

Cysteine 230 Is Essential for the Structure and Activity of the Cytotoxic Ligand TRAIL*

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Unlike other tumor necrosis factor family members, the cytotoxic ligand tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)/Apo-2L contains an unpaired cysteine residue (Cys²³⁰) in its receptor-binding domain. Here we show that the biological activity of both soluble recombinant TRAIL and cell-associated, full-length TRAIL is critically dependent on the presence of Cys²³⁰. Mutation of Cys²³⁰ to alanine or serine strongly affected its ability to kill target cells. Binding to its receptors was decreased by at least 200-fold, and the stability of its trimeric structure was reduced. In recombinant TRAIL, Cys²³⁰ was found engaged either in interchain disulfide bridge formation, resulting in poorly active TRAIL, or in the chelation of one zinc atom per TRAIL trimer in the active, pro-apoptotic form of TRAIL.

Ligands and receptors of the tumor necrosis factor (TNF)¹ family function to trigger cell growth, differentiation, activation, and apoptosis and are mainly implicated in the maintenance and function of the immune system (1). The TNF-related apoptosis-inducing ligand (TRAIL/Apo-2L) displays cytotoxic activity toward a number of transformed cell lines but not against most primary cells, suggesting that it may be a useful anti-tumor agent (2, 3). Recent reports demonstrating that TRAIL is able to counteract progression of human tumor xenografts in a murine model support this hypothesis (4, 5).

There are several receptors for TRAIL that all belong to the TNF receptor family. These include TRAIL-R1/DR4, TRAIL-R2/DR5, TRAIL-R3/DcR1, and TRAIL-R4/DcR2, which display high sequence homology in their extracellular domains and have TRAIL as their only known ligand. Osteoprotegerin (OPG), however, belongs to a distinct subfamily and binds to both TRANCE/RANKL/OPGL and TRAIL. The widely expressed TRAIL-R1 and TRAIL-R2 contain an intracellular death domain and can signal apoptosis; the remaining receptors are believed to function as decoys and either do not contain a functional death domain (TRAIL-R4), have a glycolipid anchor (TRAIL-R3), or exist in a soluble form (osteoprotegerin) (for a review on TRAIL-

Rs, see Ref. 6).

The extracellular domains of TNF receptor family members have an elongated shape stabilized by multiple disulfide bridges, which is reflected at the level of the primary structure by a characteristic pattern of Cys residues (7). In contrast, ligands are formed of anti-parallel β -sheets folding into the so-called “jellyroll” structure, which in some cases is stabilized by a single disulfide bridge (8, 9). TRAIL is remarkable in that it contains an unpaired Cys residue (Cys²³⁰) at the corresponding position where some other ligands have a disulfide bridge. TRAIL, alone or in complex with TRAIL-R2, has been crystallized recently by three different groups (10–12). Although Cys²³⁰ appears to be important in the organization of the different structures, it has been found either (*a*) as a free Cys residue, (*b*) involved in disulfide bridge formation with Cys²³⁰ of another monomer (and as a free Cys in the remaining monomer), or (*c*) as a zinc chelator, together with the Cys residues of the remaining two monomers.

In this study, we show that Cys²³⁰ is required for optimal TRAIL activity and for stabilization of the TRAIL trimeric structure. We confirm the presence of one zinc atom per trimer of TRAIL, and we find that the presence of the interchain disulfide bridge decreases its specific activity, suggesting that Cys²³⁰ in the active TRAIL coordinates a zinc atom, rather than forming a disulfide bridge.

EXPERIMENTAL PROCEDURES

Materials—Anti-FLAG M2 antibody and M2-agarose were purchased from Sigma. Anti-HA mouse IgG1 monoclonal antibody 16B12 was from Babco (Richmond, CA). Anti-mouse IgG1 γ 1 chain antibodies coupled to peroxidase were purchased from Southern Biotechnology Associates (Birmingham, AL). Fas:Fc, CD40:Fc, TRAIL-R1:Fc, TRAIL-R2:Fc, TRAIL-R3:Fc and TRAIL-R4:Fc were obtained from Alexis (San Diego, CA). Thermolysin from *Bacillus thermoproteolyticus* (~37,500 Da) was purchased from Fluka (Buchs, Switzerland) and NaCl (Suprapure grade, <0.005 parts/million zinc) from Merck.

Cells—293T cells, Jurkat T lymphoblastic leukemia and BJAB Burkitt lymphoma cell lines were maintained as described previously (13).

TRAIL Expression Vectors and Expression of Recombinant TRAIL—Bacterial FLAG-TRAIL and FLAG-BAFF expression vectors encode the following proteins: MRGSDYKDDDDKGPQVQLQ followed by either aa 95–281 of hTRAIL or aa 83–285 of hBAFF (13). Mammalian expression vector for HA signal-FLAG-TRAIL, HA signal-HA-TRAIL, and FLAG-tagged full-length TRAIL encode the following predicted mature proteins, respectively, DYKDDDDKGPQVQLQ followed by aa 95–281 of hTRAIL, YPYTPDYAGPGQVQ followed by aa 95–281 of TRAIL, and MDYKDDDDKEF followed by aa 1–281 of hTRAIL. These or related vectors have been described previously (14, 15). Site-directed mutagenesis of Cys²³⁰ to Ala or Ser was performed by a polymerase chain reaction-based method and was confirmed by sequencing. Expression of FLAG-TRAIL and FLAG-BAFF was achieved by induction in M15 pRep4 bacteria (Qiagen) for 16 h at 18 °C. Bacteria were resuspended in TBS (10 mM Tris-HCl, pH 7.5, 140 mM NaCl) and lysed in a French press. FLAG-TRAIL and FLAG-BAFF recovered in the soluble fraction were affinity purified on M2-agarose, eluted with 50 mM citrate-NaOH pH 2.7, immediately neutralized with Tris-HCl, pH 9, and the buffer was exchanged for either TBS, PBS, or 140 mM Suprapure NaCl by

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¹ The abbreviations used are: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; BAFF, B cell activation factor of the TNF family; aa, amino acid; HA, hemagglutinin; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; wt, wild type.

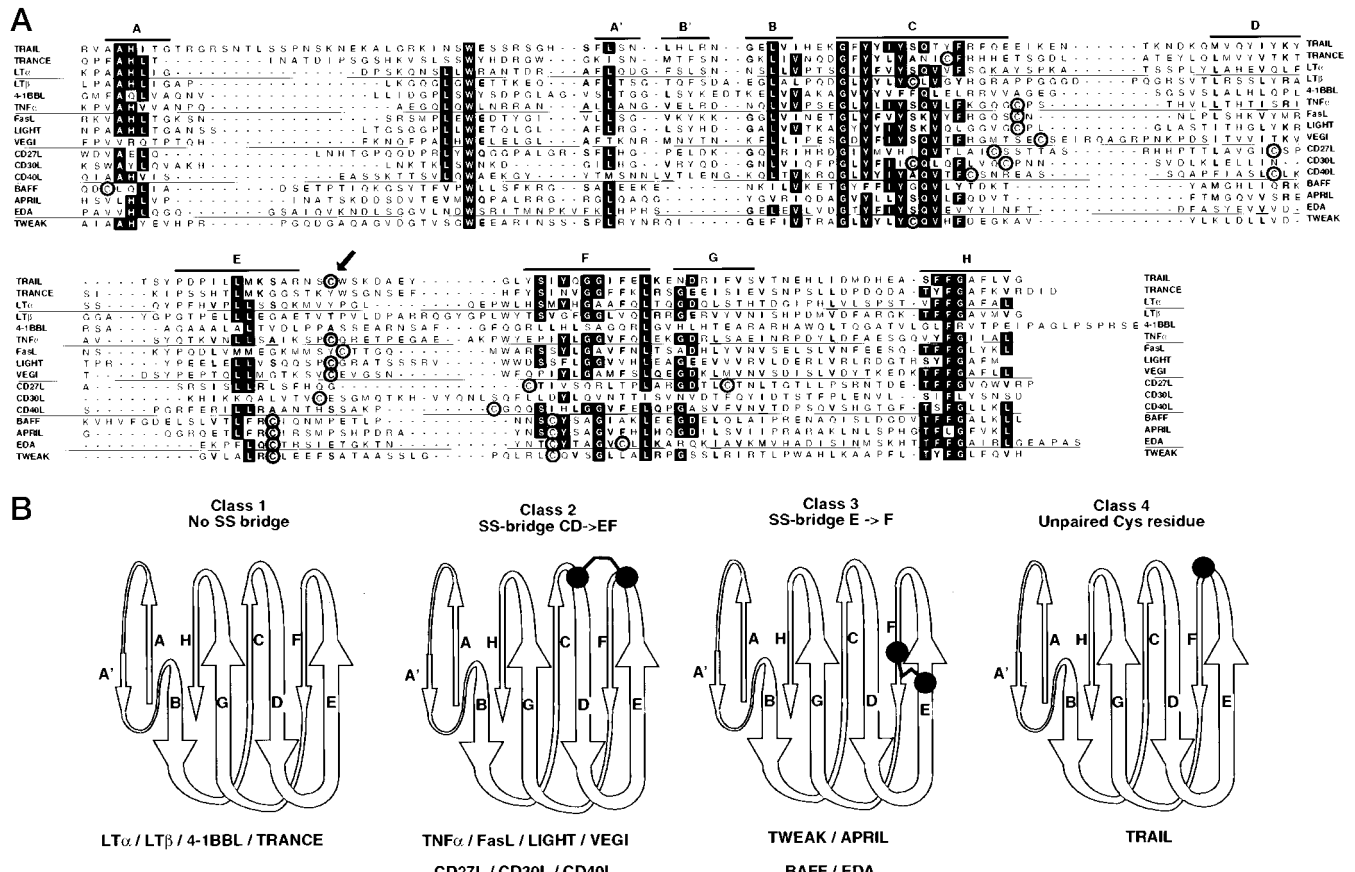


FIG. 1. Alignment of TNF family ligands and schematic representations showing the position of predicted disulfide bridges. *A*, comparison of the extracellular protein sequences of TRAIL and other members of the TNF ligand family. Identical and homologous residues are represented in *black* and *shaded boxes*, respectively. Cysteine residues are *circled*. The *arrow* points at the unpaired Cys²³⁰ residue of TRAIL. Positions of β -strands are indicated *above* the sequence. *B*, some ligands of the TNF family have no disulfide bridge (*class 1*). Ligands with one disulfide bridge fall into two classes. In *class 2*, the disulfide bridge links the CD loop with the EF loop (the location of the disulfide bridge in the loop can vary). In *class 3*, a predicted disulfide bridge links β -sheet E with β -sheet F. TRAIL, the only member of *class 4*, resembles *class 2* ligands but lacks the Cys residue in the CD loop (replaced by a Glu residue). The unpaired Cys residues occurring in β -strands C, D, or F of some ligands are not shown. *LT*, lyphotoxin.

repeated centrifugation in protein concentrators (Centricon-10 or -30, Amicon, Easton, TX). To study the pH sensitivity of TRAIL, bacterial pellets corresponding to 20 ml of culture were lysed in 1 ml of a universal pH buffer containing 30 mM each sodium citrate, sodium borate, sodium phosphate, and sodium barbital at pH 5–9. Bacterial lysates were centrifuged for 5 min at 10,000 $\times g$, and supernatants, containing TRAIL at about 100 $\mu\text{g/ml}$, were collected. For FLAG-TRAIL produced by transient transfection of 293T cells in serum-free Opti-MEM medium (14), cell supernatants were concentrated 20 times in Centricon-10.

Cytotoxic Assays—Cytotoxic assays for soluble FLAG-TRAIL were performed in the presence of 2 $\mu\text{g/ml}$ anti-FLAG M2 as described previously (15).

For chromium release assays using transfected 293T cells, 2×10^6 BJAB cells were labeled for 1 h at 37 $^{\circ}\text{C}$ with 50 μCi of $\text{Na}_2^{51}\text{CrO}_4$. 10^4 labeled BJAB cells were co-cultured with the indicated ratio of transfected 293T cells, in the presence or absence of 10 $\mu\text{g/ml}$ TRAIL-R2:Fc or Fas:Fc. Maximum release was obtained by lysing cells in 700 mM HCl. Released [^{51}Cr] was monitored after 15 h, and the percentage of specific chromium release was calculated as follows: (measured release – spontaneous release)/(maximum release – spontaneous release).

Receptor-binding ELISA—The binding of FLAG-TRAIL (purified or in crude bacterial extracts) to plate-bound TNF-R1:Fc and TRAIL-R1(R2)(R3)(R4):Fc was detected with anti-FLAG M2 antibody and secondary reagents as described previously (14).

Gel Permeation Chromatography—Purified wt and mutant TRAILs expressed in *Escherichia coli* were loaded onto a Superdex-200 HR10/30 column and eluted in PBS at 0.5 ml/min with on-line monitoring at 280 nm. The identity of the eluted peaks was confirmed by Western blotting using anti-FLAG M2. The column was calibrated with a set of standard proteins for gel filtration (Amersham Pharmacia Biotech).

Immunoprecipitations—Soluble wt or mutated TRAIL carrying

FLAG or HA tags were co-transfected in 293T cells. After 3 days, cell supernatants were pre-cleared with Sepharose 6B and immunoprecipitated with anti-FLAG M2-agarose. Beads were washed 4 times with PBS and analyzed by Western blotting with anti-FLAG or anti-HA antibodies followed by peroxidase-coupled anti-mouse IgG $\gamma 1$ chain.

For the precipitation of TRAIL with TRAIL-R2:Fc, 15 μl of protein A-Sepharose (Amersham Pharmacia Biotech) was mixed with 20 μg of *E. coli*-produced TRAIL and 10 μg of TRAIL-R2:Fc (or CD40:Fc in the control) in a final volume of 70 μl , for 30 min at room temperature. The reaction was poured into an empty mini-column, washed 3 times with 200 μl of PBS, and eluted with 20 μl of 0.1 M citrate/NaOH, pH 2.7. The unbound fractions, final washes, and neutralized eluates were analyzed by SDS-PAGE under non-reducing conditions and Coomassie Blue staining.

Mass Spectrometry—TRAIL in a matrix of sinapinic acid (Aldrich) was analyzed in a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer (Voyager-DE, Perspective Biosystem, Framingham, MA) equipped with a nitrogen laser ($\lambda = 317$ nm) to desorb and ionize the sample. Ion masses were assigned based on an external mass calibration using peptides of known molecular mass. Relative intensities in the matrix-assisted laser desorption/ionization spectra are not proportional to relative abundance of the species, due to intrinsic variability in the desorption process.

Atomic Absorption—Proteins in 140 mM Suprapure NaCl were loaded on a 5-ml HiTrap desalt column (Amersham Pharmacia Biotech) equilibrated and eluted at 0.5 ml/min in 140 mM Suprapure NaCl. Fractions (500 μl) were analyzed for protein content by the bicinchoninic acid assay (Pierce) using bovine serum albumin as a standard and for zinc content by electrothermal atomization atomic absorption using a Perkin-Elmer Analyst 800 spectrometer equipped with a graphite furnace, using the external standard method and Zeeman background correction. The light source was an Intensitron hollow cathode lamp

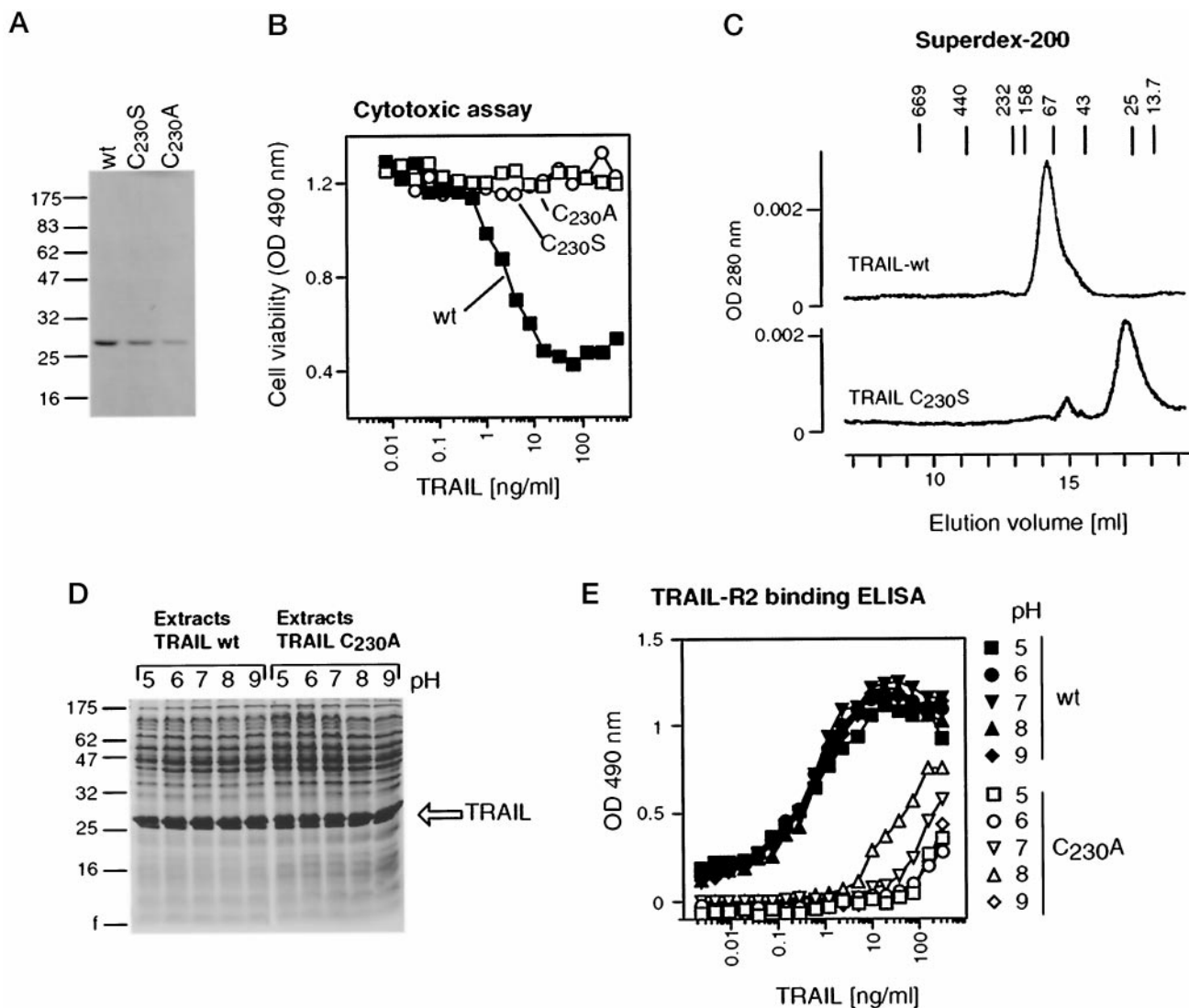


FIG. 2. Cys²³⁰ is required for activity and pH stability of recombinant TRAIL expressed in bacteria. *A*, Coomassie Blue-stained gel showing affinity purified recombinant soluble FLAG-TRAIL (wt and mutants) expressed in *E. coli*. *B*, cytotoxic activity of wt and mutated TRAILS. Jurkat cells were incubated for 16 h in the presence of anti-FLAG antibody and of the indicated concentrations of recombinant TRAILS shown in *A*. *C*, recombinant soluble TRAILS were analyzed by gel permeation chromatography and detected on line at 280 nm. The elution positions of molecular mass markers (in kDa) are indicated at the top of the figure. The presence of FLAG-TRAIL in the main peaks was confirmed by Western blotting (not shown). *D*, soluble extracts of bacteria expressing wt or C230A TRAIL were prepared at various pH values and were analyzed by SDS-PAGE and Coomassie Blue staining. The band of overexpressed TRAIL is indicated. *E*, receptor binding ELISA for wt and C230A TRAILS. TRAIL wt and C230A contained in crude bacterial extracts prepared at different pH values (see *D*) were allowed to bind in PBS to coated TRAIL-R2:Fc or to the TNF-R1:Fc negative control. Values obtained for the negative control have been subtracted in the results presented.

operated at 15 mA, using the 213.9 nm emission line for zinc. The furnace temperature program was as follows: 30 s at 110 °C, 110–130 °C over 15 s, 30 s at 130 °C, 130–300 °C over 15 s, 30 s at 300 °C, 5 s at 1800 °C (measure), and 3 s at 2450 °C (regeneration). The molar ratio of zinc has been calculated using the following molecular masses: 23.8 (TRAIL), 24.2 (BAFF), and 37.5 kDa (thermolysin).

Dialysis and Proteolytic Assay—Purified wt TRAIL expressed in *E. coli* (100 µg per condition) was dialyzed for 7 days at 4 °C against 1000 volumes of TBS, pH 7.5, supplemented or not with 1 mM EGTA, EDTA or *ortho*-phenanthroline. Buffers were replaced after 24 h. Dialyzed TRAILS were assayed for TRAIL-R2 binding and for zinc content as described above. Thermolysin was dialyzed for 24 h under otherwise identical conditions. Its proteolytic activity was measured as follows: thermolysin (3 µg/ml) was incubated for 0, 5, 10, 20, and 30 min at 37 °C in TBS containing 4 mg/ml azocasein (Sigma). Reactions were stopped by the addition of an equal volume of ice-cold trichloroacetic acid at 5%. After centrifugation, absorbance of supernatants containing soluble azocasein peptides was measured at 366 nm.

RESULTS AND DISCUSSION

TRAIL Contains an Unpaired Cys Residue—Members of the TNF family can be classified into three main classes on the

basis of the disulfide bridge pattern in their extracellular TNF homology domains. LT α , LT β , 4-1BBL, and TRANCE have no disulfide bridges. The crystal structures of TNF α and CD40L reveal the presence of a disulfide bridge between the CD and EF loops (8), and FasL, LIGHT, VEGI, CD30L, and CD27L have a predicted disulfide bridge at corresponding positions. Finally, TWEAK, EDA, APRIL, and BAFF have a predicted disulfide bridge between β -strands E and F (Fig. 1). In addition, several ligands display unpaired Cys residues that are mainly located in the middle of β -strands C (mLT α , LT β , TWEAK, TRANCE, and CD30L), D (CD40L), or F (EDA). TRAIL is unique in that both the human and murine sequences contain a single Cys residue in the TNF homology domain that is located in the EF loop at the exact same position as one of the Cys of the disulfide bridge of TNF α , FasL, and LIGHT. However, the corresponding position in the CD loop of TRAIL is a Glu residue instead of the expected Cys residue. As the role of this unpaired Cys residue of TRAIL was unclear, we decided to study its function in more detail.

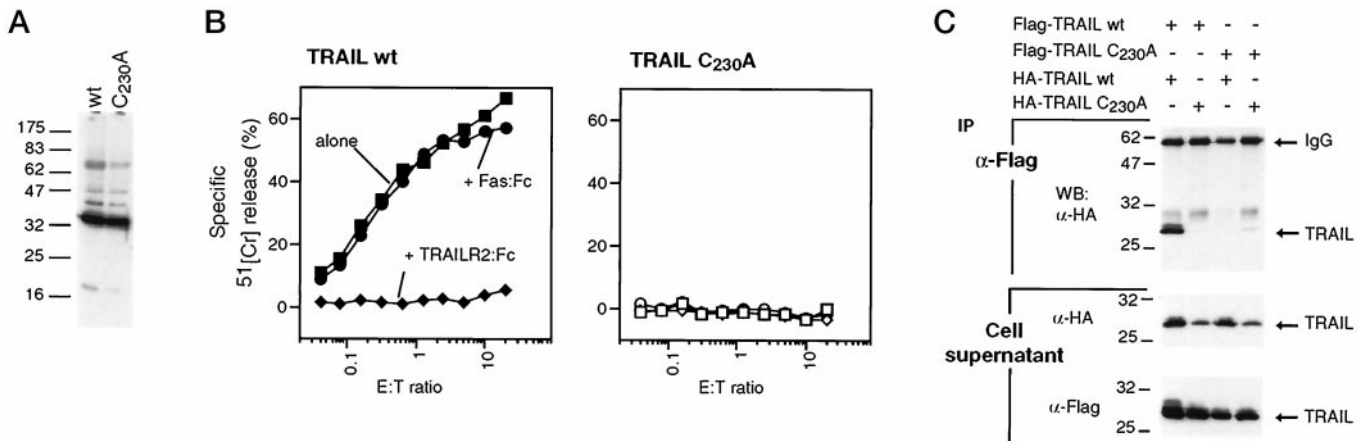


FIG. 3. Cys²³⁰ is required for the activity and trimerization of TRAIL expressed in mammalian cells. *A*, expression of full-length TRAILs in 293T cells. 293T cells were transiently transfected with expression constructs for N-terminally FLAG-tagged full-length TRAIL (wt or C230A), and cell extracts were analyzed under reducing conditions by Western blotting with anti-FLAG. *B*, Cys²³⁰ is required for the activity of full-length TRAIL in mammalian cells. 293T cells were transfected with full-length TRAIL as described in *A* and incubated at the indicated ratio with ⁵¹Cr-labeled BJAB cells, in the presence or in the absence of Fas:Fc or TRAIL-R2:Fc. Non-transfected 293T cells did not induce specific lysis of target cells (not shown). *C*, 293T cells were transiently transfected with expression constructs for secreted soluble TRAILs as indicated. FLAG-TRAILs in the cell supernatants were immunoprecipitated (IP) with anti-FLAG-agarose, and the presence of co-immunoprecipitating HA-TRAILs was assessed by Western blotting (*upper part*). Expression of the various proteins in the cell supernatants is also shown (*lower part*).

Cys²³⁰ of TRAIL Is Essential for TRAIL Activity and pH Stability—Soluble forms of wild type (wt) and mutated (C230A and C230S) FLAG-tagged recombinant soluble TRAILs were produced in *E. coli* (Fig. 2A), and their activities were assayed on the TRAIL-sensitive Jurkat cell line. TRAIL wt was cytotoxic when cross-linked with anti-FLAG antibodies, as described previously (Fig. 2B) (15). In contrast, TRAILs C230A and C230S were totally inactive under the same conditions (Fig. 2B). The interaction between wt TRAIL and TRAIL-R1, -R2, -R3, and -R4 was readily demonstrated in an ELISA, whereas TRAILs C230A and C230S showed very little specific binding and only at high concentrations (data not shown). The integrity of the quaternary trimeric structure of TRAIL was determined by gel permeation chromatography. We found that wt TRAIL eluted from the column with an apparent mass of about 80 kDa, which is compatible with a trimeric structure. In contrast, TRAIL C230S and C230A were markedly smaller and eluted with an apparent mass of about 28 kDa corresponding to a monomer (Fig. 2C and data not shown). As the affinity purification procedure included an acid elution step, we assessed acid lability of TRAIL. Bacteria expressing comparable levels of wt or mutated TRAIL were lysed at different pH values (Fig. 2D), and TRAIL-R2 binding activity of these extracts was subsequently measured in PBS (Fig. 2E). In bacterial lysates prepared at pH 8 or pH 7, wt TRAIL bound TRAIL-R2 200–1000-fold better than mutated TRAIL. In addition, a clear pH sensitivity effect was observed for mutant, but not for wt TRAIL, in lysates prepared at higher or lower pH values (Fig. 2E). Mutation of Cys²³⁰ therefore affects both the activity and stability of TRAIL.

Full-length Membrane-bound C230A TRAIL Is Inactive in Mammalian Cells—We confirmed the effect of Cys²³⁰ mutation on TRAIL activity in a distinct experimental setup, using N-terminally FLAG-tagged, full-length TRAIL expressed in mammalian cells. Whenever TRAIL was expressed in mammalian cells, we only used the C230A mutation because the C230S mutation generated a predicted N-glycosylation site in the sequence of TRAIL, which was indeed utilized during biosynthesis. Both wt and C230A TRAILs were expressed at comparable levels (Fig. 3A), but only cells transfected with wt TRAIL displayed significant cytotoxic activity on the target BJAB cells (Fig. 3B). The observed cytotoxic activity was completely abrogated by soluble TRAIL-R2, but not by the soluble Fas control,

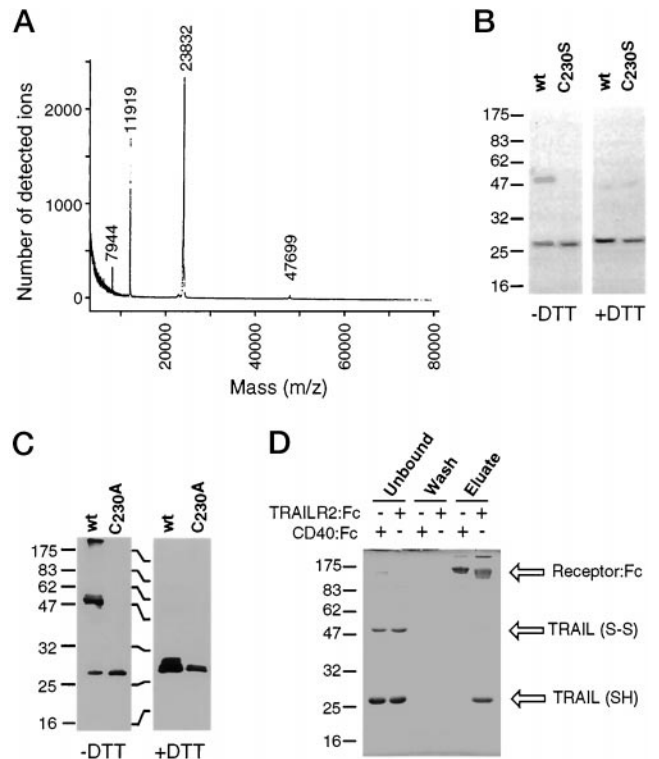


FIG. 4. Interchain disulfide bond formation in TRAIL is dependent on Cys²³⁰ and reduces the affinity of TRAIL for TRAIL-R2. *A*, FLAG-tagged recombinant soluble TRAIL expressed in *E. coli* was analyzed by mass spectrometry. Ions at 23,832, 11,919, and 7944 represent the single, double, and triple charged ions of TRAIL, respectively. The ion at 47,699 corresponds to dimeric TRAIL. *B*, FLAG-tagged recombinant soluble TRAIL expressed in *E. coli* was analyzed by SDS-PAGE under reducing and non-reducing conditions and stained with Coomassie Blue. DTT, dithiothreitol. Presence of the FLAG tag in the 27- and 54-kDa bands was confirmed by Western blotting (not shown). *C*, secreted FLAG-tagged soluble TRAIL expressed in 293T cells was analyzed by SDS-PAGE under reducing or non-reducing conditions and detected by Western blotting. *D*, FLAG-tagged recombinant soluble TRAIL expressed in *E. coli* was immunoprecipitated with substoichiometric amounts of TRAIL-R2:Fc (or CD40:Fc as a control) and protein A-Sepharose. Proteins in the unbound fraction (*Unbound*), the last wash (*Wash*), and the eluate (*Eluate*) were analyzed by SDS-PAGE under non-reducing conditions and revealed by Coomassie Blue staining. Molecular mass markers are in kDa.

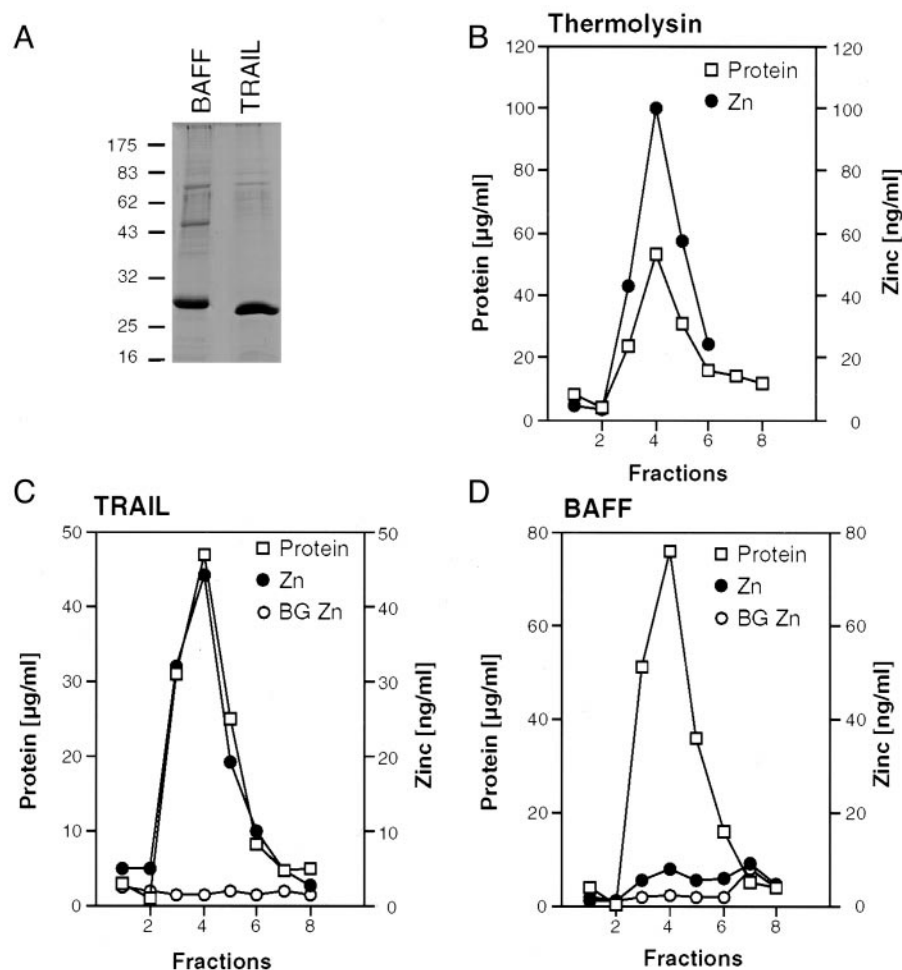


FIG. 5. Recombinant soluble TRAIL contains a zinc atom. *A*, Coomassie Blue staining of TRAIL and BAFF used in *C* and *D*. Both ligands were expressed in *E. coli*. Molecular mass markers are in kDa. *B*, thermolysin was loaded onto a HighTrap desalting column and eluted in 140 mM NaCl. Fractions were analyzed for both protein (open squares) and zinc contents (closed circles). *C*, TRAIL was analyzed as described in *B*. The flow-through of the last wash in Centrikon-30 (see “Materials and Methods”) was also analyzed (BG zinc, open circles). *D*, as for *C*, except that BAFF was analyzed.

confirming that the observed cytotoxicity was a direct effect of TRAIL expression. Thus, Cys²³⁰ is essential for TRAIL cytotoxicity, regardless of the construct and expression system used.

A defect in the quaternary structure of TRAIL C230A was observed in the mammalian expression system by co-immunoprecipitation of secreted soluble TRAILS. Wild type TRAILS expressed with two different tags co-precipitated, showing their oligomerization capacity. In contrast, TRAIL C230A failed to interact either with itself or with wt TRAIL (Fig. 3C). We conclude that Cys²³⁰ plays an essential role in the assembly and/or maintenance of the quaternary structure of TRAIL.

Formation of an Interchain Disulfide Bridge Decreases TRAIL Activity—In proteins, cysteine residues can be involved in posttranslational events such as internal thioester and disulfide bridge formation, isoprenylation, fatty acid acylation, and metal coordination, or they can remain as a free SH group displaying a structural or catalytic role. The measured molecular mass of TRAIL (23,832 ± 10 Da, predicted 23,839.8 Da) and its soluble behavior during Triton X-114 phase separation excluded that Cys²³⁰ would carry large or hydrophobic substituents (Fig. 4A and data not shown). However, a minor proportion of the recombinant TRAIL produced in *E. coli* migrated as a dimer by SDS-PAGE under non-reducing conditions, indicating the presence of an interchain disulfide bridge. A minor species of 47,699 ± 20 Da, corresponding to a TRAIL dimer, was also detected by mass spectrometry (Fig. 4, A and B). When TRAIL was expressed in mammalian cells, disulfide-linked TRAIL was by far the major product (Fig. 4C), but dimers were never detected in Cys²³⁰ mutants, indicating that Cys²³⁰ is directly implicated in disulfide bridge formation. In our hands, soluble TRAILS expressed in mammalian cells (with

high disulfide bridge content) have been consistently less cytotoxic than those produced in *E. coli* (with low disulfide bridge content), and we have measured a 20–30-fold lower specific activity for their binding to TRAIL-R2. This may indicate that disulfide-linked Cys²³⁰ is not favorable for TRAIL activity. Indeed, we found that non-disulfide-linked TRAIL bound recombinant receptor better than disulfide-linked TRAIL (Fig. 4D).

Interestingly, both wt and C230A full-length TRAIL expressed in mammalian cells formed dithiothreitol-sensitive dimers, which migrated with slightly different mobilities, raising the possibility that additional interchain disulfide bridges may occur in the membrane proximal portion of the extracellular domain of TRAIL, where 2 additional Cys residues are located (data not shown). Whether this results in the cross-linking of membrane-bound TRAIL trimers remains to be determined.

Trimeric TRAIL Contains Stoichiometric Amounts of Zinc—Cysteine residues are known to coordinate zinc atoms to proteins, and as the Cys residues of TRAIL are suitably positioned to coordinate a metal ion (10–12), we measured the zinc content in recombinant TRAIL by atomic absorption. Proteins were prepared in a low zinc solution and size-fractionated just prior to analysis to remove the ubiquitous unbound zinc ions. We measured 1.08 mol of zinc per mol of positive control (the zinc-metalloprotease thermolysin), 0.027 mol of zinc per mol of negative control (BAFF, another TNF family member (13)), and 0.326 mol of zinc per mol of TRAIL, *i.e.* 0.98 mol of zinc per TRAIL trimer, whereas TRAIL C230S only contained background levels of zinc (Fig. 5 and data not shown). Taken together, our data strongly suggest that the Cys²³⁰ residues

coordinate one zinc atom per trimeric TRAIL, therefore stabilizing TRAIL in the correct conformation for receptor binding and activity. Surprisingly, dialysis of TRAIL for as long as a week against any of the chelating agents EGTA, EDTA, or *ortho*-phenanthroline did not significantly alter its binding to TRAIL-R2, whereas the proteolytic activity of the positive control thermolysin was completely abolished after only 1 day of these treatments. Atomic absorption analysis indicated that zinc was still present in dialyzed TRAIL, suggesting that zinc is tightly associated with TRAIL and/or not accessible within the structure (data not shown). However, we cannot exclude that different experimental conditions may achieve zinc removal from TRAIL, as published recently (16).

Our results are strongly supported by the crystallographic study of Hymowitz *et al.* (11), which shows that a tetrahedral zinc atom is coordinated to Cys²³⁰ of the three TRAIL monomers and to an interior chloride ion. No zinc atoms were reported in the other two available structures (10, 12), but it is noteworthy that in both cases TRAIL was obtained by refolding from inclusion bodies, which may preclude uptake of a zinc atom. In one case, an S-S bridge occurred between two Cys²³⁰ of TRAIL co-crystallized with TRAIL-R2 (12). Although disulfide-linked TRAIL binds to its receptor less efficiently than zinc-containing TRAIL (Fig. 4D), it is conceivable that it may play a physiological role under certain conditions. TRAIL with a C230S mutation has been described as being indistinguishable from wt TRAIL in its ability to immunoprecipitate TRAIL-R2 (12), which is in discrepancy with our results. However, immunoprecipitation may not reveal a change in affinity, especially if one of the components is present in large excess. It is also possible that refolded wt TRAIL lacking the zinc atom may display the same decreased activity as the Cys²³⁰ mutant.

In addition to TRAIL, several TNF family members display unpaired Cys residues in their receptor-binding domain. The Cys residue present in the β -sheet C of TWEAK, LT β , and CD30L corresponds to and probably fulfills the same structural role as Ser¹⁸⁶ of TRAIL, which faces to the interior of the monomer and is engaged in a hydrogen bond with the backbone oxygen of Phe²⁷⁴ in β -sheet H. Cys¹⁹⁴ of CD40L in β -sheet D is located on the outer surface of the trimer (9) and is not conserved in murine CD40L. Finally, the unpaired Cys residues of EDA and TRANCE are predicted to be distant in the trimeric

structure based on sequence alignment with TNF family members of known structures (Fig. 1A). The metalloprotein TRAIL thus remains unique within the TNF family. Comparison of the primary and three-dimensional structure of TNF α (8) and TRAIL (11, 12) shows that both ligands have very good overall structural homology, except in the three following loops: AA' (which is exceptionally long in TRAIL), CD, and EF. In TNF α , loops CD and EF are linked by a disulfide bridge (see Fig. 1B), whereas loop EF of TRAIL is arranged differently to allow correct positioning of the unpaired Cys residue to chelate a zinc atom.

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