The CD95 (APO-1/Fas) Receptor Activates NF-κB Independently of Its Cytotoxic Function*

(Received for publication, October 24, 1995, and in revised form, January 30, 1996)

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Engagement of the CD95 (APO-1/Fas) receptor induces apoptosis in a variety of cell types. However, the nature of the cytotoxic signal and the intermediate messenger molecules remain to be elucidated. In an effort to understand CD95-mediated signaling, we assessed possible changes in the DNA binding activity of NF-κB as a result of CD95 engagement in various tumor cells. By performing electrophoresis mobility shift assays, we show that CD95 can stimulate the DNA binding activity of NF-κB in a variety of cells, irrespective of their sensitivity or resistance to CD95-mediated cytotoxicity. Moreover, deletion of 37 carboxyl-terminal residues from the cytoplasmic domain of CD95, which abrogates CD95-mediated apoptosis, only marginally affects NF-κB activation. Taken together, these observations indicate that CD95 has a function that involves activation of NF-κB and that appears to be unrelated to its role as an inducer of apoptotic cell death.

CD95 (APO-1/Fas) is a member of the TNF receptor superfamily and is currently recognized as the principal cell surface receptor involved in the transduction of signals that induce apoptosis in lymphocytes and in a variety of tumor cells (1). CD95-mediated apoptosis can be triggered following engagement of the CD95 receptor by a specific ligand (CD95L) expressed on activated cytotoxic T cells (1) and by specific anti-CD95 monoclonal antibodies (mAb). Naturally occurring loss of function mutants of CD95 and CD95 ligand in lpr/lpr and gld mice, respectively (2, 3), are associated with lymphoproliferative and autoimmune disease attesting to the importance of the role that CD95 plays in the maintenance of lymphoid tissue homeostasis. Although CD95-mediated apoptosis is currently a field of intense study, little is known about the signaling pathway along which CD95-mediated cytotoxicity is transduced. The cytoplasmic domain of CD95 contains a segment known as the "death domain," homologs of which are present within the cytoplasmic domain of the TNF receptor p55, and several recently isolated intracellular proteins that interact with CD95 (4–7). While expression of the death domain is required for CD95-mediated cytotoxicity (8), the full spectrum of its putative functions remains to be determined, since related sequences have now been observed in molecules that are not known to be involved in mediating cell death (9). Furthermore, despite significant relatedness between CD95 and TNFRp55, the bulk of evidence currently available indicates that induction of cell death by these two receptors proceeds along different pathways (10).

Expression of CD95 in any given cell type does not invariably indicate that the cell is sensitive to CD95-mediated cytotoxicity. Sensitivity of cells to the death signal induced by CD95 has been shown to be highly variable and to depend in part on their metabolic state (11). Thus, CD95-mediated cell death appears to be regulated in cell type- and possibly activation/differentiation stage-specific fashion. Interestingly, the function of CD95 does not appear to be exclusively restricted to the induction of apoptosis. One study has shown that engagement of CD95 by mAb can trigger T cell activation (12), suggesting that CD95 may have functional properties other than cytotoxic signal transduction which remain to be fully explored.

It has been suggested that signals that induce apoptotic cell death may require activation of "death genes" or repression of "survival genes" (13, 14). Because apoptosis mediated by CD95 occurs within minutes in some cell types (10), the second possibility appears more likely. It has been shown, for example, that glucocorticoid-induced apoptosis of human leukemic cells is caused by transrepression (14), and a number of mechanisms have been proposed to underlie transcription factor-mediated inhibition of gene expression (15). In most cases, repression of transcription involves DNA binding or release by factors which can be visualized by electrophoretic mobility shift assays (EMSAs). We therefore used EMSAs to address transcription factor DNA binding activity during CD95-mediated apoptosis. One potentially attractive candidate transcription factor is NF-κB, because it is inducible and can both repress and activate transcription of numerous genes whose products are critical to a variety of biological processes, most notably in inflammation and immune responses (16). Using cell lines that are naturally sensitive or resistant to CD95-dependent apoptosis, we show that engagement of CD95 can activate the binding of NF-κB to its specific DNA binding site in a cell type-dependent manner. However, there appears to be no direct relationship between activation of NF-κB and the cytotoxic function of CD95. This notion is supported by the observation that deletion of a portion of the death domain within the intracytoplasmic region of CD95, which results in abrogation of CD95-mediated cytotoxicity only minimally affects NF-κB activation.

**EXPERIMENTAL PROCEDURES**

Cell Lines and Reagents—The cell lines Jurkat and T24 (from ATCC) were cultured in RPMI 1640 and in Dulbecco's modified Eagle's medium, respectively, supplemented with 5% fetal bovine serum (Irvine Scientific, Santa Ana, Ca). The stable transfectants MC-CD40-Fas, MC-NEO (described in Ref. 10) and MC-CD40-FasR1-7R, were maintained in Dulbecco's modified Eagle's medium, 5% fetal bovine serum supplemented with 1 mg/ml of G418 (Life Technologies, Inc.). To trigger the CD95 signal, Jurkat and T24 cells were incubated for 1 h with...
anti-CD95 mAb (50 ng/ml) (Upstate Biotechnology, Inc., Lake Placid, NY), while the MC-CD40-Fas and related mutant transfectants were incubated for 1 h with soluble CD8-CD40L. CD8-gp39 fusion protein generated in COS cell as described previously (10). Cycloheximide (10 \( \mu \)g/ml) and DPI (diphenylene iodonium, 25 \( \mu \)M) (Sigma) were added individually to the culture medium for 1 h alone or before the addition of anti-CD95 mAb. DDC (diethyldithiocarbamate, 1 mM) was added to the cell culture medium in combination with DPI 1 h prior to the addition of anti-CD95 mAb. Treatment of cells with TNF-\( \alpha \) (30 ng/ml) (Upstate Biotechnology, Inc.) when mentioned, was for 1 h.

Development of Cytoplasmic Domain Deletion Mutants—The deletion mutants were developed from the CD40-Fas chimeric cDNA by specific oligonucleotide primer–driven polymerase chain reaction as described (10). The reverse primers were designed to contain a NotI site and to generate Fas cytoplasmic domain deletion mutants truncated at residue 311, 282, 247, 215, and 181 and designated R7, R4, R3, R2, and R1, respectively. The common forward primer contained a BamHI site for in-frame fusion to the extracellular domain–specific sequences of CD40 cDNA. The polymerase chain reaction reactions were done using a 30-cycle protocol composed of 94°C/1 min, 58°C/2 min, 72°C/3 min segments. The amplified segments were digested with BamHI and NotI ligated and ligated to the BamHI/NotI-cut pCDM8 CD40-Fas expression vector (10). Fusion protein deletion mutants were stably introduced into MC melanoma cells as described (10).

DNA Gel Mobility Shift Assays—T24, J urkat, and MC melanoma–derived cell lines were treated as described above, and nuclear protein extracts were prepared as described (17). The nuclear extracts (10 \( \mu \)g) were preincubated for 20 min at 25°C with poly(dI-dC)-poly(dI-dC) (80 \( \mu \)g/ml) in a buffer containing 25 \( \mu \)M HEPES-KOH (pH 7.9), 40 \( \mu \)M NaCl, 1 \( \mu \)M EDTA, 5% glycerol, and 1 \( \mu \)M dithiothreitol. Following preincubation, radiolabeled double-stranded oligonucleotide (10,000 cpm) was added in each reaction and incubated for 20 min at 25°C. For supershift experiments, the reactions were incubated for 45 min at 25°C, with EMSA buffer or antibody against p65 or p50 as indicated in the legend of Fig. 2. The reactions were immediately loaded onto 5% TGE (50 mM Tris (pH 7.5), 380 mM glycine, 2 mM EDTA) native polyacrylamide gels, and the electrophoresis was performed at 4°C for 1 h. The gel was prepared by annealing a 5'-radiolabeled single-stranded oligonucleotide with a 3-fold molar excess of the complementary oligonucleotide in 0.15 M NaCl. The annealing mixture was heated to 65°C for 2 min and cooled to room temperature for 30 min. The double-stranded oligonucleotide was purified on a 15% native polyacrylamide gel and eluted overnight in water. The sequence of the oligonucleotides that corresponds to the NF-\( \kappa \)B consensus DNA binding site was: forward, 5' AGTTGAGGGGACTTTCCCAGGC 3'; reverse, 5' GCCTGG-GAAAGTCCCCCTAACT 3'.

RESULTS AND DISCUSSION

CD95-mediated Activation of NF-\( \kappa \)B in CD95-resistant and CD95-sensitive Cell Lines—To verify the possibility that CD95 may activate the nuclear translocation of the transcription factor NF-\( \kappa \)B, we performed DNA mobility gel shift analysis using the consensus binding site for NF-\( \kappa \)B as a probe and nuclear extracts from the human bladder carcinoma T24. The T24 cell line is naturally resistant to CD95-mediated apoptosis, but can be rendered sensitive by exposure to the protein synthesis blocker cycloheximide (CHX) or the flavoprotein inhibitor diphenylene iodonium (DPI) (Fig. 1A and Ref. 18). Engagement of CD95 by monoclonal antibody on T24 cells induces the binding of NF-\( \kappa \)B to its specific DNA binding site (Fig. 1B).

Several DNA-protein complexes (bands numbered 1–5 in all of the figures), revealed by gel mobility retardation, appear, which suggests that different members of the NF-\( \kappa \)B/Rel-related family bind the NF-\( \kappa \)B consensus sequence. The specificity of the transcription factor binding is indicated by the observation that all of the complexes disappear when the nuclear extracts are preincubated with a molar excess of cold double-stranded oligonucleotide probe (data not shown). The most prominent complex (indicated as band 2) represents the classic NF-\( \kappa \)B (p50/p65) as shown by supershift experiments using
Jurkat cells were treated with TNF-α-specific antibody against p65 or p50 (Fig. 2). As a control, most likely due to the effect of DPI treatment alone (Fig. 1), death by increasing intracellular O2. Dithiocarbamate (DDC) induces resistance to CD95-mediated killing. NF-κB activation in T24 cells that are sensitized to CD95, on the other hand, is not related to the sensitivity of the cells but rather depends on the reagent used to induce sensitivity. Thus, whereas both CHX and DPI sensitize T24 cells to CD95-mediated cytotoxicity, CHX augments while or with DPI and anti-CD95 mAb (Fig. 1B).

Although T24 cells are resistant to cytotoxic signals transduced by CD95 and TNF receptors (Fig. 1 and data not shown), engagement of both receptors in these cells results in the activation of NF-κB. To determine whether sensitization of T24 cells to CD95 might alter NF-κB binding activity, electrophoretic mobility shift assays were performed using lysates of T24 cells that had been triggered with anti-CD95 antibody following exposure to CHX or DPI. Both CHX and DPI pretreatment augmented T24 cell sensitivity to CD95-mediated cytotoxicity (Fig. 1A). Pretreatment of the T24 cells with cycloheximide increased the effect of anti-CD95 mAb on NF-κB DNA binding activity (Fig. 1B). However, this is probably due to the additive effect of cycloheximide and CD95 engagement, since cycloheximide alone can activate NF-κB (Fig. 1B and Ref. 20). DPI pretreatment of the T24 cells, on the other hand, inhibits the effect of anti-CD95 mAb on NF-κB binding activity (Fig. 1B). The low degree of NF-κB activation in this case is most likely due to the effect of DPI treatment alone (Fig. 1B).

We have shown previously that exposure of cells to diethylthiocarbamate (DDC) induces resistance to CD95-mediated death by increasing intracellular O2 concentration as a result of the inhibition of CuZn-superoxide dismutase (18). Preincubation of T24 cells with DPI and DDC has been observed to result in the restoration of resistance to CD95 from DPI-induced sensitivity (Fig. 1A and Ref. 18). To determine whether this reversal of phenotype correlates with any detectable modification in NF-κB binding, lysates of T24 cells triggered with anti-CD95 mAb following pretreatment with DPI and DDC were subjected to EMSAs. The NF-κB DNA binding activity was not observed to be increased, in contrast to anti-CD95 mAb treatment alone. The complexes obtained were comparable with the ones seen following treatment of cells with DPI alone with the ones seen following treatment of cells with DPI alone.

FIG. 2. Identification of the classical NF-κB-DNA complex by supershift assays in T24 and Jurkat cells. Nuclear extracts from T24 and Jurkat cells treated with anti-CD95 mAb and TNF-α, respectively, were incubated with the probe as indicated under “Experimental Procedures” and further incubated for 45 min at 25°C with EMISA buffer (lanes 1 and 4) or with antibody against p65 (lanes 2 and 5) or against p50 (lanes 3 and 6). The retarded complexes and nonspecific binding activity are indicated as in Fig. 1B.

specific antibody against p65 or p50 (Fig. 2). As a control, Jurkat cells were treated with TNF-α or with H2O2, both of which are known to activate the p50/p65 form of NF-κB (19). The retarded complex numbered 2 is also observed to undergo a supershift by an antibody against p65 or p50, supporting the notion that it represents the classical NF-κB (Fig. 2 and data not shown). Treatment of T24 cells with TNF-α resulted in the generation of the same complexes as those observed following anti-CD95 antibody treatment, but the activation appeared to be significantly stronger (Fig. 1B).

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FIG. 3. Determination of CD95-induced NF-κB DNA binding activity in Jurkat cells. Nuclear extracts from Jurkat cells treated with medium alone (lane 2), TNF-α (lane 3), anti-CD95 mAb (lane 4), cycloheximide (lane 5), cycloheximide and anti-CD95 mAb (lane 6), as described under “Experimental Procedures,” were subjected to EMSAs using radiolabeled NF-κB consensus binding site double-stranded oligonucleotide. The retarded complexes obtained and nonspecific binding activity to the probe are indicated as in Fig. 1B.

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FIG. 4. Determination of CD95-induced NF-κB DNA binding activity in MC-CD40-Fas cells. Nuclear extracts from MC-CD40-Fas treated with medium alone (lane 2) or with CD8-CD40L (CD8-gp39) fusion protein (lane 3) generated in COS cells as described under “Experimental Procedures” were subjected to EMSAs using radiolabeled NF-κB consensus binding site double-stranded oligonucleotide. The retarded complexes obtained and nonspecific binding activity to the probe are indicated as in Fig. 1B.

or with DPI and anti-CD95 mAb (Fig. 1B).

The above observations indicate that engagement of CD95 by mAb in T24 cells can activate NF-κB. Importantly, however, there appears to be no correlation between the NF-κB activation and cell sensitivity or resistance to CD95. Although engagement of CD95 in untreated, resistant cells activates NF-κB, activation is inhibited by pretreatment with a combination of DPI and DDC, which does not alter cell resistance to CD95-mediated killing. NF-κB activation in T24 cells that are sensitized to CD95, on the other hand, is not related to the sensitivity of the cells but rather depends on the reagent used to induce sensitivity. Thus, whereas both CHX and DPI sensitize T24 cells to CD95-mediated cytotoxicity, CHX augments while...
DPI inhibits CD95-induced NF-κB activation.

To address the relationship between engagement of CD95 and NF-κB activation in different cell types, we performed DNA mobility gel shift analysis using two cell lines that are naturally sensitive to CD95, the T cell leukemia Jurkat, which constitutively expresses CD95 and TNFR, and the human melanoma MC stably transfected with the CD40-Fas fusion protein (10). The CD40-Fas fusion protein consists of the extracellular domain of CD40 and the intracellular and transmembrane domains of CD95. CD95-mediated cell death can be conveniently induced by soluble recombinant CD8-CD40L (CD8-gp39) fusion protein overproduced in COS cells (10). When Jurkat cells were treated with anti-CD95 mAb, no activation of NF-κB DNA binding was observed, in contrast to TNF-α treatment (Fig. 3). Cycloheximide treatment alone was observed to activate NF-κB (mainly complex 3) in Jurkat cells, but no increase in the binding activity was seen following incubation of cycloheximide-treated cells with anti-CD95 mAb (Fig. 3). The effect of cycloheximide appears to be nonspecific, possibly resulting from the inhibition of IκB, the natural repressor of NF-κB (21). Contrary to Jurkat cells, MC40-Fas transfectants display activation of NF-κB binding activity following stimulation with soluble CD40L (Fig. 4).

These observations support the notion that triggering of CD95 augments the DNA binding activity of NF-κB in some but not all cell types and that there is no correlation between activation of binding and cell sensitivity to CD95-mediated killing. Moreover, these results may explain the recent report by Schulze-Osthoff et al. (22) that triggering CD95 overexpressed in the CD95 and TNF-sensitive cell line L929 does not activate NF-κB, whereas engagement of the TNFR by TNF-α does.

Determination of CD95 Intracytoplasmic Domain Sequences Involved in NF-κB DNA Binding Activation—To determine which segment of the intracellular domain of CD95 is associated with stimulation of NF-κB DNA binding activity, we used the CD40-Fas chimeric cDNA construct that served to generate MC-CD40-Fas transfectants (10) to develop a panel of Fas cytoplasmic domain deletion mutants. The deletion mutants, R1–R4 and R7, were truncated at residue 181, 215, 247, 282, and 311, respectively (Fig. 5A), and stable transfectants expressing each mutant were generated and transfectants expressing comparable levels of fusion protein selected for further study. DNA mobility gel shift analysis was performed using nuclear extracts from the different transfectants stimulated with soluble CD40L for 1 h. Engagement of CD40-FasR7
induced NF-κB DNA binding activity at comparative levels to those associated with stimulation of wild type CD40-Fas, with the exception of complex 4 whose binding appeared to be reduced (Fig. 5B). However, sensitivity of MC40-FasR7 and MC40-Fas wild type the cytotoxic signal of Fas, as measured by cytotoxicity assays, was comparable (Fig. 5A). Triggering of CD40-FasR4 activated the binding of NF-κB, though to a slightly lower degree than did engagement of CD40-FasR7 (Fig. 5B), but failed to induce apoptosis in the corresponding transfectants (Fig. 5A). These observations are consistent with results obtained using T24 and Jurkat cells showing that the stimulation of NF-κB binding activity is not related to sensitivity to CD95-mediated killing. In this regard, it is noteworthy that the CD40-FasR4 mutant lacks a significant portion of the death domain required for transducing the death signal, indicating that a functional CD95 death domain is not necessary for the activation of NF-κB. Activation of DNA binding by the major NF-κB complex (band 2) was greatly reduced in MC-CD40-FasR3 and MC-CD40-FasR2 transfectants (Fig. 5B) and was undetectable in MC-CD40-FasR1 transfectants (Fig. 5B). However, some activity of complexes 3 and 4 was observed in MC-CD40-FasR1 cells. These results suggest that although the stretch between amino acids 247 and 282 of the CD95 intracytoplasmic domain contains residues that are important for the transduction of signals required to stimulate the DNA binding activity of the NF-κB complex that corresponds to p50/p65, some NF-κB activity that implicts other complexes appears to be induced by mutants in which the 247–282 residue stretch has been deleted. In addition, the segment of the intracytoplasmic domain of CD95 that is required for activation of NF-κB (amino acids from 181 to 215) is not part of the death domain, indicating that at least the classical NF-κB (complex 2) is not involved in the transduction of the death signal. The possibility that some of the NF-κB activation, most notably the faint complexes 3 and 4, may be due to signaling by the cross-linking of a CD40-associated molecule is unlikely, because engagement of a TNF-Fas fusion protein in melanoma transfectants resulted in comparable NF-κB activation (data not shown). Furthermore, activation of the major NF-κB complex clearly appears to be intracellular Fas sequence-dependent.

The functional significance of NF-κB activation by CD95 is presently unknown. However, the present observations support the notion that functions other than triggering of apoptosis are mediated by CD95, which may be relevant to studies on the interaction of CD95 with intracellular proteins. Our results demonstrate the presence of several complexes by EMSAs following CD95 triggering that probably consist of different NF-κB/Rel related factors present in various homo- or heterodimeric combinations. The putative effects of these factors and the identity of their target genes can presently be only speculative. It is possible, for example, that some of the observed complexes may selectively activate or repress gene transcription or alternatively have both stimulatory and inhibitory effects on different genes in the same cell (23). Characterization of these factors and their target genes may help to uncover hitherto unrecognized functions of CD95.

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