Identification and Characterization of a Ligand-independent Oligomerization Domain in the Extracellular Region of the CD95 Death Receptor*

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The CD95 death receptor plays an important role in several physiological and pathological apoptotic processes involving in particular the immune system. CD95 ligation leads to clustering of the receptor cytoplasmic “death domains” and recruitment of the zymogen form of caspase-8 to the cell surface. Activation of this protease through self-cleavage, followed by activation of downstream effector caspases, culminates in cleavage of a set of cellular proteins resulting in apoptosis with disassembly of the cell. It is very well known that the extracellular region of the CD95 receptor is required for CD95L interaction and that the death domain is necessary for the induction of the apoptotic signaling. Here, we identified and characterized a novel CD95 ligand and death domain-independent oligomerization domain mapping to the NH2-terminal extracellular region of the CD95 receptor. In vitro and in vivo studies indicated that this domain, conserved among all soluble CD95 variants, mediates homo-oligomerization of the CD95 receptor and of the soluble CD95 proteins, as well as hetero-oligomerization of the receptor with the soluble variants. These results offer new insight into the mechanism of apoptosis inhibition mediated by the soluble CD95 proteins and suggest a role of the extracellular oligomerization domain in the regulation of the non-signaling state of the CD95 receptor.

Apoptosis is a cell death process that can be elicited by a variety of stimuli, such as growth factor deprivation, ionizing radiation, and other DNA-damaging agents, or by triggering specific cell surface receptors, the death receptors. Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily, which is characterized by similar cysteine-rich extracellular domains (1). The death receptors TNF-R1 (also called p55 or CD120a), CD95 (also called Fas or Apo-1), DR3 (also called Wal-1, Apo-3, LARD, or TRAMP), DR4 (also called TRAIL-R1), DR5 (also called Apo-2, TRAIL-R2, TRICK2, or KILLER) (2), and DR6 (3) are also characterized by the presence of a death domain within the cytoplasmic region critical for the initiation of apoptotic signaling (4, 5).

Notably, CD95 ligand (CD95L) and CD95 receptor interactions play an important role in the regulation of the immune response and in the control of peripheral B and T cell survival that is critical to the maintenance of immune cell homeostasis (6). This system has also been implicated in maintaining immunoprivilege in the eye and testis (7, 8), attenuating immunosurveillance against certain types of tumors, such as melanoma and hepatocellular carcinoma (9). Moreover, alterations in the control of apoptosis, also mediated by the CD95/CD95L system, are involved in the pathogenesis of several diseases such as Hashimoto thyroiditis (10), fulminating hepatitis (11, 12), and toxic epidermal necrolysis (Lyell’s syndrome) (13). Thus, it is particularly interesting to elucidate the mechanisms that regulate CD95 and CD95L interactions.

A common feature of the death receptors is that, upon binding to cognate ligands or agonistic antibodies, the receptors homo-oligomerize through the death domain and recruit adaptor and effector cysteine proteases to the cell surface (2). Cross-linking of CD95 results within seconds in receptor aggregation and formation of the death inducing signaling complex (DISC) (14) containing trimerized CD95, FADD/MORT1 (15, 16) (a death domain-containing adaptor protein), and caspase-8 (FLICE/MACH/Mch5) (17, 18) (a member of a family of cysteine proteases, the caspases, crucial players of the apoptotic process) (19). Aggregation and self-activation of caspase-8 (20–22) in turn results in cleavage and activation of other caspases and cleavage of several intracellular substrates leading to the cytoplasmic and nuclear morphological modifications hallmark of the apoptotic process (19). Thus, the common view today is that the extracellular region of the CD95 receptor is crucial for CD95L interaction while the intracytoplasmic death domain is required for CD95 receptor trimerization and for induction of apoptotic signaling. However, characterization of CD95 isoforms indicates an NH2-terminal extracellular domain as a potential novel ligand-independent oligomerization domain.

Several Fas/Apo-1 mRNAs have been identified by reverse transcriptase (RT)-PCR in human activated peripheral mononuclear cells and in hematopoietic and non-hematopoietic tumor cell lines (23–26). Such variants derive by alternative splicing of the primary transcript, and a descriptive nomenclature based on the exons deleted has been previously proposed (26). With the exception of FasΔExo6 (also called FasΔTM), in
all other variants (FasΔExo4; FasΔExo4,6; FasΔExo3,4; FasΔExo3,4,6) the deletions result in an altered reading frame with the introduction of a premature termination codon. Thus, these splicing variants code for smaller CD95 proteins with deletions in the extracellular region and with a novel sequence of 21 or 38 amino acids at the COOH-terminal end (24, 26). Such soluble CD95 isoforms are actively secreted from the cells and block in vitro the apoptosis induced by both the agonistic antibody, CH-11, or by the natural CD95L in CD95-positive sensitive cell lines (24, 26). Interestingly, all isoforms with deletions in the extracellular regions are lacking the CH-11 agonistic antibody (27) and the CD95L (28–30) binding sites, and thus they cannot interfere with the apoptotic process by simple competition. Alternatively, we proposed that the soluble isoforms bind to the CD95 receptor, thus interfering with homotrimer formation and with apoptotic signaling (the “inactive heterotrimer” model) (26, 31). Remarkably, the NH2-terminal 49 amino acids of the mature CD95 receptor is the only region conserved among all soluble isoforms and thus is likely to be implicated in this functional anti-apoptotic property.

In this study we provide evidence that a CD95L-independent oligomerization domain does exist in the extracellular region of the CD95 receptor and that this maps to the NH2-terminal 49 amino acids of the receptor. Moreover, the ability of the CD95 receptor to self-associate through such a domain does not require an intact cytoplasmic death domain. The oligomerization domain, conserved among all CD95 variants, may mediate both homo-oligomerization of the CD95 receptor and of the soluble CD95 proteins and hetero-oligomerization of the receptor with the soluble variants modulating the apoptotic signaling.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—The human T leukemia cell line HuT78 and the human embryonic kidney 293T cells were cultured, respectively, in RPMI 1640 and in Dulbecco’s modified Eagle’s medium supplemented with 10 mM Heps, antibiotics, and 5% fetal bovine serum. The BW cells were maintained in RPMI with the addition of nonessential amino acids, sodium pyruvate, 0.1 mM β-mercaptoethanol, and 500 μg/ml active G418 (Geneticin; Life Technologies, Inc.).

**Transfection, Immunoprecipitation, and Western Blotting Analyses**—The human T leukemia cell line HuT78 and the human embryonic kidney 293T cells were transiently transfected in 293T cells: pcDNA3FLAG-CD95, pre-}

**Construction of LexA and VP16 Fusion Proteins and Two-hybrid System Assays**—The DNA fragments corresponding to various fragments of the human CD95 receptor were obtained by PCR amplification using primers containing restriction sites for cloning (Table I, restriction sites are underlined, bold indicates the stop codons, and numbering is according to Itoh et al. (35)). CD95Extra, CD95Extra1–42, CD95Extra1–69, and CD95Intra DNA fragments were ligated into the EcoRI and BamHI sites of the pEG202 expression vector in frame with the LexA open reading frame and then the DNAs excised with EcoRI and SalI and subcloned into pBTM116 in frame with LexA. The sCD95 DNA was cloned directly in the BamHI site of pBTM116. Moreover, CD95Extra, CD95Intra, sCD95, and 49NCD95 PCR products were also cloned in the BamHI site of pVP16. The 49NCD95 BamHI and NotI insert of pVP16 was subcloned in pEG202 and the DNA excised with BamHI and SalI and subcloned into pBTM116 in frame with LexA. We used the following combinations of primers, indicated in Table I, to produce DNA fragments coding for the CD95 mutant proteins indicated in Fig. 1: (a) GR99 or GR131 and GR102, CD95Extra; (b) GR137 and GR102, CD95Extra1–42; (c) GR143 and GR102, CD95Extra1–69; (d) GR103 or GR141 and GR104, CD95Intra; (e) Recombinant β-galactosidase II and LexA. All results were confirmed by DNA sequencing. Two-hybrid interaction assays in L40 strain cells with pBTM116 and pVP16 constructs were performed essentially as described (36). Clones that formed on His-deficient media (His+) were tested for the ability to trans-activate a lacZ reporter gene by liquid culture β-galactosidase assays with ONPG (o-nitrophenyl-β-D-galactopyranoside) as substrate following standard protocols. β-Galactosidase was estimated from absorbance at 420 nm following standard protocols.

**Construction of GST Fusion Proteins and Preparation of E. coli Proteins**—The DNA fragments, described above, corresponding to CD95Extra, CD95Intra, CD95Extra1–42, and CD95Extra1–69 were cloned into the EcoRI and Xhol sites of pGEX-4T-1 in frame with the open reading frame of GST, sCD95 and 49NCD95 were PCR-amplified, respectively, with the GR101/GR119 or the GR101/GR105 primer pairs (Table I) and cloned in the BamHI site of the pGEX-4T-1. Constructs were transformed into E. coli strain BL21 (DE3), and fusion proteins expressed by induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside for 4 h at 37 °C. Cells were lysed on ice by mild sonication and then subjected to centrifugations at 4 °C. The supernatants were subjected to centrifugation at 4 °C. The supernatant was centrifuged by centrifugation and the supernatant was incubated with the thrombin protease to liberate the protein of interest from the GST moiety following manufacturer’s instructions. Such proteins following extensive dialysis against 1 × PBS at 4 °C were used in apoptosis inhibition assays and in gel filtration experiments. The sCD95 GST-free protein follow-
ing dialysis in sodium borate buffer (0.1 M, pH 8.8) was also biotinylated. Briefly, a purified cCD95 protein (1–3 mg/ml in sodium borate buffer) was incubated at room temperature for 4 h with N-hydroxysuccinimide biotin (50 pmol of biotin/mg of protein). The reaction was stopped by the addition of 4 µl of 1 M NH4Cl/50 µg of ester and incubation for 10 min at room temperature. Finally, the protein was extensively dialyzed against 1× PBS to remove uncoupled biotin.

In Vitro Transcription and Translation—The CD95-Extra EcoRI-BclI DNA fragment was PCR-amplified with the GR100, which includes a translation start site, and the GR102 synthetic oligonucleotides and cloned into the EcoRI and BamHI sites of the pBluescript II KS vector (Stratagene) (Table I). CD95L was PCR-amplified with the GR57 and GR88 oligonucleotides (numbering is according to Mita et al. (37); Table I) and cloned into the XbaI site of pcDNA3 expression vector. AU1FADD pCDNA3 plasmid was kindly donated by Dr. V. M. Dixit. The linearized plasmids were transcribed in vitro using T3 or T7 RNA polymerase under conditions recommended by the Promega Protocols and Application Guide. RNA was purified, ethanol-precipitated, and stored at −70 °C. To generate radiolabeled proteins, in vitro translation was performed using 1–2 µg of CD95Extra and FADD RNA/50-µl reaction for 120 min at 30 °C using a rabbit reticulocyte lysate (Promega) and L-[35S]methionine (800 Ci/mmol; Amersham Pharmacia Biotech). For CD95L in vitro translation, wheat germ extract (Promega) was used in a 50-µl reaction for 120 min at 25 °C. The labeled proteins were analyzed by SDS-PAGE under reducing conditions. Gels were fixed, treated with 1 M sodium salicylate for 30 min, dried, and fluorographed.

In Vitro Protein-Protein Interaction Assay—The assay was performed essentially as described previously (38). An aliquot (0.1–0.2 µl) of in vitro translated material was subjected directly to SDS-PAGE, or the in vitro translated protein reactions (10–20 µl) were diluted in 200 µl of binding buffer and incubated with the respective GST fusion proteins immobilized on glutathione-Sepharose (approximately 5 µg of protein on 10 µl of beads) diluted in 100 µl of binding buffer (20 mM Hepes (pH 7.4), 50 mM NaCl, 5 mM dithiothreitol, 0.1% Nonidet P-40, 10 mg/ml BSA) for 2 h at 4 °C. The beads were then washed four times in 150 mM NaCl, 16 mM Na2HPO4/7H2O, 4 mM NaH2PO4 (pH 7.3), 0.1% Nonidet P-40 at 4 °C. The bound proteins were then eluted with SDS-containing sample buffer, and one-fifth of each sample was subjected to SDS-PAGE and fluorography. In the competition assays, 1 µg of cold sCD95, GST-cleaved product, was used in the binding reactions. The binding assays were also performed by incubating a biotinylated sCD95 protein with the GST fusion proteins. The bound proteins were analyzed on SDS-PAGE transferred onto nitrocellulose membranes, incubated with streptavidin-horseradish peroxidase, and developed using ECL chemiluminescence system.

Binding Assay of GST-CD95 Fusion Proteins to Mammalian Cells—Cromatography purified GST-CD95 fusion proteins (250 µg of GST-CD95Extra, GST-cCD95, GST-48CD95, and GST-CD95ExtraΔ1-42, in 50 µl of PBS) were labeled with 125I (400 Ci/µl in 4 µl of NaOH, Nycodenz, Amersham Pharmacia Biotech) for 30 min in a vial coated with IODOSYN® (Pierce) (40 µg/vial) (39). After labeling, peptides were purified from free 125I using a gel filtration chromatography column (G25, PD10, Amersham Pharmacia Biotech) eluted with PBS. The fraction containing the radiolabeled peptide was stored at 4 °C with 1% human serum albumin until binding assay. For binding assays, HuT78 or BWTA or BW263F3 cells (1 × 106 cells in 20 µl of PBS + 1% human serum albumin) were incubated in duplicate in a 96-well plate at 4 °C for 30 min with increasing concentrations of radiolabeled peptide (final concentrations ranging from 10−14 to 10−7 M). Nonspecific binding was assessed by repeating the same binding in presence of 10−6 M unlabeled peptide. After incubation, cells were harvested and centrifuged for 1 min at 10,000 rpm in a 10-ml conical vial (Beckman) over a layer of 150 µl of dibutylphthalate oil and mineral light oil (87:5.12:5, v/v) (40). After centrifugation, vials were frozen by dipping in liquid N2 and the tip of the vial was then cut and counted for radioactivity. Scatchard plot analysis was performed on the available results using the Ligand 2.0 (Biosoft) computer program (kindly donated by Imperial Cancer Research Fund, London, United Kingdom).

Results

Expression of CD95 Mutants in Yeast and in Escherichia coli

To express a series of CD95 mutants in yeast as fusion proteins with the LexA DNA-binding domain or the VP16 transcriptional activation domain, the pBTM116 and the pVP16 proteins with the LexA DNA-binding domain or the VP16 transcriptional activation domain, the pBTM116 and the pVP16 proteins.
activity of the recombinant E. coli GST-CD95Extra, GST-sCD95, and GST-49NCDD95, a protein corresponding to the NH2-terminal 49 amino acids of the mature CD95 receptor, were tested in apoptosis inhibition assays. We have previously demonstrated that natural human soluble CD95 isoforms block apoptosis in vitro induced by the agonistic antibody, CH-11, and by the soluble CD95L in sensitive CD95-positive cells such as the human T cell leukemia line, HuT78 (26). The E. coli recombinant proteins were interfering with apoptosis induction as assayed by propidium iodide staining and cytofluorimetric analysis. The FACScan flow cytometer profiles are shown in Fig. 2, where the percentage of hypoploid nuclei clearly indicates the apoptotic inhibitory role of both the GST fusion proteins as well as the GST-free products. These results show that the E. coli recombinant proteins are functionally active and behave as the natural sCD95 variants.

**Binding of 125I-Labeled sCD95 to Human CD95-positive Mammalian Cells**

We previously proposed that sCD95 produced by alternative splicing of the primary mRNA transcript may interfere with CD95-mediated apoptosis by inducing “inactive heterotrimer” formation through binding to the full-length CD95 receptor (26, 31). In order to investigate whether sCD95 directly interacts with CD95, 125I-labeled GST fusion proteins were incubated with HuT78, BWtTAFF3 (a parenteral murine cell line lacking endogenous human CD95), and BWtTAFF3 (a human CD95-positive BW transfected), previously described (42). BWtTAFF3 expresses a human CD95 receptor with a single point mutation Val/Asn254 in the death domain, equivalent to the naturally occurring mutation in the murine receptor that leads to a lymphoproliferation (lpr) syndrome in mice. The mutation has been shown to greatly reduce self-association of the death domains and binding to FADD (43). The three cell lines were incubated with 125I-labeled proteins, and Scatchard plot analysis of binding assay results was performed. As shown in Fig. 3, a specific and saturable binding of GST-CD95Extra to BWtTAFF3 cells was observed (Fig. 3A); similar results were observed with GST-49NCDD95 (Fig. 3B) and with GST-sCD95 (Fig. 3C); the apparent binding parameters are summarized in Table II. In contrast the GST-CD95ExtraΔ1–42 did not bind to the BWtTAFF3 cells, and none of the GST fusion proteins interacted with the human CD95-negative BWtTAFF murine cell line (data not shown). Finally, GST-CD95Extra, GST-sCD95, and GST-49NCDD95 but not GST-CD95ExtraΔ1–42 also specifically bound to HuT78 cells (see Table II). These results suggest that an interacting and possibly an oligomerization domain exists in the extracellular region of the CD95 receptor. To further investigate this hypothesis, several other in vitro and in vivo experimental approaches have been used.

**Identification and Mapping of a Novel Interacting Domain in the Extracellular Region of the CD95 Receptor**

**Two-hybrid Interaction Assays—LexA and VP16 constructs**

The expression of both the lacZ and HIS3 reporter genes driven by a minimal GAL1 promoter fused to multimerized LexA binding sites were used as selection criteria. Thus, interactions of the fusion proteins in the transformants were assayed by growth on histidine-deficient media and by positivity in β-galactosidase colorimetric assays. The CD95Extra, the smallest soluble isoform, sCD95 (PasExo3,4,6) (24), and the mutant containing only the NH2-terminal 49 amino acids of the mature protein, 49NCDD95, depicted in Fig. 1A strongly interacted with CD95Extra, sCD95, and 49NCDD95 proteins (Table III). In contrast, CD95ExtraΔ1–42 and CD95ExtraΔ1–69 lacking, respectively, the NH2-terminal 42 amino acids and the NH2-terminal 69 amino acids containing the entire first cysteine-rich domain (CR1) of the mature protein expressed in the pBTM116 vector.
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failed completely to interact with CD95Extra, sCD95, and 49NCD95. The control bait consisting of the intracytoplasmic tail of the CD95 receptor, CD95Intra, did not associate with the CD95 extracellular mutants but only with itself. In Table III, a representative β-galactosidase colorimetric assay is shown (for details, see "Experimental Procedures"). Taken together, the two-hybrid interaction assays strongly suggested the presence of a domain in the extracellular region of the CD95 receptor able to mediate homo-interactions. The mapping experiments described above, moreover, suggested that this domain resides between residues 1 and 42 of the extracellular region of the mature CD95 receptor or that deletion of this region destroys the interacting domain.

In Vitro Binding Assays—To further investigate the interactions observed in yeast, we took advantage of an affinity chromatography approach. In the assays, GST-CD95 fusion proteins (depicted in Fig. 1A), coupled to glutathione-Sepharose beads, were used to test for binding of 35S-labeled human CD95Extra and CD95L proteins produced by in vitro transcription and in vitro translation of the corresponding sequences. The labeled in vitro translation products (Fig. 4, A and B, lane 9) were incubated with GST-CD95 fusion proteins or GST resin, washed under low stringency conditions, and the bound material was eluted and analyzed by SDS-PAGE. The in vitro translated CD95Extra product was retained by the GST-CD95Extra, GST-sCD95, and GST-49NCD95 resins (Fig. 4A), but not by GST alone. To test the specificity of the interaction, we determined the ability of CD95Extra to bind to mutants with deletion in the NH2-terminal region: GST-CD95ExtraΔ1–42 and GST-CD95ExtraΔ1–69. No detectable amount of CD95Extra was retained by such GST fusion proteins or by our negative control GST-CD95Intra. Moreover, in competition experiments the interaction of CD95Extra and GST-sCD95 was specifically blocked by an E. coli GST-free recombinant sCD95 protein (see "Experimental Procedures" for details). As expected this competitor had no effect on FADD and GST-CD95Intra interaction (Fig. 4C).

We have previously proposed that sCD95 were unable to bind to CD95L (26). To verify this hypothesis, a 35S-labeled CD95L protein produced by in vitro transcription and translation was incubated with the GST fusion proteins (Fig. 4B) and as expected no interaction was detected with either GST-sCD95 or GST-49NCD95. However, GST-CD95Extra and the two NH2-terminal deletion CD95 mutants strongly interacted with CD95L (Fig. 4B). Thus, in agreement with some reports (28, 29) but in contrast with another (30), the CD95L binding sites of the CD95 receptor do not include the first CR1 domain. Moreover, in competition experiments the interaction of CD95L and GST-CD95Extra was specifically blocked by an E. coli GST-free recombinant CD95Extra but not by the sCD95 (Fig. 4B). As control for the binding assays, a third in vitro translated product was produced corresponding to the full-length FADD, a protein that through the death domain specifically interacts with the intracytoplasmic tail of the CD95 receptor. As expected, a specific interaction of FADD was observed only with GST-CD95Intra and not with GST or GST-CD95Extra (Fig. 4C).

The in vitro transcribed and translated 35S-labeled sCD95 protein co-migrated in the SDS-PAGE with the reticulocyte lysate, and we were unable to obtain a good protein production and a satisfactory gel resolution. Thus, for the affinity chromatographic assays with sCD95, a different experimental approach was used. sCD95 protein produced as GST fusion protein in E. coli, cleaved with thrombin to remove the GST, and labeled with biotin was incubated with the GST-CD95 fusion protein mutants coupled to the beads. The eluted bound material was analyzed by SDS-PAGE, followed by blotting with streptavidin-peroxidase. As shown in Fig. 4D, sCD95 interacted only with GST fusion proteins containing an intact NH2-terminal CD95 receptor domain.

Overall, the results demonstrate that the NH2-terminal 49 amino acids of the mature human CD95 receptor are both necessary and sufficient for the interaction.

The NH2-terminal 49 Amino Acids of the CD95 Receptor Mediate Homo- and Hetero-oligomerization

Gel Filtration Experiments with Recombinant CD95 Proteins—The two-hybrid interaction assays and the chromatographic assays with GST fusion proteins suggest that the NH2-terminal extracellular domain may induce oligomerization of the CD95 receptor and of sCD95 besides formation of heterooligomers. Gel filtration experiments were then conducted to determine whether CD95 recombinant proteins containing an intact NH2-terminal domain were in a oligomeric status. Recombinant GST-CD95 fusion proteins produced in E. coli were purified and extensively dialyzed, and 500 ng were applied to a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech). The absorption at 220 nm of the eluted material was recorded on-line; subsequently the position of GST-CD95Extra, CD95Extra, GST-CD95ExtraΔ1–42, and sCD95 in the elution
fractions was determined by immunoblot analysis. The fractions loaded on SDS-PAGE gels: 12% for GST-CD95Extra, CD95Extra, and GST-CD95ExtraΔ1–42 and 16% for sCD95 were immunoblotted with the M24 antibody, specific for the NH2-terminal 49 amino acids of the CD95 receptor (26) or the GST polyclonal antibody for GST-CD95Extra or sCD95 and GST-CD95ExtraΔ, 18 kDa (CD95Extra), 8 kDa (sCD95), and 40 kDa (GST-CD95Extra) as expected molecular mass of the recombinant proteins in the monomeric state should be approximately 44 kDa (GST-CD95Extra or sCD95 and GST-CD95ExtraΔ1–42 fractions, 1000DGalactosidase assay

### Table II

| Protein          | GST-CD95Extra | GST-SCD95 | GST-49NCD95 | GST-CD95ExtraΔ1–42 |
|------------------|---------------|----------|------------|-------------------|
| $K_v$ (nM)       | 54 ± 17       | 9.3 ± 6.4| 20 ± 540   | ND'               |
| $K_v$ (nM)       | 10 ± 1.5      | 15 ± 9.3 | 19 ± 3.4   | ND                |
| HuT78            | 6.0 ± 1.7     | 0.24 ± 4.4| 8.0 ± 9.6  | ND                |
| (Sites/cell) × 10^3 | 29 ± 4.5     | 33 ± 2.2 | 35 ± 16    | ND                |

* ND, not detected.

### Table III

β-Galactosidase assay

The numbers indicate the β-galactosidase units, where 1 unit was defined as follows: 1000 × OD_{429 nm}/(t × OD_{600 nm}), where $t$ = elapsed time (in min) of incubation and $V$ = total volume (μl) of the reaction.

| Protein | pBTM116 CD95Extra | pBTM116 sCD95 | pBTM116 49NCD95 | pBTM116 CD95Intra |
|---------|-------------------|---------------|-----------------|-------------------|
| pVP16   |                   |               |                 |                   |
| CD95Extra | 0.095            | 0.101         | 0.097           | 0.011             |
| sCD95   | 0.106            | 0.077         | 0.079           | 0.012             |
| 49NCD95 | 0.074            | 0.108         | 0.098           | 0.022             |
| CD95Intra | 0.010            | 0.010         | 0.011           | 0.120             |
| CD95ExtraΔ1–42 | 0.019       | 0.008         | 0.009           | 0.017             |
| CD95ExtraΔ1–69 | 0.014       | 0.008         | 0.013           | 0.008             |

The extracellular NH2-terminal domain of the CD95 receptor is an oligomerization domain. In vitro transcription and translation were used to produce proteins labeled with [35S]methionine (CD95Extra (A), CD95L (B), and FADD (C)). After in vitro translation, either 0.1 μl of programmed reticulocyte or wheat germ lysates were subjected directly to SDS-PAGE analysis or 10 μl of the lysates were purified on GST or CD95 mutant Sepharose beads and an aliquot (one-fifth of the binding reaction) was subjected to SDS-PAGE. In D, sCD95, a biotinylated GST-free E. coli protein, was incubated with GST and CD95 mutants bound to Sepharose beads and was detected by immunoblot with streptavidin-peroxidase followed by ECL detection. In the competition experiments unlabeled sCD95 or CD95Extra were added to the binding reactions at 100-fold excess. Molecular size markers (Wide or Low Markers (Sigma) and Protein Molecular Weight Standards, Low Range (Life Technologies, Inc.)) migrated as indicated.

Fig. 4. The extracellular NH2-terminal domain of the CD95 receptor is an oligomerization domain. In vitro transcription and translation were used to produce proteins labeled with [35S]methionine (CD95Extra (A), CD95L (B), and FADD (C)). After in vitro translation, either 0.1 μl of programmed reticulocyte or wheat germ lysates were subjected directly to SDS-PAGE analysis or 10 μl of the lysates were purified on GST or CD95 mutant Sepharose beads and an aliquot (one-fifth of the binding reaction) was subjected to SDS-PAGE. In D, sCD95, a biotinylated GST-free E. coli protein, was incubated with GST and CD95 mutants bound to Sepharose beads and was detected by immunoblot with streptavidin-peroxidase followed by ECL detection. In the competition experiments unlabeled sCD95 or CD95Extra were added to the binding reactions at 100-fold excess. Molecular size markers (Wide or Low Markers (Sigma) and Protein Molecular Weight Standards, Low Range (Life Technologies, Inc.)) migrated as indicated.

formation of new discrete bands ranging approximately between 120 and 180 kDa corresponding apparently to dimeric and trimeric receptors, even though a faint smear of higher molecular weight complexes was observed as well. The experiments with the BwTAFF3 transfectants (Fig. 6A) suggested that the cytoplasmic death domain was not required for such oligomerization and that the extracellular and the transmem-
brane region of the CD95 receptor were sufficient. Moreover, the higher molecular weight bands were not due to co-immunoprecipitation of FADD and caspase-8 proteins as shown by Western blot analyses (Fig. 6).

Such proteins co-immunoprecipitated with the receptor only in the DISC-induced HuT78 cells and not in the cross-linked ones (Fig. 6B, lane 7). Notably, immunoprecipitation of cell lysates with the DX2 and with the Apo-1 antibodies in the absence of chemical cross-linking agent very often revealed the presence of a SDS-stable faint band, more evident with the Apo-1 antibody, of approximately 120 kDa.

Co-immunoprecipitation of CD95 Receptor and sCD95 in Vivo—To demonstrate the interaction of sCD95 and CD95 in vivo in mammalian cells, 293T cells were transiently transfected with HA epitope-tagged sCD95 (HA-sCD95) and FLAG epitope-tagged CD95 or a FLAG-CD95 receptor mutant lacking the N-terminal 42 amino acids of the mature protein. Expression of the FLAG-tagged and HA-tagged constructs were shown by immunoprecipitation with an anti-FLAG or anti-HA antibodies, followed by immunoblotting with the IP antibody. As shown in Fig. 7, immunoprecipitation of FLAG-CD95 coprecipitated HA-sCD95; likewise, immunoprecipitation of HA-CD95 coprecipitated FLAG-CD95, further confirming this interaction. Notably, the FLAG-CD95Δ1−42 mutant did not co-precipitate with the sCD95 protein, suggesting that hetero-oligomerization requires an intact NH2-terminal extracellular domain in the CD95 receptor. Moreover, immunofluorescence studies demonstrated that removal of the NH2-terminal 42 amino acids did not abrogate the transport of the CD95Δ1−42 receptor to the cell surface (data not shown).

CD95L- and Death Domain-independent Oligomerization of the Full-length CD95 Receptor in Vivo—Cells co-expressing wild type and mutant receptors were generated. 293T cells were transiently transfected with a FLAG epitope-tagged CD95 and a HA-tagged CD95 receptor mutant lacking the death domain described previously, FasΔExo8 (a CD95 receptor with a deletion of amino acids 202–319 and, due to the altered reading frame, the addition of three amino acids, MLT, following residue 201 (34). Expression of the FLAG-tagged and HA-tagged constructs were shown by immunoprecipitation (IP) with an anti-FLAG (α-FLAG) or anti-HA (α-HA) antibodies followed by immunoblotting with the IP antibody. As shown in Fig. 8, immunoprecipitation of FLAG-CD95 coprecipitated HA-CD95Δ202−319; likewise, immunoprecipitation of HA-CD95Δ202−319 coprecipitated FLAG-CD95. On the other hand, as expected, the FLAG-CD95Δ1−42 mutant did not coprecipitate with the HA-CD95Δ202−319 receptor. Such co-immunoprecipitation experiments clearly demonstrate a ligand- and a death domain-independent oligomerization of the CD95 receptor in vivo mediated by an intact NH2-terminal domain.

**DISCUSSION**

The results presented in this study show that the amineterminal extracellular domain of CD95 is a homotypic oli-
A Novel Oligomerization Domain in the CD95 Receptor

Gomerization domain as assessed by co-immunoprecipitation analyses, two-hybrid system, affinity chromatography with GST matrix, chemical cross-linking, and gel filtration experiments. This novel CD95 oligomerization domain is conserved among all CD95 variants; thus, it may mediate interactions involving both the receptor and the variants modulating the apoptotic process in a very flexible way. In fact, our data indicate that this oligomerization domain is responsible for a CD95L- and a death domain-independent homo-oligomerization of the CD95 receptor and for hetero-oligomerization of the CD95 receptor with the soluble CD95 variants both in vitro and in vivo. A CD95 miniprotein corresponding to the NH2-terminal 49 amino acids was found to engage in complex formation with itself, with CD95 receptor and CD95 mutants maintaining an intact amino-terminal domain, suggesting that the oligomerization domain is both necessary and sufficient to mediate such interactions.

Five soluble CD95 variants derived by alternative splicing of the primary transcript have been previously identified and characterized. All share a domain of 49 amino acids mapping at the NH2-terminal extracellular region of the CD95 receptor, including the same oligomerization domain here described. Thus, it is likely that such CD95 variants modulate the apoptotic signaling by interfering with CD95 oligomerization and consequently with the DISC formation. The co-immunoprecipitation studies in 293T cells clearly indicate that sCD95 does interact with the full-length receptor in vivo. Alternative splicing of pre-mRNA is a widely used mechanism to increase the coding potential of genes (44). In most cases, alternative splicing gives rise to protein isoforms sharing extensive regions of identity and varying only in specific domains, thus allowing for the fine modulation of protein function. Interestingly, several members of the TNF receptor family and of the respective ligand family, including CD95, have been shown to be expressed as both membrane-bound and soluble molecules. Frequently, soluble proteins compete with the receptor for ligand binding thus blocking the receptor-mediated signaling. Here we show that sCD95 may block the apoptotic signaling by a direct physical interaction with the membrane-bound CD95 receptor and with the initiation of the cytoplasmic signaling.

Fig. 6. A, chemical cross-linking of human CD95 receptor in transfected murine cells. BWTA and BWTAFF3 cells were divided into two aliquots; one was pretreated with the chemical cross-linker Sulfo-EGS, and one remained untreated. Subsequently, cells were lysed and CD95 was immunoprecipitated (IP) using the monoclonal antibody DX-2 or a control antibody. Immunocomplexes were analyzed by SDS-PAGE (7.5%) followed by immunoblotting with the anti-CD95 antibody M24. The secondary antibody detected the IP antibodies: light chain, heavy chain, and a putative dimer of the heavy chain. The heavy chain of the control antibody has a slightly higher molecular weight compared with DX-2. In lanes 1 and 2, CD95-specific bands were detected. The positions of the molecular weight standards (BenchMark prestained protein ladder, Life Technologies, Inc.) are indicated. B, chemical cross-linking of CD95 receptor in untransfected human T cells. HuT78 cells were divided into three aliquots, one was pretreated with the chemical cross-linker Sulfo-EGS, one with the DISC-inducing antibody Apo-1, and one remained untreated. Subsequently, cells were lysed and CD95 was immunoprecipitated using DX-2, APO-1, or a control antibody. Aliquots of each immunoprecipitate were loaded onto three separate SDS-PAGE gels and analyzed by immunoblotting with M24 (anti-CD95), anti-FADD and C15 (anti-caspase-8) antibodies. As discussed for panel A, the secondary antibody reacted with IP antibodies. In lanes 2 and 6 (anti-CD95 immunoblotting), monomeric and cross-linked higher molecular bands of CD95 are visible. In the anti-FADD and anti-caspase-8 blots, specific signals were detected only in the DISC-induced sample (lane 7). The C15 antibody stained the caspase-8 and lower molecular weight double bands that should correspond to the p43 cleaved fragment bound to the DISC (20). The positions of the molecular weight standards (BenchMark prestained protein ladder, Life Technologies, Inc.) are indicated.
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FIG. 7. In vivo association of CD95 and sCD95. 293T cells were co-transfected with HAsCD95 and FLAG epitope-tagged CD95 or a CD95 receptor mutant lacking the NH2-terminal 42 amino acids. Lysates were immunoprecipitated with anti-FLAG and anti-HA monoclonal antibodies and analyzed by SDS-PAGE and immunoblotting as indicated. Co-immunoprecipitation of FLAG-CD95 and HA-sCD95 is in lane 3. Molecular weight standards (High or Low Marker, Sigma) are indicated.

FIG. 8. CD95L- and death domain-independent in vivo aggregation of the CD95 receptor. 293T cells were co-transfected with a HA-CD95 truncated receptor lacking the death domain (HACD95Δ202–319) and FLAG epitope-tagged CD95 or a CD95 receptor mutant lacking the NH2-terminal 42 amino acids. Lysates were immunoprecipitated with anti-FLAG and anti-HA monoclonal antibodies and analyzed by SDS-PAGE and immunoblotting as indicated. Co-immunoprecipitation of FLAG-CD95 and the truncated receptor HA-CD95Δ202–319, lacking the death domain, is in lane 5. Molecular weight standards (High Marker, Sigma) are indicated.

Notably increased expression of sCD95 has been reported in several human diseases, i.e., in nonhematopoietic human malignancy (45), in angioimmunoblastic T-cell lymphoma (46), in myocarditis and in patients with congestive heart failure (47, 48), and in some patients with rheumatoid arthritis or systemic lupus erythematosus (49, 50, 23). However, it should be noted that increased levels of sCD95 in autoimmune diseases is still controversial; this increase occurred infrequently or was not detected in the serum of patients with rheumatoid arthritis or systemic lupus erythematosus in other studies (51, 52).

While the role of the hetero-oligomerization of CD95 and sCD95 is likely to be an apoptotic counteracting one, the function of the CD95 receptor homo-oligomerization is still unclear.

In the current model of death receptor activation, CD95L-induced oligomerization functions to bring together the death intracellular domains, which then recruit adapter and caspase-like proteins to transduce the apoptotic signaling. Our data suggest that CD95 in unstimulated cells may be constitutively oligomerized and that ligand binding probably induces conformational changes of oligomerized subunits relative to each other, bringing together intracellular domains that are separated in the basal state. In addition, it is also possible that CD95L induces the formation of oligomers different from the one present in the basal state. The gel filtration experiments suggested that CD95 proteins containing an intact NH2-terminal domain are capable of forming very large oligomers. In contrast, the studies performed with chemical cross-linkers indicated that the CD95 receptor may form apparently mainly dimers and trimers, although a faint smear of higher molecular weight aggregates was observed in the Western blots. This difference may be due to artificially high migration rate of the chemically cross-linked complex on SDS-PAGE gels. Alternatively, the oligomeric state of CD95 may be variable and in vivo may be influenced by the presence of other molecules that bind to the CD95 receptor or by constraints mediated by the transmembrane localization of the protein. The variable and sometimes low amounts of oligomers observed in chemical cross-linking experiments suggested either a low percentage of oligomeric receptors or instability of the receptor complexes in our experimental conditions. Thus, the fraction of receptors in the oligomeric state in vivo on the cell surface is unknown. It is possible that all CD95 receptors in the plasma membrane are oligomeric or that only a portion of the receptor population may oligomerize, and that modulation of oligomerization by unknown factors may regulate responsiveness to CD95L. Interestingly, the TNF-R1 extracellular domain has been crystallized in the absence of TNF as dimers (53, 54).

Induction of apoptosis in the absence of CD95L by overexpression of the CD95 cytoplasmic domain or induction of apoptosis by overexpression of a CD95 receptor lacking the NH2-terminal 42 amino acids (data not shown) suggest that the extracellular oligomerization domain of CD95 is not required to initiate signaling and that self-association of the death domain is necessary and sufficient and occurs in the absence of an intact extracellular oligomerization domain.

The cell must have some mechanism to keep the CD95 receptor apart. Modification of the CD95 self-aggregation properties has been proposed as a mechanism to modulate the apoptotic signal. In particular, sialylation of surface CD95 has been suggested to regulate sensitivity toward ligand-mediated cell death functioning as a repellent (55). On the other hand, it can be proposed that oligomerization of the CD95 receptor through the extracellular domain, likewise the TNF-R1, may enforce a non-signaling state, possibly by inhibiting productive self-association of intracellular death domains. Moreover, intracytoplasmic interaction of the CD95 receptor or of the CD95 receptor with sCD95 might modulate cell surface expression of the receptor and consequently sensitivity to CD95L-mediated cell death.

In conclusion, a novel oligomerization domain has been identified and characterized in the extracellular region of the CD95 receptor. This domain has been mapped to the NH2-terminal portion of the receptor and is distinct from the CD95L interaction binding sites. Moreover, our results indicate that two
oligomerization domains are present in the CD95 receptor, one mapping to the extracellular region of the receptor and one, involved in apoptotic signaling, mapping to the intracytoplasmic region, the death domain. The extracellular oligomerization domain probably has a role in the regulation of the non-signaling state of the CD95 receptor and also mediates interactions with the sCD95 counteracting the apoptotic signaling.

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