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_J Immunol_ 1998; 161:2201-2207; ;
http://www.jimmunol.org/content/161/5/2201
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Fas (CD95/APO-1) is a transmembrane protein of the TNF/neuron growth factor receptor family. Ligation of Fas by specific Abs or Fas ligand (FasL/CD95 ligand) induces rapid apoptotic cell death in a variety of cell types. Despite progress in understanding the death signals transduced from Fas, very little is known with regard to the mechanisms by which Fas expression is regulated. Using our previously established murine T cell hybridoma model A1.1, we show that specific protein kinase C (PKC) inhibitors could block activation-induced Fas expression and apoptosis. The activation of PKC with PMA or 1-oleoyl-2-acetyl-sn-glycerol could mimic the TCR signal by inducing the expression of Fas but not FasL. PKC-dependent Fas expression was also observed in several murine and human tumor cell lines. Since the inhibition of Ca$^{2+}$ redistribution by an inhibitor of intracellular Ca$^{2+}$ mobilization, 8-(diethylamino)-octyl-3,4,5-trimethoxybenzoate hydrochloride, inhibited TCR-induced FasL but not Fas, the expression of Fas appears to be independent of Ca$^{2+}$ mobilization. Significantly, expression of the newly identified Fas-regulatory gene, TDA531, was found to be dependent upon the activity of PKC. PKC activation only induced Fas expression in cells expressing wild-type TDA531. Thus, Fas expression is likely mediated by PKC through TDA531.


Activation of the TCR leads to the expression of a series of genes related to either apoptosis or cell proliferation (1, 2). Whether cell proliferation, differentiation, or apoptosis is favored depends upon the cellular stage and the extracellular milieu (2–4). The activation of immature T cells during thymic development induces apoptosis (5–7). Although TCR stimulation of resting peripheral T cells usually leads to proliferation and clonal expansion, primary activated mature T cells (8–10) and peripheral mature T cells from HIV-infected patients (11) are also sensitive to activation-induced cell death (AICD). Thus, AICD in T cells is a fundamental mechanism for maintaining immune tolerance and cellular homeostasis during T cell development, immune responses, and disease (12, 13).

Similar to immature thymic lymphocytes and activated mature T cells, T cell hybridomas also undergo AICD following TCR cross-linking (14–17). Recent studies have demonstrated that AICD in T cell hybridomas is dependent upon the induction of Fas (CD95) and Fas ligand (FasL/CD95 ligand) (18–20). Importantly, competitive inhibition of the interaction between Fas and FasL with soluble Fas proteins effectively inhibited AICD (19). Therefore, Fas and FasL play a fundamental role in AICD in T cells and T cell hybridomas.

The activation of Fas through its interaction with FasL or with specific anti-Fas Abs triggers the activation of a cascade of pro- tease; these proteases, in turn, execute the apoptotic process (21). Interestingly, the apoptosis signals after Fas ligation are independent of macromolecular synthesis (22). Therefore, the expression of Fas is the key checkpoint for cells to commit to AICD. In many cellular systems, the expression of Fas is strictly controlled (23). An overexpression of Fas rendered cells highly sensitive to FasL-mediated killing (24, 25). However, the regulation of Fas expression is poorly understood. Thus, elucidation of the regulatory mechanisms for Fas expression could provide important information for a better understanding of the molecular mechanisms of apoptosis.

Ligation of the TCR by specific Ag peptide presented by the MHC on APCs initiates a biochemical cascade that involves the activation of protein tyrosine kinases and protein tyrosine phosphatases (1, 2). These enzymes change the phosphorylation status of a number of intracellular substrates participating in the signal transduction cascade, which leads to the activation of protein kinase C (PKC) and to Ca$^{2+}$ mobilization (13). Since PKC activation and Ca$^{2+}$ redistribution have been shown to be critical in TCR-mediated activation signals, we examined the role of these signals in Fas and FasL expression during AICD. An increase in cytosolic Ca$^{2+}$ has been shown to be fundamental in the regulation of FasL expression, since the activation-induced expression of FasL could be completely inhibited by cyclosporin A (CsA) and FK506 (26). Nevertheless, the role of PKC in the regulation of Fas and FasL expression is not clear. We report that the activation of PKC without intracellular Ca$^{2+}$ mobilization is sufficient to induce Fas expression. Interestingly, FasL expression requires both PKC activation and intracellular Ca$^{2+}$ redistribution.

Materials and Methods

Cells, reagents, and Abs

Murine T cell hybridoma (A1.1) cells (27), B cell lymphoma CH31 (a gift of Dr. David Scott, Holland Laboratory of the American Red Cross), T cell hybridoma KCIT and its mutants (kindly provided by Dr. Yongwon Choi, Rockefeller University, New York, NY), and human Jurkat cells were
maintained in RPMI 1640 medium (Life Technologies, Gaithersburg, MD) that was supplemented with 2 mM L-glutamine, 50 mM 2-ME, 10% heat-
inactivated FBS (Sigma, St. Louis, MO), and 10 mM gentamicin. In ad-
dition, rIL-2 was added to the CTLL2 culture at a concentration of 100
U/ml. Cultures were incubated at 37°C in humidified atmosphere with 5%
CO2. Ab to CD3 was produced by a hamster B cell hybridoma, 145-2C11
(obtained from Dr. Jeffery Bluestone, University of Chicago, Chicago, IL).
K. Cell lysates were incubated at 50°C for 4 h followed by the addition of
1 mM Tris (pH 8.0), 1.6% sodium lauryl sarcosinate, and 1 mg/ml proteinase
K). DNA integrity was also analyzed by determining the DNA con-
tent in the nuclei by flow cytometry as described previously (28). Cells
were fixed with 70% ethanol for 30 min at 4°C, followed by two washes
with PBS. The fixed cells were then incubated in PBS containing pro-
pidium iodide (Sigma) at 50 µg/ml and RNase (Boehringer Mannheim,
Indianapolis, IN) at 0.1 mg/ml at room temperature for 30 min. DNA
content was determined by flow cytometry on a FACScan (Becton Dick-
inson, Sunnyvale, CA). The FL2 intensity was plotted as histograms on
a linear scale.

**IL-2 ELISA**
The TCR activation-induced production of IL-2 in culture supernatants was
measured using a Cytoscreen Immunooassay Kit obtained from BioSource
(Camarillo, CA). The murine rIL-2 included in the kit was diluted to gen-
terate a standard curve. An ELISA was performed according to the manu-
facturer’s instructions. Briefly, culture supernatants were diluted in the
standard diluent buffer included in the kit. Standard dilutions and samples
were incubated on the first anti-IL-2 Ab-coated microtiter plates for 1.5
h at 37°C. After washing with the washing buffer (PBS plus 0.2% Tween 20),
the bound IL-2 was detected with a biotinylated second anti-IL-2 Ab and
streptavidin-peroxidase. The amount of IL-2 was determined by the addi-
tion of tetramethyl benzidine.

**Analysis of functional Fas expression**
Activation-induced Fas expression on murine T cell hybridomas was as-
essed by determining the sensitivity to killing by anti-Fas Ab (JO2) or by
L cells expressing sense FasL but not antisense FasL (kindly provided by
Dr. T. A. Ferguson, Washington University School of Medicine, St. Louis,
MO). Briefly, cells were incubated with JO2 or L cells after appropriate
treatments. Apoptosis was determined by flow cytometric DNA content
analysis as described above.

**3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay**
Cell viability was measured by MTT conversion (29). Briefly, cells were
incubated in complete RPMI 1640 medium in 96-well plates. After treat-
ments as indicated, 10 µl of MTT (5 mg/ml in H2O) was introduced, and
cells were incubated at 37°C for 4 h followed by addition of 100 µl of
acid-isopropanol (0.04 N HCl). After the reduced MTT crystals were dis-
solved, the plate was immediately scanned by an ELISA reader with a
595-nm filter.

**Northern blotting**
Total RNA was isolated with affinity columns (Qiagen, Chatsworth, CA)
according to the protocol recommended by the manufacturer. RNA sam-
pies were fractionated on 1% agarose/2.2 M formaldehyde denaturing gel
and transferred onto a Nytran membrane (Schleicher and Schuell, Keene,
NH). The DNA probes (mouse Fas and Fasl, were provided by Dr.
Shigekazu Nagata, Osaka Bioscience Institute, Osaka, Japan; TDAG51
was obtained from Dr. Yongwon Choi) were labeled by random priming
(Boehringer Mannheim) according to the manufacturer’s instructions. Pre-
hybridization and hybridization were conducted at 42°C in a solution con-
taining 5× SSC (1× SSC is 1.5 M NaCl, and 0.15 M sodium citrate), 2.5
mM EDTA, 0.1% SDS, 5× Denhardt’s solution, 2 mM sodium pyrophos-
phate, 50 mM sodium phosphate, and 50% formamide. After washing with
0.2× SSC and 0.1% SDS at 56°C for 1 h, hybridization signals were
detected by autoradiography.

**Results**

**Both PKC activation and Ca2+ influx are required for AICD**
Murine T cell hybridoma A1.1 cells commit to AICD in a Fas- and
FasL-dependent manner (15, 19). AICD occurs in a period of 8 h,
with a sequential induction of Fas at 2 h and FasL at 4 h following
TCR cross-linking (19). Ligation of the TCR initiates a signal transduction cascade in which Ca2+ influx and the activation of
PKC have been shown to be the critical points in activation-in-
cuced cytokine production and proliferation in T cells. Thus, the
combination of PMA and Ca2+ ionophore mimics the signals from the
TCR (13). Here, we examined the role of PKC in the regulation of
Fas and Fasl expression during AICD.

We initially tested the requirement of PKC activation and Ca2+
influx for AICD in T cell hybridomas. As shown in Figure 1, treat-
ing A1.1 cells with the Ca2+ ionophore, ionomycin, plus ei-
er PMA or OAG readily induced apoptosis as detected by
genomic DNA fragmentation (Fig. 1A) and MTT viability assay
(Fig. 1B). When applied individually, neither ionomycin, PMA,
or OAG was sufficient to induce apoptosis. Similar to the activ-
ation of primary T cells (30), both PKC activation and Ca2+
influx are required for AICD in the A1.1 hybridoma.

To further investigate the role of PKC in the pathway leading to
AICD, we examined the effect of several relatively specific PKC
inhibitors (31). We found that these inhibitors could effectively
block TCR ligation-induced AICD in A1.1 hybridomas as dem-
onstrated by DNA content analysis (Fig. 1C) and genomic DNA
oligonucleosomal fragmentation (Fig. 1D). Furthermore, we have
performed PKC isoform typing and found that α, γ, δ, ε, ι, μ,
were present in A1.1 cells, while isoforms β, ξ, and θ, were not
found (Fig. 1E). Thus, several PKC isoforms are present in T cell
hybridoma A1.1 cells and are required for activation-induced
apoptosis.

**Activation of PKC alone sensitizes T cell hybridomas to anti-
Fas-induced apoptosis**
The activation-induced expression of Fas and FasL is absolutely
required for AICD in T cell hybridomas, since soluble Fas protein
could completely block AICD (19). Unactivated A1.1 cells express
a low level of Fas, which are insensitive to anti-Fas Ab- or FasL-
expressing fibroblast-induced apoptosis (Fig. 2). Interestingly, al-
though the application of PMA or OAG alone could not induce
apoptosis in A1.1 cells (Fig. 1), these treatments primed A1.1 to
express a low level of Fas, which are insensitive to anti-Fas Ab- or FasL-
expressing fibroblast-induced apoptosis (Fig. 2). Interestingly, al-
though the application of PMA or OAG alone could not induce
apoptosis in A1.1 cells (Fig. 1), these treatments primed A1.1 to
undergo increased Fas ligation-induced apoptosis as detected by
DNA content analysis (Fig. 2). Specifically, A1.1 cells were treated with
PMA (Fig. 2A) or OAG (Fig. 2B) for 2 h and then incubated with L cells that had been transfected with either sense FasL or antisense FasL (Fig. 2A) or with or without anti-Fas (J02, Fig. 2B) for 12 h. PKC activation with PMA or OAG sensitized
A1.1 cells to Fas ligation-induced apoptosis as assessed by DNA
content analysis. There are two possible explanations to account
for this result, i.e., PKC activation either increases Fas expression
or sensitizes the T cell hybridoma cells to Fas-mediated death
signals.
PMA alone induces Fas expression

We have reported that AICD in T cell hybridomas requires de novo synthesis of macromolecules, since apoptosis could be suppressed by the inhibition of either protein or RNA synthesis (15). However, recent studies have shown that the apoptosis triggered by anti-Fas Ab is independent of macromolecular synthesis once the Fas receptor is expressed (22). Since treatment with phorbol esters sensitized cells to anti-Fas killing (Fig. 2), the effect of PKC activation might induce Fas expression.

Therefore, we characterized the phorbol ester induction of Fas expression. A1.1 cells were treated with PMA in the presence or absence of ionomycin for 3 h, and RNA was isolated. Electrophoretically fractionated RNA was examined for Fas and FasL expression by Northern blot hybridization. As shown in Figure 3A, the expression of Fas mRNA could be induced by phorbol ester alone, whereas the expression of FasL required both the activation of PKC and Ca^{2+} mobilization. To further confirm the role of PKC activation in Fas expression, we treated A1.1 cells with different concentrations of PMA (0.1 ng-1000 ng/ml). The maximal induction of Fas expression was observed at 5 ng/ml. The expression level was comparable with the level induced by CD3 ligation (Fig. 3B). On the other hand, when the same blots were examined for the expression of FasL, we found that PMA alone does not have any effect on the induction of FasL. PMA plus ionomycin or anti-CD3, however, induced the expression of FasL. Experiments with OAG, which is a membrane permeable analogue of 1,2-diacylglycerol (32), showed results that were similar to those obtained with PMA (data not shown).
Next, we isolated RNA from A1.1 cells that had been treated with PMA for different times; we found that the PMA-induced Fas expression was detectable at 2 h posttreatment. This increase was sustained for at least 12 h (data not shown), while the Fas expression that was induced by anti-CD3 diminished at 5 to 6 h postactivation. This sustained expression of Fas induced by PMA is compatible with the extended effect of PMA on PKC activation (33, 34).

To examine whether the activation of PKC alone could induce Fas in cell types outside the T cell lineage, we treated various cell lines with PMA for 3 h and analyzed Fas expression by Northern blot hybridization. We found that PMA could induce Fas expression in a murine B cell lymphoma, CH31, in a human breast cancer cell line, MDA231, in a human ovarian cancer cell line, Hey, in a human cervical cancer, HeLa, and in a human prostate cancer, DU145 (data not shown). Since it has recently been shown that treating Jurkat cells with PMA increases their resistance to Fas ligation-induced apoptosis (34), we have tested whether PMA also induces the expression of Fas in these cells. As shown in Figure 3C, we found indeed that Jurkat cells were also induced to increase the expression of Fas. Therefore, the effect of PMA on the sensitivity of Jurkat cells to Fas-mediated apoptosis is not exerted by regulating Fas expression; rather the effect of PMA is exerted by interfering Fas signals. Nevertheless, our results in A1.1 cells are consistent with the recent observation of Wong et al. (35), who have shown that PKC treatment increased the sensitivity of T cell hybridomas to Fas-mediated apoptosis (the double-band pattern of human Fas has been shown previously). Thus, PKC plays a general role in regulating Fas expression.

Expression of Fas is completely blocked by PKC inhibitors

Although it has been well-established that the activation of T cells through the TCR activates both PKC and Ca\(^{2+}\) influx, our studies on the effect of activation of PKC with PMA do not necessarily establish a physiologic role for PKC during TCR signaling. To confirm the role of PKC in activation-induced Fas expression through the TCR, we treated A1.1 cells with anti-CD3 in the presence or absence of the PKC inhibitor, H7, or its analogue, HA1004, for 3 h; total RNA was isolated. H-7 has a greater ability to inhibit PKC than cyclic nucleotide-dependent kinase. Conversely, HA1004 has a greater ability to inhibit cyclic nucleotide-dependent kinase than PKC (34). As shown in Figure 5, Northern blot analysis revealed that H7 completely blocked activation-induced Fas expression, while HA1004 had no effect on TCR activation-induced Fas expression in A1.1 cells. Similar results were also obtained with the relatively specific PKC inhibitors calphostin C and Go\(_{6983}\) (data not shown).

Ca\(^{2+}\) is not required for Fas expression

We have previously reported that blocking Ca\(^{2+}\) -dependent calcineurin with CsA completely inhibits AICD in T cell hybridomas. As reported recently (26), cyclosporin could inhibit activation-induced FasL but not Fas expression (data not shown). However, cyclosporin has no effect on the Fas expression induced by TCR ligation or PMA treatment (data not shown). Thus, calcineurin and downstream events, while important for FasL expression, are not required for Fas expression. Since cyclosporin does not have any effect on cytosolic Ca\(^{2+}\) levels and thus does not interfere with the interaction between PKC and Ca\(^{2+}\), we tested the effect of an inhibitor of intracellular Ca\(^{2+}\) redistribution, TMB-8 (36), on TCR cross-linking-induced AICD. We found that TMB-8 completely blocked AICD (Fig. 4A) and FasL expression. However, TMB-8 did not inhibit the activation-induced expression of Fas (Fig. 4B). Thus, activation-induced Fas expression is independent of the redistribution of intracellular Ca\(^{2+}\).

Expression of Fas regulator TDAG51 is associated with Fas expression

PKC signaling in T cells is a complex process involving multiple enzyme cascades. Although our data clearly demonstrated...
a vital role for PKC in AICD, the PKC downstream effectors have not yet been elucidated. It has recently been reported that TDAG51, a newly identified potential transcription factor, is required for activation-induced Fas expression in T cell hybridomas (17). Thus, we determined the relationship between PKC and TDAG51 in our system. When the expression of TDAG51 was analyzed in A1.1 cells, we found that this gene is not expressed in unactivated A1.1 cells. However, its expression is induced upon activation through the TCR or by PMA alone. Similar to Fas, the expression of TDAG51 also has a requirement for PKC (Fig. 5A). In addition, we examined the expression of Fas in another T cell hybridoma, KCIT, and its mutant bearing the mutation of TDAG51. We found that PMA induced Fas expression in KCIT cells, but not in the TDAG51 mutant. Interestingly, transfecting the mutant with wild-type TGAG51 restored its response to PMA-induced Fas expression (Fig. 5B). Therefore, TDAG51 is a downstream effector in the PKC-mediated pathway leading to the induction of Fas expression.

Discussion

It is clear that the expression of Fas and FasL is responsible for apoptosis in some cell types, not only for the elimination of activated T cells after mounting a proper immune response (12, 37) but also for the maintenance of immune-privileged sites (38, 39). AICD in T cells requires de novo macromolecular synthesis, including the up-regulation of Fas and FasL, whose interaction then activates the apoptosis program (19–21). Since it has been shown that Fas ligation-induced apoptosis is independent of macromolecule synthesis (22), the activation of Fas and FasL genes is a critical step in initiating AICD. It has been well-established in T cell hybridomas that the expression of Fas and FasL increases after activation (19, 17). Furthermore, blocking the interaction of Fas and FasL with Fas fusion proteins could completely block AICD (19). Alternatively, T cells can be activated by calcium ionophore and phorbol ester. This combination implies that both calcium redistribution and the translocation of PKC are required for T cell activation (30). Since the signals leading to the expression of Fas still remain largely unknown, we investigated the role of Ca2+ and PKC in the regulation of Fas expression. Our data show that Fas expression is solely dependent upon PKC activation and does not require Ca2+ redistribution. This effect of PKC is exerted through TDAG51. PKC-mediated Fas expression was also observed in the murine B cell lymphoma CH31 and in several human cancer cell
treated with 2000 nM of PMA for 12 h. We found that this treatment blocked activation-induced apoptosis in these cells (data not shown). Although this treatment blocked activation-induced FasL expression, it did not show effects on Fas expression. Since the Ca^{2+} PKC isoforms are more sensitive to PMA depletion, we conclude that activation-induced Fas expression does not require Ca^{2+} based on these findings and the above data combined with our observation that PMA alone could induce maximal Fas expression. Thus, our data strongly suggest that FasL expression requires both PKC and Ca^{2+}.

We have shown that the diacylglycerol analogue OAG, which stimulates PKC, induces apoptosis in A1.1 cells when added together with ionomycin. Further, we have shown that the inhibition of PKC could prevent activation and inhibit anti-CD3-induced Fas expression and apoptosis in the T cell hybridoma. This observation indicates that PKC is involved in activation-induced Fas expression. When we down-regulated PKC function by preincubating cells with high concentrations of PMA, we were able to show again that activation-induced Fas expression required PKC (data not shown).

TDAG51 is a newly identified potential transcription factor that is involved in the regulation of activation-induced Fas expression in the T hybridoma KMIs-8.3.5. This hybridoma undergoes Fas/Fasl-dependent apoptosis upon activation. A variant of KMIs-8.3.5 bearing a mutation in TDAG51 expresses Fasl, TNFR-1, and IL-2, but not Fas (17). Transfection of the active gene restored activation-induced Fas expression in this mutant. Thus, TDAG51 may play an essential role in the induction of apoptosis by coupling TCR stimulation to Fas expression. Our data show that TDAG51 expression could also be activated solely by PKC; it is very likely that the effect of TDAG51 acts downstream of PKC. The transfection of TDAG51 does not appear to induce Fas expression (17). Thus, it is possible that other factors are required. Studies are in progress to determine how PKC regulates TDAG51 and Fas expression.

**Acknowledgments**

We thank Drs. David W. Scott, Wendy Davidson, Achsah D. Keegan, and Juliann G. Kiang for critical discussions. This is publication number 46 of the Department of Immunology, Holland Laboratory.

**References**


