Protein Kinase C Regulates FADD Recruitment and Death-inducing Signaling Complex Formation in Fas/CD95-induced Apoptosis*

Received for publication, May 30, 2001, and in revised form, August 20, 2001
Published, JBC Papers in Press, October 1, 2001, DOI 10.1074/jbc.M104919200

Mireia Gómez-Angelats and John A. Cidlowski‡
From the Laboratory of Signal Transduction, Molecular Endocrinology Group, NIEHS, National Institutes of Health, Research Triangle Park, North Carolina 27709

Activation of protein kinase C (PKC) triggers cellular signals that inhibit Fas/CD95-induced cell death in Jurkat T-cells by poorly defined mechanisms. Previously, we have shown that one effect of PKC on Fas/CD95-dependent cell death occurs through inhibition of cell shrinkage and K⁺ efflux (Gómez-Angelats, M., Bortner, C. D., and Cidlowski, J. A. (2000) J. Biol. Chem. 275, 19609–19619). Here we report that PKC alters Fas/CD95 signaling from the plasma membrane to the activation of caspases by exerting a profound action on survival/cell death decisions. Specific activation of PKC with 12-O-tetradecanoylphorbol-13-acetate or bryostatin-1 induced translocation of PKC from the cytosol to the membrane and effectively inhibited cell shrinkage and cell death triggered by anti-Fas antibody in Jurkat cells. In contrast, inhibition of classical PKC isotypes with Gö6976 exacerbated the effect of Fas activation on both apoptotic volume decrease and cell death. PKC activation/inhibition did not affect anti-Fas antibody binding to the cell surface, intracellular levels of FADD (Fas-associated protein with death domain), or c-FLIP (cellular FLICE-like inhibitory protein) expression. However, processing/activation of both caspase-8 and caspase-3 and BID cleavage were markedly blocked upon PKC activation and, conversely, were augmented during PKC inhibition, suggesting a role for PKC upstream of caspase-8 processing and activation. Analysis of death-induced signaling complex (DISC) formation was carried out to examine the influence of PKC on recruitment of both FADD and procaspase-8 to the Fas receptor. PKC activation blocked FADD recruitment and caspase-8 activation and thus DISC formation in both type I and II cells. In contrast, inhibition of classical PKCs promoted the opposite effect on the Fas pathway by rapidly increasing FADD recruitment, caspase-8 activation, and DISC formation. Together, these data show that PKC finely modulates Fas/CD95 signaling by altering the efficiency of DISC formation.

The balance between negative and positive life signals that cells are exposed to is a fundamental process in cellular homeostasis. Apoptosis is a cell death process that plays a key role in the regulation of the immune system, and excessive apoptosis has been shown to occur in diseases such as AIDS, in which there is a massive removal of CD4⁺ helper T-cells. In contrast, defective apoptosis can have detrimental effects on the regulation of the immune response or on the emergence of hyperplasia among immune system cell lineages (1). Fas/CD95 is a member of tumor necrosis factor receptor superfamily that is highly expressed in the plasma membrane of a variety of cell types, including lymphoid cells. Apoptosis triggered by activation of members of the tumor necrosis factor receptor superfamily is a critical process for the maintenance of T- and B-cell homeostasis and thus essential for regulation of immune responses. Specifically, removal of activated and self-reactive T- and B-lymphocytes has been shown to require activation of Fas/CD95 (1). Activation of Fas/CD95 upon ligation with its natural ligand, the cytokine FasL (Fas ligand), or by agonistic antibodies such as CH-11 initiates a rapid signaling process that induces cells to undergo apoptosis. Binding of FasL, or anti-Fas antibody to Fas/CD95 results in rapid recruitment of the adaptor protein FADD (Fas-associated protein with death domain, also called Mort-1) to the receptor through interaction of its C-terminal death domain with the cytosolic death domain of the receptor (2–4). Several lines of evidence have demonstrated that this recruitment plays a pivotal role in the cell death pathway. For example, FADD-deficient T-cells (5) or lymphoid cells transfected with dominant-negative FADD have an abrogated Fas signaling cascade (6). Similarly, FADD−/− mice have been shown to display defects in apoptotic pathways in the immune system (7).

The N terminus of FADD contains a death effector domain that, upon activation of the Fas receptor, recruits cytosolic procaspase-8 through the death effector domains located in the N-terminal domain of the proenzyme (8). The process of Fas/CD95 receptor interaction with FADD and procaspase-8 was termed death-inducing signaling complex (DISC) formation by Kischkel et al. (4). Immediately upon recruitment, procaspase-8 becomes autoproteolytically cleaved to an active dimer in a process presumably induced by physical proximity of procaspase-8 molecules (9). Active caspase-8 initiates amplification of the death cascade through cleavage and activation of both downstream caspases (10) and pro-apoptotic molecules such as BID (11, 12).

Resistance to Fas/CD95-induced cell death in immune system cells can result in the generation of severe pathologies such as autoimmunity and hematological malignancies (2). In many instances, resistance to apoptosis arises from constitutive or up-regulated expression of an array of proteins and signaling molecules that counteract death signals initiated by activation of the Fas/CD95 receptor. In this context, PKC activation has

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Laboratory of Signal Transduction, Molecular Endocrinology Group, NIEHS, NIH, 111 Alexander Dr., Research Triangle Park, NC 27709. Tel.: 919-541-1564; Fax: 919-541-1367; E-mail: cidlowsk@niehs.nih.gov.

1 The abbreviations used are: DISC, death-inducing signaling complex; PKC, protein kinase C; PMA, 12-O-tetradecanoylphorbol-13-acetate; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.
been shown to inhibit Fas/CD95-induced apoptosis of Jurkat T-cells (13, 14) by likely controlling early stages of the cell death program (15, 16). The PKC family is composed of at least 12 broadly expressed serine/threonine kinase isotypes that have been subdivided in three subgroups based on their regulation (17, 18). The classical PKCs include the α, βI, βII, and γ isotypes and require Ca\(^{2+}\) and diacylglycerol for maximal activation. The novel PKCs include the δ, ε, η, and ζ isotypes and are Ca\(^{2+}\)-independent, but require diacylglycerol mobilization for their activation. The atypical group is represented by the ζ, η, and λ isotypes and are both Ca\(^{2+}\)- and diacylglycerol-independent. Finally, PKCγ and protein kinase D (human and mouse isoforms, respectively) constitute their own group due to their distinct structure (19, 20). Each PKC isotype is the expression product of an individual gene, except for βI and βII, which arise from alternative RNA splicing. PKCs are expressed in a tissue-specific manner and become activated in response to specific hormonal and growth factor stimuli. PKC expression in T-cells includes the α, δ, ε, ζ, and η isotypes, which exhibit different degrees of activation depending on the nature of the stimulus (21, 22).

FLIP (FLICE-like inhibitory protein, also termed Casper/FADD IgG1 and rabbit polyclonal anti-human BID antibodies were purchased from Transduction Laboratories and BIOSOURCE (San Diego, CA), respectively. Monoclonal anti-Fas/CD95 IgG1 antibody (B-10) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal anti-human c-FLIP\(_{\text{int}}\). IgG2a antibody was from Abpotech, and rabbit polyclonal anti-FLIP\(_{\text{c}}\) antibody (H-202) was from Santa Cruz Biotechnology. Horseradish peroxidase-linked secondary anti-mouse IgG and anti-rabbit IgG were purchased from Amersham Pharmacia Biotech. Horseradish peroxidase-linked anti-rat IgG antibody was from Southern Biotechnology (Birmingham, AL). The rest of the reagents were purchased from either Sigma or Pierce unless indicated.

Fluorescence-activated Cell Sorter Analysis—Changes in cell volume and plasma membrane integrity were assessed by flow cytometry analysis using a Becton Dickinson FACSort. 2 ml of cell suspension was used per sample prior to addition of the vital dye propidium iodide (10 μg/ml final concentration; Sigma). For cell volume analysis, 10,000 cells were examined for each sample by excitation with a 488-nm argon laser. Changes in cell size were examined on a forward light scatter versus side light scatter dot plot using CellQuest software as previously described (32). Since a decrease in forward scatter concomitant with an increase in side scatter in a given cell population is indicative of cell shrinkage, gates were drawn to analyze the percent of shrunk versus normal sized cells. For cell viability, forward scatter versus propidium iodide fluorescence (FL-3) dot plot histograms were used to quantify the percentage of cells with high propidium iodide fluorescence, which is a measure of plasma membrane integrity. In the control sample, a gate was drawn around the normal population and used to compare the percentage of viable (low propidium iodide) versus nonviable (high propidium iodide) cells for each condition. Expression of surface Fas receptor was determined with an anti-Fas antibody conjugated to FITC (FITC-labeled anti-iG1 antibody AP0-1-1, Kamiya Biomedical Co.). Jurkat cells (2 × 10\(^5\) cells) were incubated in the presence or absence 20 nM PMA, 10 nM bryostatin-1, or 2.5 μM Gö6976 for 0 h and collected by centrifugation at 1500 × g for 5 min. Pelleted cells were washed once with ice-cold phosphate-buffered saline (PBS), resuspended in 100 μl of ice-cold PBS containing nothing or 20 nM PMA, 10 nM bryostatin-1, or 2.5 μM Gö6976. 5 μl of FITC-labeled CD95 were then added to the cells while gently mixing the suspension. Cells were kept on ice for 30 min, and 1 μl of PBS was added prior to flow cytometry analysis of cells on FL-1 (488 nm excitation and 550 nm emission). Flow cytometry histograms were used to analyze the distribution of FITC-labeled CD95 fluorescence versus cell number under the different conditions.

Subcellular Fractionation and Cell Extract Preparation—For subcellular fractionation studies, 2 × 10\(^7\) Jurkat cells per treatment were preincubated for 30 min in the presence or absence of PMA, bryostatin-1, or Gö6976 and with or without anti-Fas antibody CH-11 for 15 min. Cells were centrifuged at 1500 × g for 5 min, washed with PBS; and resuspended in 1 ml of buffer containing 20 μM Tris-HCl (pH 7.5), 5 mM EDTA, 0.3% β-mercaptoethanol, and a mixture of protease inhibitors (Complete™, Roche Molecular Biochemicals). Cells were homogenized by being passed 30 times through a 25-gauge needle and centrifuged at 280,000 × g for 1 h. The resultant supernatants (cytosolic fraction) were stored at −80°C, and the residual pellet was resuspended in the same buffer supplemented with 1% Triton X-100, sonicated for 1 min, and stored at −80°C as the membrane fraction. Prior to Western blot analysis, protein concentration was assayed by the method of Bradford using the Bio-Rad system, and all cell extracts were normalized to equal protein concentration. For crude Jurkat lysate preparation, cells were collected and washed with cold PBS. The cells were immediately placed on ice and resuspended for 20 min in lysis buffer (20 μM Tris-HCl (pH 7.5), 2 mM EDTA, 150 mM NaCl, and 0.5% Triton X-100) containing protease inhibitors (Complete™). Cells were then disrupted with a 25-gauge needle and centrifuged at 16,000 × g for 15 min, and protein concentration was determined prior to protein normalization.

Western Blot Analysis—20–50 μg of protein/sample were equally diluted in Laemmli loading buffer supplemented with β-mercaptoethanol and denatured at 99°C for 5 min, followed by 2 h of electrophoresis on 12 or 4–20% Tris/glycine gels (Novex, San Diego, CA) at 120 V. Gels were then electrophoretically transferred to nitrocellulose membranes (Schleicher & Schuell) at 42 V for 1.5 h and stained with Ponceau S to verify equal amounts of protein loading. Membranes were blocked in 10% nonfat dry milk for 1 h. Antibodies were diluted in TBS containing 0.05% Tween and 1% dry milk, and membranes were blotted with the corresponding antibody for 1 h at room temperature. Blots were washed three times with TBS containing 0.05% Tween and 1% dry milk and incubated for 1 h with the corresponding horseradish peroxidase-linked secondary antibodies diluted 1:5000 in TBS containing 0.05% Tween.
PKC-dependent Modulation of DISC Formation

and 0.5% milk. After three washes with TBS, membranes were visualized with the ECL chemiluminescence detection system and exposed to Hyperfilm ECL (Amersham Pharmacia Biotech). In some cases, blots were stripped with Re-Blot™ (Chemicon International, Inc.) and reprobed for actin expression.

DISC Analysis and c-FLIP Immunoprecipitation—DISC formation analysis was carried out as previously described (33) with minor modifications. Briefly, 1.5 × 10⁶ cells suspended at a density of 5 × 10⁷ cells/ml were pretreated with PKC modulators for 30 min. Cells were treated with or without 1 µg/ml anti-Fas antibody CH-11 (mouse IgM) and incubated at 37°C for the indicated times. Cells were collected and pelleted at 4°C, washed with ice-cold PBS, and resuspended in 1 ml of a phosphate-based immunoprecipitation buffer (20 mM Na⁺ phosphate, 150 mM NaCl, 50 mM NaF, 0.5 mM Na₂VO₄, and 1% Triton X-100 (pH 7.2) supplemented with protease inhibitors (Complete™). Protein from post-nuclear supernatants was normalized among all samples and used for immunoprecipitation of the Fas/CD95 receptor. Extracts from all control groups (Fas-ununtreated cell extracts) were supplemented with 1 µg of anti-Fas antibody CH-11, and all lysates were subsequently incubated at 4°C overnight with 40 µl of protein A-Sepharose beads coated with rabbit anti-mouse IgM antibody. Beads were washed five times with 1 ml of cold immunoprecipitation buffer and resuspended in 30 µl of Laemmli buffer containing β-mercaptoethanol, and the denatured samples were run on 4–20% Tris/glycine gels. Western analyses were performed as described above, and the blots were reprobed with rat monoclonal anti-human Fas/CD95 antibody and horseradish peroxidase-linked anti-mouse IgG1 antibody. Occasionally, re-stripped blots were reprobed with rabbit monoclonal anti-human c-FLIPS/L, IgG2a and horseradish peroxidase-linked anti-mouse IgG1 antibody. Blots were stripped and reprobed with a mixture containing both anti-caspase-8 and anti-FADD antibodies, followed by exposure to horseradish peroxidase-linked anti-mouse IgG1 antibody. Occasionally, re-stripped blots were reprobed with rat monoclonal anti-human c-FLIPS/L, IgG2a and horseradish peroxidase-linked anti-rat IgG1 antibodies. c-FLIPS/L was immunoprecipitated using rabbit polyclonal anti-c-FLIPα antibody. Briefly, cells were plated, treated, and processed as described for DISC analysis. Post-nuclear supernatants were precleared for 1 h at 4°C with 4 µl of rabbit antiserum and with 25 µl of protein A-Sepharose beads. Precleared supernatants were first incubated overnight at 4°C in the presence of 1 µg of rabbit anti-human c-FLIPS/L antibody and then with 25 µl of protein A-Sepharose for an additional hour. Beads were washed five times with 1 ml of cold immunoprecipitation buffer, and samples run as described above. Blots were then probed using rat monoclonal anti-human c-FLIPS/L, IgG2a antibody.

RESULTS

PKC Modulates Fas/CD95-induced Cell Shrinkage and Cell Death in Jurkat Cells—A pharmacological approach was first used to evaluate the modulation of apoptosis by PKC activation and inhibition. Jurkat cells were preincubated for 30 min in the presence or absence of the specific PKC modulators PMA (20 nM), bryostatin-1 (10 nM), and G66976 (2.5 µM) prior to addition of 50 ng/ml anti-Fas antibody CH-11 for 3 h. PMA is a tumor promoter phorbol ester recognized to be a general activator of PKC that rapidly induces general activation and translocation of PKC isoforms to the plasma membrane (34). Bryostatin-1 is a macrocyclic lactone that potently activates the diacylglycerol-sensitive classical and novel PKC subfamilies in lymphoid cells (35). The efficacy of both PMA and bryostatin-1 in activating PKC was determined by examination of changes in the relative amount of PKCα in both particulate and cytosolic subcellular fractions induced by these compounds. After 30 min of PMA or bryostatin-1 treatment, the membrane fraction of Jurkat cells exhibited relative enrichment of PKCα (Fig. 1A, with parallel depletion of the iso type in the cytosolic fraction. This effect occurred in the absence or presence of anti-Fas antibody, indicating that both compounds similarly stimulated PKCα activity. Anti-Fas antibody, which does not interfere with PKC stimulation G66976, a sulfonamide (sulfanilamide-derived indolocarbazole) with selectivity for binding and inhibiting the catalytic subunit of PKC (36), was chosen as a selective PKC inhibitor on the basis of previous reported effects on Fas-induced cell death (16). This compound did not affect the PKC translocation pattern since it interacts with the catalytic subunit of the enzyme (37) and thus inhibits PKC activity rather than affecting the activation/translocation process. Actin expression (Fig. 1A, lower panels) was used as an internal control in the experiments, revealing that the observed pattern of PKCα translocation was not a result of the fractionation procedure. After preincubation of Jurkat cells with PKC modulators, cell shrinkage, which is a defining feature of apoptosis (38), was analyzed at 3 h after exposure to anti-Fas antibody. As shown in Fig. 1B, treatment of Jurkat cells with anti-Fas antibody alone (50 ng/ml) resulted in a shift of the cell population to lower values of forward scatter and side scatter light, indicative of the loss of cell volume. However, PKC activation with PMA (20 nM) or bryostatin-1 (10 nM) inhibited the loss of cell volume triggered by anti-Fas antibody, whereas PKC inhibition with G66976 (2.5 µM) exacerbated the effect of anti-Fas antibody on cell shrinkage. Plasma membrane integrity (viability) was examined in parallel with forward scatter analyses, revealing that with this dose of anti-Fas antibody CH-11, the cell population remained viable after 3 h (data not shown). Given that G66976 has been reported to be highly selective for the PKCα and PKCβ isotypes (37), these results suggest that inhibition of the α and/or β isotype may likely be involved in altering the effectiveness of anti-Fas antibody in inducing cell shrinkage in Jurkat cells. Cell death analysis was carried out at a 18-h time point by examination of the ratio of propidium iodide-positive (nonviable) cells in the cell population. As shown in Fig. 1C, treatment of cells with PMA or bryostatin-1 induced resistance to Fas-induced cell death after 18 h, whereas PKC inhibition with G66976 significantly augmented the number of nonviable cells upon anti-Fas antibody exposure. Treatment of cells with 4a-phorbol 12-myristate 13-acetate, a phorbol ester with no efficacy for activating PKC, did not result in inhibition of anti-Fas antibody-induced cell death. These results suggest that PKC can modulate the Fas-induced cell death in Jurkat cells from incipient stages of the cell death pathway.

PKC Does Not Alter Levels of Fas/CD95 or FADD—To further explore the role of PKC in the Fas pathway, cell-surface expression of the Fas/CD95 receptor was analyzed after 3 h of treatment with the cells with PMA, bryostatin-1, and G66976 at the indicated doses. After this period of time, FITC-labeled anti-Fas/CD95 antibody was added, and cells were analyzed by flow cytometry for FITC fluorescence as described under “Materials and Methods.” Analysis of the FITC fluorescence versus cell number histograms shown in Fig. 2A revealed that the levels of Fas/CD95 expression did not change upon treatment of cells with PKC modulators for 3 h and were identical to those in control cells. In addition, since APO-1-1 binding to the Fas receptor was equal in all the treatments, these results suggest that PKC modulators do not induce detectable chemical changes in the Fas receptor. Since FADD is well known to rapidly mediate downstream effects of Fas/CD95 activation upon ligation to the receptor, total levels of FADD in cell lysates from cells treated under various PKC modulating conditions and with anti-Fas antibody for 3 h were analyzed by Western blotting. The expression pattern of FADD (Fig. 2B) was similar among all the groups, suggesting that the principal mechanism of action of PKC on apoptosis does not rely on an alteration of the levels of cellular FADD protein.

Procaspase-8 Processing and Subsequent Downstream Proteolytic Cleavage of Proprocaspase-3 and BID Are Controlled by PKC—Upon activation of the Fas/CD95 receptor, recruitment of FADD to the Fas receptor occurs via mutual interaction of death domains present in both proteins. This effect leads to the recruitment of cytosolic procaspase-8 to the Fas/CD95-FADD complex and subsequent transactivation/cleavage of procaspase-8, which is the most upstream caspase in the Fas/caspase-8, which is the most upstream caspase in the Fas pathway.
We have previously shown that one of the effects of PKC activation in this context is the inhibition of Fas-induced caspase-8 and caspase-3 cellular activities and that, conversely, inhibition of PKC leads to an increase in the activity of the two enzymes (16). To determine whether this PKC modulatory effect is exerted on unprocessed/non-cleaved or, alternatively, on activated/cleaved caspases, we analyzed Fas-induced processing of procaspase-8 and procaspase-3 in cells cultured in the presence or absence of PMA, bryostatin-1, and Go6976. As shown in Fig. 3 (upper panel), an antibody recognizing both intact and cleaved caspase-8 cognates revealed that, after exposure of cells for 3 h to anti-Fas antibody, the levels of the active form of caspase-8 (p41/43 fragments) were detectable, with a concomitant reduction in the pro form (p52/54) of the enzyme. However, treatment of cells with PMA and bryostatin-1 inhibited cleavage of procaspase-8; and conversely, the presence of Go6976 augmented the ratio of cleaved to intact caspase-8. An identical profile was observed when caspase-3 was analyzed (Fig. 3, lower panel), probably reflecting the upstream inhibition of caspase-8 processing and abrogation of its activation.

BID is a known cytosolic substrate of caspase-8 that, upon activation of the protease, is rapidly cleaved, leading to the generation of a truncated form of BID, tBID (11). Cleaved/truncated BID (p15) translocates to the mitochondria (39) upon its myristoylation (40), where it triggers initiation of mitochondrial disruption and further amplification of the death cascade. Analysis of BID in Jurkat cells was carried out using an antibody that recognizes both intact and truncated forms of BID (p22 and p15, respectively). Western blot analysis showed that cleavage of BID in anti-Fas antibody-treated Jurkat cells was detectable at 3 h of Fas receptor stimulation (Fig. 4). However, BID fragmentation was notably inhibited in the presence of either PMA or bryostatin-1 and, conversely, was promoted by PKC inhibition with Go6976. The data correlate precisely with observed profiles of caspase-8 processing (Fig. 3, upper panel) and inhibition of its activation (1). These results also suggest that at least one of the modulatory effects of PKC occurs up-

**Fig. 1.** Effect of PMA, bryostatin-1, and Go6976 on PKCα translocation and Fas-induced cell shrinkage and cell death. **A**, Jurkat cells were pretreated for 30 min with 20 nM PMA, 10 nM bryostatin-1 (Bryo), or 2.5 μM Go6976 and then with 1 μg/ml anti-Fas antibody CH-11 for 15 min. Cytosolic and membrane fraction extracts were prepared as described under “Materials and Methods” and analyzed by Western blotting using antibodies against PKCα. Blots were stripped and reprobed for actin to verify equal protein enrichment among fractions. **B**, after pretreatment with PKC modulators, Jurkat cells were treated with 50 ng/ml anti-Fas antibody CH-11 for 3 h, harvested, and examined in a FACSsort flow cytometer. A dot plot showing the cell size distribution (forward scatter versus side scatter) was used to analyze normal and shrunken subpopulations under each condition. The percentage of shrunken cells quantified in each treatment is shown on each plot. **C**, cell viability was then determined by flow cytometry analysis of propidium iodide (PI)-positive cells after 18 h of treatment with anti-Fas antibody CH-11. All data are representative of at least four independent experiments.
PKC-dependent Modulation of DISC Formation

Involvement of c-FLIP in Anti-Fas Antibody-induced DISC Formation in Jurkat Cells—c-FLIP has been shown to mediate resistance to Fas/CD95 apoptosis in both B- and T-lymphocytes (27, 29, 41, 42). We therefore hypothesized that a likely mechanism exerted by PKC at the plasma membrane level could be afforded via modulation of DISC formation by a potential PKC-modulated and c-FLIP-dependent mechanism. To examine a potential interaction between the formation of DISC and FLIPS/L, we first determined optimal conditions of Fas/CD95 immunoprecipitation and DISC formation using anti-Fas IgM antibody CH-11 by analyzing both dosage and time course responses of DISC assembly in Jurkat cells. As shown in Fig. 5A, recruitment of both caspase-8 and FADD upon activation of the Fas receptor in Jurkat cells occurred as early as 1 min after addition of 1 μg/ml anti-Fas antibody to the cells, and the complex remained stable for at least 15 min. In addition, using the 15-min time point as a reference, a dose of 1 μg/ml anti-Fas antibody was shown to be the most effective in promoting DISC formation compared with the other 0.05, 0.25, 2.5, and 5 μg/ml doses tested. Thus, in all subsequent experiments, cells were treated with 1 μg/ml anti-Fas antibody for 15 min prior to DISC formation analysis. Expression of c-FLIPS/L in Jurkat cells was analyzed by immunoprecipitation. A rabbit polyclonal antibody recognizing both short and long isoforms of human c-FLIP was used to immunoprecipitate c-FLIP proteins from Jurkat cell lysates. To ensure maximal specificity of the immunoprecipitation procedure, immunoprecipitated FLIPS/L was then probed with rat monoclonal anti-human FLIPS/L antibody. As shown in Fig. 5B (left panel) FLIPS/L was found constitutively expressed in Jurkat cells, and changes in c-FLIP levels were not detected when comparing lysates from anti-Fas antibody-treated cells versus anti-Fas antibody-treated cells in the presence of PMA. We next examined the potential recruitment of FLIP to the Fas receptor by analyzing the presence of co-immunoprecipitated c-FLIP in Fas/CD95 immunoprecipitates. As shown in Fig. 5B (right panel), FLIP<sub>L</sub> was found not to be associated with Fas/CD95 under any of the tested conditions. FLIP<sub>S</sub> was, however, consistently found to be interacting with the Fas/CD95 receptor in cells that were not exposed to anti-Fas antibody. Interestingly, activation of cells with anti-Fas antibody led to a rapid (15 min or less) disappearance of the FLIP<sub>S</sub> band, suggesting that, in Jurkat cells, FLIP<sub>S</sub> is presumably interacting in a constitutive fashion with the Fas/CD95 receptor and can be displaced from the receptor upon activation of cells with anti-Fas antibody. No differences in this pattern of FLIP<sub>S</sub> translocation between anti-Fas antibody-treated cells versus anti-Fas antibody- plus PMA-treated cells could be found, however. These results also suggest that c-FLIP is unlikely to be a primary target of PKC modulation.

PKC Inhibits FADD Recruitment and DISC Formation—We...
PKC-dependent Modulation of DISC Formation

Transduction of Fas-dependent death signals occurs as a result of activation of the self-associated Fas/CD95 receptor (45) and immediate recruitment of FADD to the cytoplasmic domain of receptor (2–4). The Fas pathway is activated at very precise stages of the immune response and becomes particularly critical for the deletion of immunoreactive T-cells following a successful immune response (46). Thus, repressive effects on stages of the cell death program may lead to the survival of a potentially reactive and unwanted T-cell population. However, cells are continuously exposed to a number of additional stimuli such as cytokines, growth factors, and oncogene expression that are able to signal up-regulation of survival pathways and to counteract the action of pro-apoptotic proteins (47, 48). Due to the increase in the number of pathologies associated with inhibition of apoptosis, it is important to dissect signaling mechanisms that trigger apoptosis resistance phenotypes.

The loss of cell volume is a distinctive feature of apoptotic cell death that can be detected in early stages of the cell death program and that, in some instances, can take place prior to effector caspase activation, mitochondrial depolarization, and DNA degradation (49). In a previous investigation, we reported

next hypothesized the existence of a PKC-dependent mechanism responsible for the regulation of procaspase-8 processing in Fas-stimulated Jurkat cells. Procaspase-8 is known to be activated after Fas/CD95 stimulation via its direct interaction with FADD, which leads to DISC assembly and initiation of the cell death program (4). Since Fas/CD95-induced processing of procaspase-8 was prevented in the presence of PKC activators and enhanced by PKC inhibition (Fig. 3, upper panel), it was plausible that the mechanism of caspase-8 processing inhibition exerted by PKC occurs at the level of DISC. Thus, analyses of DISC formation were done in extracts from Jurkat cells treated with the indicated doses of PMA, bryostatin-1, or G66976 for 30 min prior to addition of 1 μg/ml anti-Fas antibody for 15 min (for details, see “Materials and Methods”). Interestingly, the results from DISC formation assays in Jurkat cells (Fig. 6A) showed that the level of FADD co-immunoprecipitating with Fas/CD95 was significantly reduced in the lysates of cells treated with PMA and bryostatin-1. In contrast, inhibition of PKC with G66976 prior to Fas receptor stimulation led to a significant increase in the amount of both FADD and caspase-8 associated with immunoprecipitated Fas/CD95. Interestingly, not only did the amount of caspase-8 recruited to DISC increase, but also the amount of processed/cleaved fragments (p41/43) of caspase-8 in DISC increased during PKC inhibition. To exclude the possibility of a direct chemical modification of the Fas receptor and DISC components by PMA, bryostatin-1, and G66976, DISC analysis was carried out by adding these compounds to the post-nuclear supernatants. Under these conditions, the amounts of FADD and caspase-8 recruited by the Fas receptor were identical between samples from Fas-treated cells and from Fas-treated cells in which the modulators were added to the lysates prior to the immunoprecipitation reaction (data not shown).

Fas/CD95-induced apoptosis has been suggested to progress in cells through two pathways that differ in their mechanism of caspase-3 activation (43). Cell lines in which large amounts of DISC units are formed display predominance of caspase-3 activation directly by caspase-8 and are classified as type I cells. In contrast, type II cells produce a relative lower amount of DISC upon Fas activation, and caspase-3 seems to be activated by an alternative amplification pathway through the mitochondria. Inhibition of the mitochondrial pathway via overexpression of anti-apoptotic proteins such as Bcl-2 has been shown to block apoptosis in type II (but not type I) cells (43). However, the molecular bases of type I versus type II divergence are still poorly understood. Jurkat T-cells have been classified as type II cells (43); and as shown in Fig. 6A, the amount of DISC formation in this cells could be reduced by PKC activation. To examine the potential differences between type I and II cells at the level of this regulatory response, we analyzed DISC formation in H9 cells, a human T-cell lymphoma cell line that has been classified as type I (44). DISC analysis in H9 cells was carried out as described for Jurkat cells; and similar to the results in Jurkat cells, we found a significant and fast (15-min exposure to anti-Fas antibody) reduction upon Fas-induced recruitment of both FADD and caspase-8 in cells during activation of PKC with PMA (Fig. 6B). These data indicate that PKC has the potential to modulate DISC formation in both type I and II cells. Together, these results show for the first time that PKC exerts a robust influence on DISC formation in both type I and II cells by quickly modulating the most upstream signaling step upon receptor activation: FADD recruitment and DISC formation.

DISCUSSION

Transduction of Fas-dependent death signals occurs as a result of activation of the self-associated Fas/CD95 receptor (45) and immediate recruitment of FADD to the cytoplasmic domain of receptor (2–4). The Fas pathway is activated at very precise stages of the immune response and becomes particularly critical for the deletion of immunoreactive T-cells following a successful immune response (46). Thus, repressive effects on stages of the cell death program may lead to the survival of a potentially reactive and unwanted T-cell population. However, cells are continuously exposed to a number of additional stimuli such as cytokines, growth factors, and oncogene expression that are able to signal up-regulation of survival pathways and to counteract the action of pro-apoptotic proteins (47, 48). Due to the increase in the number of pathologies associated with inhibition of apoptosis, it is important to dissect signaling mechanisms that trigger apoptosis resistance phenotypes.

The loss of cell volume is a distinctive feature of apoptotic cell death that can be detected in early stages of the cell death program and that, in some instances, can take place prior to effector caspase activation, mitochondrial depolarization, and DNA degradation (49). In a previous investigation, we reported

FIG. 5. c-FLIP does not interfere with formation of DISC in Jurkat cells. A, time course (left panels) and dose-dependent (right panels) analyses of DISC formation were carried out using anti-Fas IgM antibody CH-11 (a-Fas CH11) at the indicated times after treatment of cells with 1 μg/ml antibody for time course experiments or at increasing doses from 0.05 to 5 μg/ml for 15 min for dose-response analyses. Immunoprecipitates (IP) were run on 4–20% Tris/glycine gels and analyzed by Western blotting (WB) using antibodies for FADD and caspase-8. Each Western blot is representative of three independent experiments. B, FLIPs/L was immunoprecipitated from Jurkat lysates using rabbit polyclonal anti-FLIPS/L antibody (left panel). Cells were pretreated with PMA for 30 min and subsequently stimulated with or without 1 μg/ml anti-Fas antibody CH-11 for 15 min. Immunoprecipitates were subjected to electrophoresis and probed with monoclonal anti-human FLIPs/L antibody. FLIPs recruitment to the Fas/CD95 precipitates were subjected to electrophoresis and probed with monoclonal anti-FLIPS/L antibody. Blots are representative of three independent experiments. IB, immunoblot.
PKC-dependent Modulation of DISC Formation

FIG. 6. PKC modulates FADD and procaspase-8 recruitment to DISC in type I (H9) and type II (Jurkat) cells. A, Jurkat cells were pretreated for 30 min with 20 nM PMA, 10 nM bryostatin-1 (Bryo), or 2.5 μM Go6976 and then with 1 μg/ml anti-Fas antibody CH-11 (a-Fas CH11) for 15 min. Immunoprecipitates (IP) were subjected to electrophoresis, analyzed for Fas/CD95 expression, stripped, and reprobed for caspase-8 and FADD expression analysis. Lysates obtained prior to immunoprecipitation were used to analyze levels of caspase-8 in cell extracts. B, H9 cells were pretreated with 20 nM PMA for 30 min and with anti-Fas antibody as described for DISC analysis in Jurkat cells. Blots are representative of at least six independent experiments. WB, Western blot.

that the effect of PKC on Fas-induced cell death involves modulation of cell shrinkage and K⁺ efflux at a point proximal to the Fas receptor (16). In the present study, we show for the first time that activation of PKC leads to inhibition of Fas-induced apoptosis in Jurkat T-cells by controlling the most apical intracellular signaling in the Fas pathway: recruitment of FADD to the receptor. PKC plays a significant role in T-cell function since activation of resting cells through the T-cell receptor (TCR) complex induces rapid changes in the activity and translocation of PKC isoforms in T-lymphocytes (50, 51). Moreover, PKC is responsible for up-regulating gene transcription of proteins with a pivotal role in the immune response such as interleukin-2 (52). In vivo, TCR/CD3 complex activation induces cycling of peripheral T-cells and long-term sensitizes cells to Fas-induced cell death (AICD (activation-induced cell death)) through transcriptional up-regulation of FasL (46, 53, 54). Recently, it has been shown that T-cell receptor stimulation for a limited period of time (2–3 h) exerts a protective effect on CD8⁺ T-cells in CD95-mediated apoptosis (55), which presumably contributes to an increase in the efficacy of the immune response. As shown in Fig. 1B, the modulatory effect of PKC on Fas-induced cell death was already detectable by 3 h of anti-Fas antibody treatment and extended to an 18-h time point (Fig. 1C), which did not rule out the possibility of a transcriptional mechanism behind the resistance. However, protein expression analyses after 3 h of anti-Fas antibody exposure revealed no significant changes in the pattern of either Fas/CD95 or FADD levels in Jurkat cells treated with PKC modulators. We have previously reported that PKC activation blocks the generation of caspase-8 activity induced by anti-Fas antibody (16). We now also show that a main regulatory effect of PKC on the intrinsic activity of the caspase-8 enzyme can be discarded since there was no protease processing in anti-Fas antibody-treated cells in the presence of either PMA or bryostatin-1 (Fig. 3, upper panel). In addition, inhibition of PKC with Go6976 resulted in an increase in the cellular levels of active caspase-8 (p41/p43); and in turn, this effect of PKC enhanced downstream activation of caspase-3 (Fig. 3, lower panel). To further investigate the effect of procaspase-8 inhibition by PKC, BID, another substrate of caspase-8, was analyzed. Cytosolic BID is a 22-kDa pro-apoptotic member of the Bel-2 family that, when cleaved by caspase-8 to a truncated form (tBID), is translocated to the mitochondria, where it induces cytochrome c release (11, 12). We also found that anti-Fas antibody-induced BID cleavage (Fig. 4) was significantly inhibited by PKC activation and enhanced by PKC inhibition, in correlation with caspase-8 processing shown in Fig. 3 (upper panel). One current model of PKC modulation of Fas-induced cell death attributes the potential PMA-dependent mechanism of Fas resistance in type II cells to a blockade of BID cleavage downstream of caspase-8 activation (1). It was also suggest that PMA is able to control apoptosis only in type II cells since these cells do not have the ability to form much DISC, and their main apoptotic cascade proceeds through a mitochondrial pathway. However, our results show the existence of an additional molecular mechanism controlled by PKC not only via PKC activation with PMA or bryostatin-1, but indeed in the opposite direction via PKC inhibition with Go6976. Jurkat cells have been functionally classified as type II cells in opposition to type I, which can be represented by H9 cells. However, this functional classification (type I versus type II) seems not to be precise in classifying the regulatory effect of PMA since we have shown that, in both Jurkat and H9 cells (Fig. 5, A and B), PMA inhibited anti-Fas antibody-induced DISC formation. Based on our results, we thus propose an additional mechanism promoted by PKC upstream of both caspase-8 and BID, in
that PKC mediates Fas resistance at the receptor level, which is responsible for the PKC-dependent inhibition of procaspase-8 cleavage and the subsequent reduced amount of tBID (p17 band as seen in Fig. 4). Thus, the decreased level of BID cleavage/truncation found in PKC-stimulated cells may possibly arise from the limited levels of active caspase-8 found at the level of DISC. Conversely, higher levels of tBID found upon PKC inhibition arise from the increase in the level of FADD and caspase-8 recruitment to DISC. Furthermore, the results from Western blot analysis of both procaspase-8 and procaspase-3 shown in Fig. 3 reaffirm this evidence.

Because in lymphoid cells one of the inhibitory effects of c-FLIP has been shown to occur via its recruitment to DISC (i.e. Ref. 28), we investigated potential changes of c-FLIP (Fig. 5B) and found that the mechanism of resistance promoted by PKC in Jurkat cells is independent of c-FLIP. Although treatment of cells with bisindolylamine (a series of PKC inhibitory molecules) has been reported to sensitize monocyte-derived dendritic cells to CD95-mediated apoptosis via down-regulation of c-FLIP expression (29), this does not seem to be the mechanism in Jurkat cells. Both c-FLIPα and c-FLIPβ were found to be expressed in Jurkat cells (Fig. 5B); but interestingly, only c-FLIPα was found to be constitutively interacting with the Fas/CD95 receptor as deduced from co-immunoprecipitation analysis. However, we have shown that the stimulation of cells with anti-Fas antibody displaces c-FLIPα from interacting with Fas/CD95, independent of PKC action, suggesting that c-FLIP is an unlikely molecule to be a mediator of PKC modulation of DISC assembly in Jurkat cells.

Analyses of DISC formation shown in Fig. 6 provide supporting evidence that PKC modulates anti-Fas antibody-induced apoptosis upstream of procaspase-8 recruitment and processing. As shown in Fig. 6A, recruitment of FADD to the Fas receptor was studied in Jurkat cells treated with anti-Fas antibody for 15 min. FADD recruitment was significantly reduced upon activation of cells with either 20 nM PMA or 10 nM anti-Fas antibody (Fig. 4A). In contrast, the amount of FADD recruitment in DISC was found to be increased in cells pretreated with the PKC inhibitor with specificity for the classical PKCα and PKCβ isotypes (37), whose expression has been found to be abundant in T-cells (34). Given the fact that classical PKCs like PKCδ have been typified as “anti-apoptotic” (56, 57), our results further suggest that inhibition of classical PKCs may sensitize Jurkat cells to Fas-induced death by at least facilitating recruitment of FADD molecules to DISC. As shown in Fig. 6 (A and B), inhibition of procaspase-8 recruitment to DISC was detected in both Jurkat and H9 cells pretreated with PMA, although to a lesser extent compared with the effect shown on inhibition of FADD recruitment. This effect can be explained on the basis of an amplification outcome characteristic of signal transduction pathways, e.g. a given molecule of FADD recruited to DISC can recruit/activate a number of procaspase-8 molecules. Because preincubation of cells with PKC modulators was performed for 30 min, but their modulatory action was effective after a 10-min period of incubation (data not shown), we inferred that a signaling rather than a transcriptional mechanism was involved in inhibition of FADD recruitment to DISC. Preliminary results from our laboratory indicate that the phosphorylation status of several protein components of DISC change upon activation of cells with PMA (data not shown), suggesting that PKC can modulate DISC formation via a phosphorylation-dependent mechanism.

In conclusion, our results show that PKC activation/inhibition can rapidly signal modulation of Fas-induced cell death by inhibiting the clustering of both FADD and caspase-8 with the receptor or, conversely, by the potentiation of this clustering.

Although the protein or factor that triggers this effect is unknown to date, further studies on the regulation of CD95 and FADD interaction or novel Fas receptor-binding proteins such as the recently cloned SADS (58) will undoubtedly help to unveil cellular mechanisms of resistance of Fas-induced cell death. In addition, the use of PKC modulators might prove to be of potential interest in the intervention of specific pathologies in which the Fas pathway is impaired.

REFERENCES

1. Kramer, P. H. (2000) Nature 407, 789–790
2. Beldin, M. P., Varfolomeev, E. E., Panzer, Z., Mett, I. L., Camonis, J. H., and Wallach, D. (1995) J. Biol. Chem. 270, 7795–7798
3. Chinnaiyan, A. M., O’Rourke, K., Tewari, M., and Dixit, V. M. (1995) Cell 81, 505–512
4. Kischkel, F. C., Hellbardt, S., Behrmann, I., Germer, M., Pawlita, M., Kramer, P. H., and Peter, M. E. (1995) EMBO J. 14, 5579–5588
5. Joo, P., Woo, M. S., Koo, C. J., Signorelli, P., Biemann, H. P., Hannun, Y. A., and Schaller, M. G. (2000) J. Biol. Chem. 11, 991–996
6. Chinnaiyan, A. M., Tepper, C. G., Seldin, M. F., O’Rourke, K., Kischkel, F. C., Hellbardt, S., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) J. Biol. Chem. 271, 4901–4905
7. Zhang, J., Cado, D., Chen, A., Kabra, N. H., and Winoto, A. (1998) Nature 199, 296–300
8. Musio, M., Chinnaiyan, A. M., Kischkel, F. C., O’Rourke, K., Shvechenko, A., Ni, J., Scaffidi, C., Breez, J. D., Zhang, M., Gentz, R., Mann, K., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
9. Musio, M., Stockwell, B. R., Stenicek, H. R., Salvesen, G. S., and Dixit, V. M. (1998) J. Biol. Chem. 273, 2926–2930
10. Thornbery, N. A., and Lazebnik, Y. (1998) Science 281, 1312–1316
11. Li, H., Zhu, H., Xu, C. J., and Yuan, J. (1998) Cell 94, 491–501
12. Lencz, X., Budihardjo, I., Zou, H., Slaughter, C., and Wang, X. (1998) Cell 94, 481–490
13. Mizuno, K., Noda, K., Araki, T., Imakoa, T., Kobayashi, Y., Akitah, Y., Shimomaka, M., Kishi, S., and Osho, S. (1997) Eur. J. Biochem. 253, 7–18
14. Ruiz-Ruiz, M. C., Imperiali, M., de Murcia, G., and Lopez-Rivas, A. (1997) Eur. J. Immunol. 27, 1442–1450
15. Ruiz-Ruiz, C., Robledo, G., Font, J., Izquierdo, M., and Lopez-Rivas, A. (1999) J. Immunol. 163, 4777–4780
16. Gómez-Angelats, M., Bortner, C. D., and Cidlowski, J. A. (2000) J. Biol. Chem. 275, 19609–19619
17. Mellor, H., and Parker, P. J. (1998) Biochem. J. 332, 281–292
18. Modely-Rosen, D., and Gordon, A. S. (1998) FASEB J. 12, 35–42
19. Rozengurt, E., Sinnett-Smith, J., Van Lint, J., and Valverde, A. M. (1995) Mutat. Res. 333, 153–160
20. Van Lint, J. V., Sinnett-Smith, J., and Rozengurt, E. (1995) J. Biol. Chem. 270, 1455–1461
21. Tsutsumi, A., Kubo, M., Fujii, H., Freire-Maia, J., Turch, C. W., and Ransom, M. R. (1995) J. Biol. Chem. 270, 6578–6580
22. Baier, G., Baier-Bitterlich, G., Meller, N., Coggeshall, K. M., Giampa, L., Telford, D., Isakov, N., and Altman, A. (1994) Eur. J. Biochem. 225, 195–203
23. Vermeeren, C., and Dixit, V. M. (1997) J. Biol. Chem. 272, 6578–6583
24. Golstein, Y. V., Kovalenko, A. V., Arnold, E., Varfolomeev, E. E., Brodianski, V. M., and Wallach, D. (1997) J. Biol. Chem. 272, 16841–16844
25. Preisler, M., Thome, M., Häfner, S., Schneider, P., Hofmann, K., Steiner, V., Bodmer, J. L., Schröter, M., Burns, K., Mattmann, C., Rimoldi, D., French, L. E., and Tschopp, J. (1997) Nature 388, 190–195
26. Thome, M., Schneider, P., Hofmann, K., Fickenscher, H., Meini, E., Neipel, F., Mattmann, C., Burns, K., and Tschopp, J. (1997) Eur. J. Biochem. 252, 496–502
27. Gros, A., Xin, X. M., Wang, K., Wei, J. L., Sjoelck, J., Milliman, C., Erdjument-Bromage, H., Tempst, P., and Korsmeyer, S. J. (1999) J. Biol. Chem. 274, 1156–1163
28. Wei, M. C., Lindsten, T., Moota, V. K., Weiler, S., Gross, A., Ashiya, M.,
PKC-dependent Modulation of DISC Formation

Thompson, C. B., and Korsmeyer, S. J. (2000) Genes Dev. 14, 2060–2071

41. Mueller, C. M., and Scott, D. W. (2000) J. Immunol. 165, 1854–1862

42. Wang, J., Lobito, A. A., Shen, F., Herrung, F., Winoto, A., and Lenardo, M. J. (2000) Eur. J. Immunol. 30, 155–163

43. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomasselli, K. J., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) EMBO J. 17, 1675–1687

44. Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S. J., Krammer, P. H., and Peter, M. E. (1999) J. Biol. Chem. 274, 22532–22538

45. Ju, S. T., Panka, D. J., Cui, H., Ettinger, R., el-Khatib, M., Sherr, D. H., Stanger, B. Z., and Marshak-Rothstein, A. (1995) Nature 373, 444–448

46. Fenton, R. G., Hixon, J. A., Wright, P. W., Brooks, A. D., and Sayers, T. J. (1998) Cancer Res. 58, 3391–3400

47. Bertmer, C. D., and Cidlowski, J. A. (1999) J. Biol. Chem. 274, 21953–21962

50. Monks, C. R., Kupfer, H., Tamir, I., Barlow, A., and Kupfer, A. (1997) Nature 385, 83–86

51. Keenan, C., Kelleher, D., and Long, A. (1995) Eur. J. Immunol. 25, 13–17

52. Modiano, J. F., Klop, R., Lamb, R. J., and Nowell, P. C. (1991) J. Biol. Chem. 266, 10552–10561

53. Brunner, T., Mogil, R. J., LaFace, D., Yoo, N. J., Mahboubi, A., Echeverri, F., Martin, S. J., Force, W. R., Lynch, B. H., Ware, C. F., and Green, D. R. (1995) Nature 373, 441–444

54. Dhein, J., Walczak, H., Baumler, C., Debatin, K. M., and Krammer, P. H. (1995) Nature 373, 438–441

55. Karas, M., Zake, T. Z., Yakar, S., Dudley, M. E., and LeRoith, D. (2001) Hum. Immunol. 62, 32–38

56. Kelly, M. L., Tang, Y., Rosensweig, N., Clejan, S., and Beckman, B. S. (1998) Blood 92, 416–424

57. Whelan, R. D., and Parker, P. J. (1998) Oncogene 16, 1939–1944

58. Suzuki, A., Obata, S., Hayashida, M., Kawano, H., Nakano, T., and Shiraki, K. (2001) Nat. Med. 7, 88–93
