposed in other systems, including extracellular signal-regulated kinase activation in nerve growth factor-differentiated PC-12 cells and CD3 cross-linking in T lymphoblasts (50, 60). These results suggest a novel mechanism for Fas-mediated cell death. Inhibition of PKC-mediated pathways by Fas engagement would prevent rescue or protection from apoptosis by

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2 C. Y. Chen and D. V. Faller, unpublished data.
coincident of subsequent PKC activation. Furthermore, the data indicate that cross-talk between these two opposing signals (destructive versus protective) may occur at a very early stage, and perhaps at cellular membrane level, to determine cell fate: life or death.

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