Functional analysis of tumour necrosis factor-α-related apoptosis-inducing ligand (TRAIL): cysteine-230 plays a critical role in the homotrimerization and biological activity of this novel tumoricidal cytokine

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We have determined that the mutation of the cysteine-230 residue to either glycine or serine in TRAIL (tumour necrosis factor-α-related apoptosis-inducing ligand) results in the formation of a structurally incompetent dimer and a consequent loss of apoptotic activity. Similarly, chemical modification of the thiol residues present in both reduced and Zn^{2+}-depleted trimer converts TRAIL into an inactive dimer. We postulate that cysteine-230 plays a critical role in homotrimerization of this tumoricidal cytokine.

Key words: glutathione S-transferase, soluble TNFα-related apoptosis-inducing ligand, sTRAIL, TdT-mediated dUTP nick end labelling.

INTRODUCTION

Apoptosis is an intrinsic process required for maintaining tissue homoeostasis and development in all metazoans [1–3]. The deregulation of this crucial process leads to various diseases such as cancer, autoimmune disease and neurodegenerative disorders [4–6]. One of the central pathways of apoptosis is initiated by cytokines, such as tumour necrosis factor-α (TNF-α), Fas ligand (Fas L) and tumour necrosis factor-α-related apoptosis-inducing ligand (TRAIL), upon binding to their cognate receptors. TRAIL/Apo2L is a novel cytokine since it induces apoptosis in a number of tumour cells without affecting normal cells. This property differentiates it from other members of the TNF family (i.e. TNF-α or Fas L) that are severely toxic to normal tissues. The cancer-directed, apoptotic potential of TRAIL makes it a promising candidate for therapeutic interventions. TRAIL triggers apoptosis through an interaction with death receptors called DR4 and DR5 [7,8], resulting in the initiation of a signalling cascade that leads to the activation of aspartate-specific cysteine proteases, now called caspases [8,9]. Activated caspases cleave important cellular proteins thereby inducing the signature changes of apoptosis such as membrane blebbing, chromatin condensation, DNA fragmentation and cytoplasmic shrinkage [10]. TRAIL triggers apoptosis in a number of cancer cell types without affecting normal cells [7,8]. Since various tissues express this cytokine constitutively it has been suggested that there should be mechanisms protecting them from death by TRAIL. A possible mechanism could involve the expression of decoy receptors such as DcR1, DcR2 and osteoprotegrin that have a missing or incomplete death domain [8,11]. The crystal structure of TRAIL has been determined recently [12] and it revealed that the insertion loop (amino acids 137–152) plays a crucial role in the interaction of TRAIL with its cognate receptors. The same study also showed that TRAIL monomers may form a trimer in a head-to-tail fashion.

Because of the therapeutic potential of TRAIL, considerable efforts directed at the purification and characterization of recombinant TRAIL have been undertaken. A polyhistidine-tagged soluble form of human TRAIL (114–281) has been shown to be biologically active [11,13], but a FLAG-tagged form of TRAIL (amino acids 95–281) was found to be poorly active and required the incubation with an anti-FLAG antibody for the potentiation of the biological activity [14]. In another effort a modified leucine zipper was linked to TRAIL (amino acids 114–281) in order to promote the trimerization of the ligand [15].

In this study we provide the evidence that homotrimerization is an inherent property of TRAIL and that cysteine-230 plays a critical role in this trimerization process and thereby the tumoricidal activity of this cytokine. We show that the mutation of this residue results in the formation of a structurally distorted dimer resulting in the loss of pro-apoptotic activity of this cytokine.

MATERIALS AND METHODS

Cell lines and cell culture

The colon carcinoma cell lines HT-29 and DLD-1 were originally obtained from the A.T.C.C. and grown in a medium recommended by the supplier. HCT 116 colon carcinoma cell line was maintained in a mixture (1:1) of Dulbecco's modified Eagle's medium and RPMI 1640. Media were supplemented with 2 mM glutamine, 10% fetal calf serum, 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were grown at 37 °C in a humidified atmosphere containing 5% CO_2. Normal colon cell lines CCD-18Co and CCD 841 CoN were a kind gift from M. Dhalla (King Faisal Specialist Hospital and Research Center,

Abbreviations used: TNF-α, tumour necrosis factor-α; TRAIL, TNF-α-related apoptosis-inducing ligand; sTRAIL, soluble TRAIL; GST, glutathione S-transferase; NEM, *N*-ethylmalemide; DTT, dithiothreitol.

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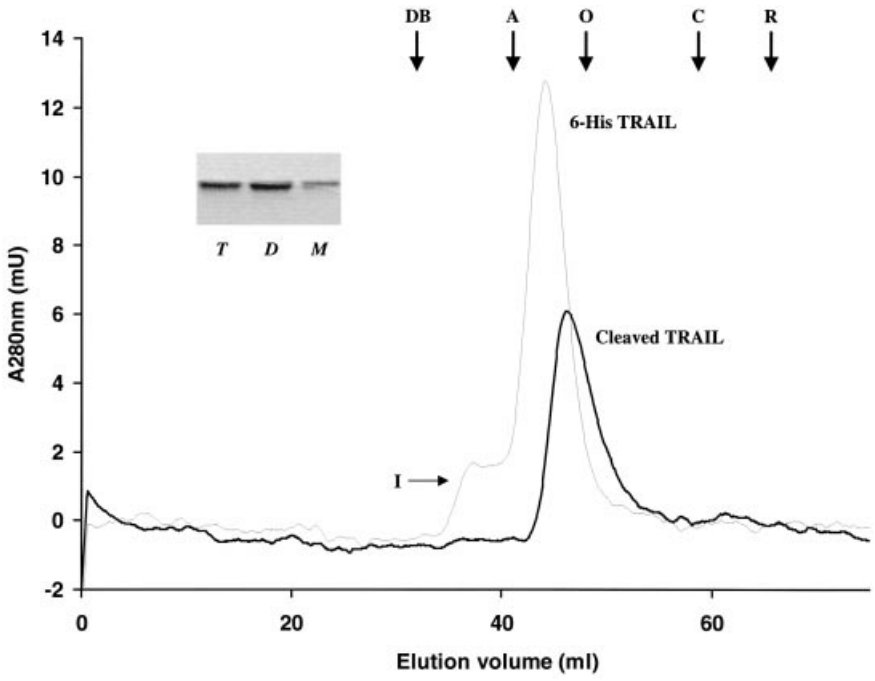

Figure 1 Size-exclusion chromatography of tagged and cleaved sTRAIL (amino acids 95–281)

Superimposed elution curves of 6-His-tagged sTRAIL analysed before and after the treatment with thrombin. I indicates the presence of a minor protein contaminant of bacterial origin. Arrows indicate the elution volumes of Dextran Blue (DB), albumin (A), ovalbumin (O), chymotrypsin (C) and ribonuclease (R). Inset: SDS/PAGE of fractions identified as trimer (*T*), dimer (*D*) and monomer (*M*). The dimeric and monomeric forms were obtained as a result of storage of diluted TRAIL.

Riyadh, Saudi Arabia) and maintained in the recommended medium.

Site-directed mutation of TRAIL

cDNA coding for TRAIL (comprising amino acids 95–281) was used as a template to introduce site-directed mutations at position 230 (cysteine-230) by two-step PCR. The mutations introduced were: cysteine to glycine and cysteine to serine. The mutant soluble TRAIL (sTRAIL) cDNAs were cloned into pET 28(b) (Novagen) or pGEX 2T (Amersham Pharmacia Biotech) in-frame with the N-terminal tags. Mutations at the desired position were verified by sequencing. Mutants of the TRAIL variant (amino acids 114–281) were constructed similarly.

Purification and size-exclusion chromatography of TRAIL

Bacterial strain BL21(DE3) (Novagen) was transformed with pET28(b) vector harbouring either sTRAIL or various mutant cDNAs to obtain 6-His-tagged proteins. Similarly, *Escherichia coli* strain DH5α was transformed with pGEX2T vector containing sTRAIL and the mutant cDNAs to obtain glutathione S-transferase (GST)-tagged chimaeras. Bacterial cultures were induced with 2 mM isopropyl β-D-thiogalactoside and grown at 25–30 °C for 2 h. Cells were lysed by sonication in PBS, pH 7.4, and the soluble material was purified on either Ni^{2+}–nitrilotriacetate–agarose (Clontech Laboratories) or GST–Sepharose (Amersham Pharmacia Biotech). Recombinant proteins were eluted from the beads according to manufacturers' recommendations. Tags were cleaved by thrombin (Sigma) and the reaction was carried out directly on beads containing bound TRAIL. The eluted tagged or untagged proteins were then subjected to size-exclusion chromatography on a Sephacryl S-100 column controlled using the AKTA-FPLC system (Amersham Pharmacia Biotech). The column was developed in PBS at a constant flow rate of 0.5 ml/min.

Table 1 Calculated and estimated (apparent native) molecular masses of different forms of TRAIL

Recombinant sTRAIL molecules contained 6-His tags. Calculated molecular mass is based on amino acid sequence. Apparent mass and Stokes radius were determined by gel filtration carried out on AKTA-FPLC. A Sephacryl S-100 column was calibrated with the low-molecular-mass kit (Amersham Pharmacia Biotech). At least four independent runs for each TRAIL form were carried out, with similar results.

	Molecular mass (kDa)		
	Calculated	Apparent native	Stokes radius (Å)
Tagged TRAIL			
Trimer	66	64	35.0
Dimer	46	38	28.3
Monomer	23	25	22.4
Cleaved TRAIL			
Trimer	63	59	34.2
Reduced tagged TRAIL			
Trimer	66	64	35.0
NEM-modified reduced tagged TRAIL			
Dimer	46	46	31.4
Tagged mutant TRAIL			
Dimer	46	46	31.4
Monomer	23	20	19.5
Cleaved mutant TRAIL			
Dimer	42	42	30.3

Chemical modification of TRAIL

Reduced sTRAIL was purified as described above, with the exception that 50 mM D,L-dithiothreitol (DTT; Roche Molecular Biochemicals) was present throughout the entire procedure. Reduced product was then analysed using the AKTA-FPLC system, where the mobile phase contained 1 mM DTT. The modification of TRAIL was obtained by incubating the purified peak of reduced TRAIL with 5 mM *N*-ethylmaleimide (NEM; Sigma) for 40 min at room temperature. The reaction mixture was then analysed using the AKTA-FPLC system, where DTT was excluded from the mobile phase.

Evaluation of TRAIL-induced apoptosis

Around 10^5 cells/well were cultured in 24-well plates overnight and then treated with FPLC-purified untagged soluble recombinant TRAIL (200 ng/ml) or with GST- or 6-His-tagged variants. Similarly, cells were also treated with FPLC-purified untagged or tagged mutant TRAIL molecules at the same as well as a higher concentration (500 ng/ml). Cells were analysed after 8 or 24 h by phase-contrast microscopy for signs of apoptosis, as described before [7]. The percentage of apoptotic cells was calculated by annexin V staining. Cells were scored as apoptotic if they were positive for annexin V but negative for propidium iodide. The degree of apoptosis was also verified by TdT-mediated dUTP nick end-labelling (TUNEL) assay by following the recommendations of the manufacturer (Roche Molecular Biochemicals).

RESULTS AND DISCUSSION

Homotrimer formation of TRAIL is an inherent property of this cytokine

While purifying recombinant TRAIL without tags on nickel resin from bacterial culture as reported by Ashkenazi et al. [11], we observed many bacterial proteins bound to the resin in addition to TRAIL. We also observed that a few tags used in the purification of proteins facilitated the non-specific oligomerization of the proteins (D. Trabzuni and M. Ahmad, unpublished work). To examine whether the homotrimerization of TRAIL is facilitated by foreign sequences present in the form of various tags or is an inherent property of TRAIL, we purified the recombinant soluble TRAIL (amino acids 95–281 and 114–281) by the virtue of GST or 6-His tags. Resin-bound TRAIL was then treated with thrombin to facilitate the removal of the N-terminal tag. The eluted, non-tagged TRAIL was subjected to further purification on a Sephacryl S-100 column. The elution of the purified protein was done with an isocratic solution of PBS as explained in the Materials and methods section. The apparent 'native' molecular mass of the eluted cytokine, as estimated by size-exclusion chromatography, was 64 kDa (Figure 1 and Table 1), but only 21 kDa (Figure 1, inset) when examined by SDS/PAGE under reducing conditions.

The shift in the apparent molecular mass of sTRAIL caused by the tag cleavage was only 3 kDa, when compared with its 6-His-tagged counterpart. Since the calculated molecular mass based on the amino acid sequence 95–281 of the TRAIL monomer is 21 kDa, we conclude that even in the absence of tags this

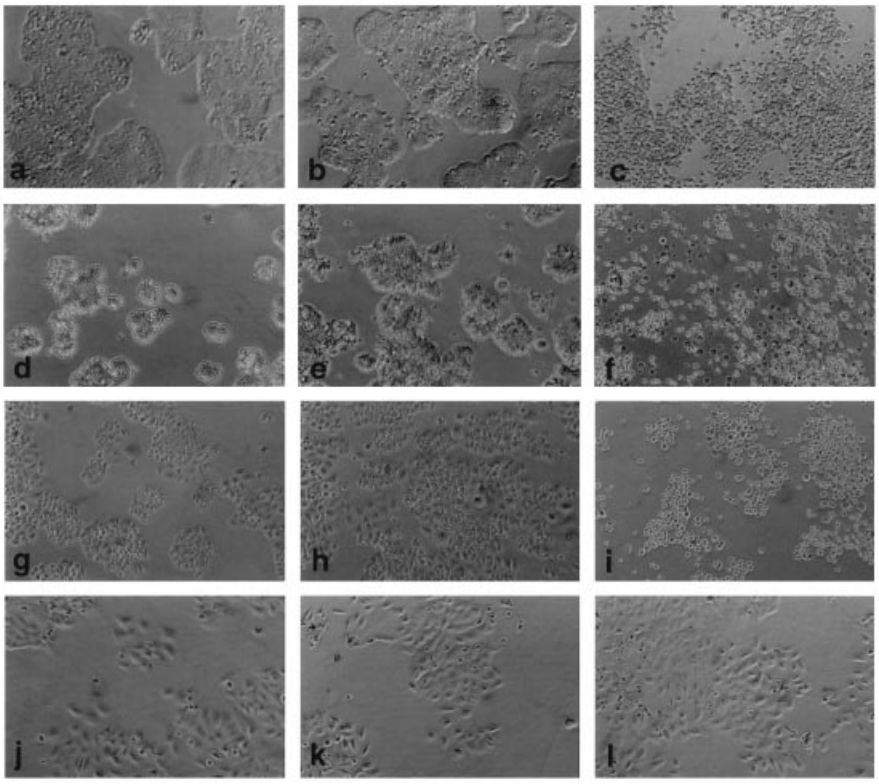

Figure 2 Microscopic evaluation of morphological changes induced by TRAIL in colon cancer and normal colon cell lines

The colon cancer cell lines HCT 116 (**a**–**c**), HT-29 (**d**–**f**), DLD-1 (**g**–**i**) and normal colon cell line CCD-18Co (**j**–**l**) were treated with equal amounts (200 ng/ml) of monomeric (**a**, **d**, **g**, **j**), dimeric (**b**, **e**, **h**, **k**) and trimeric (**c**, **f**, **i**, **l**) forms of TRAIL. The cells were analysed after 8 h of treatment. A representative field of the treated cells has been shown. Magnification, × 20.

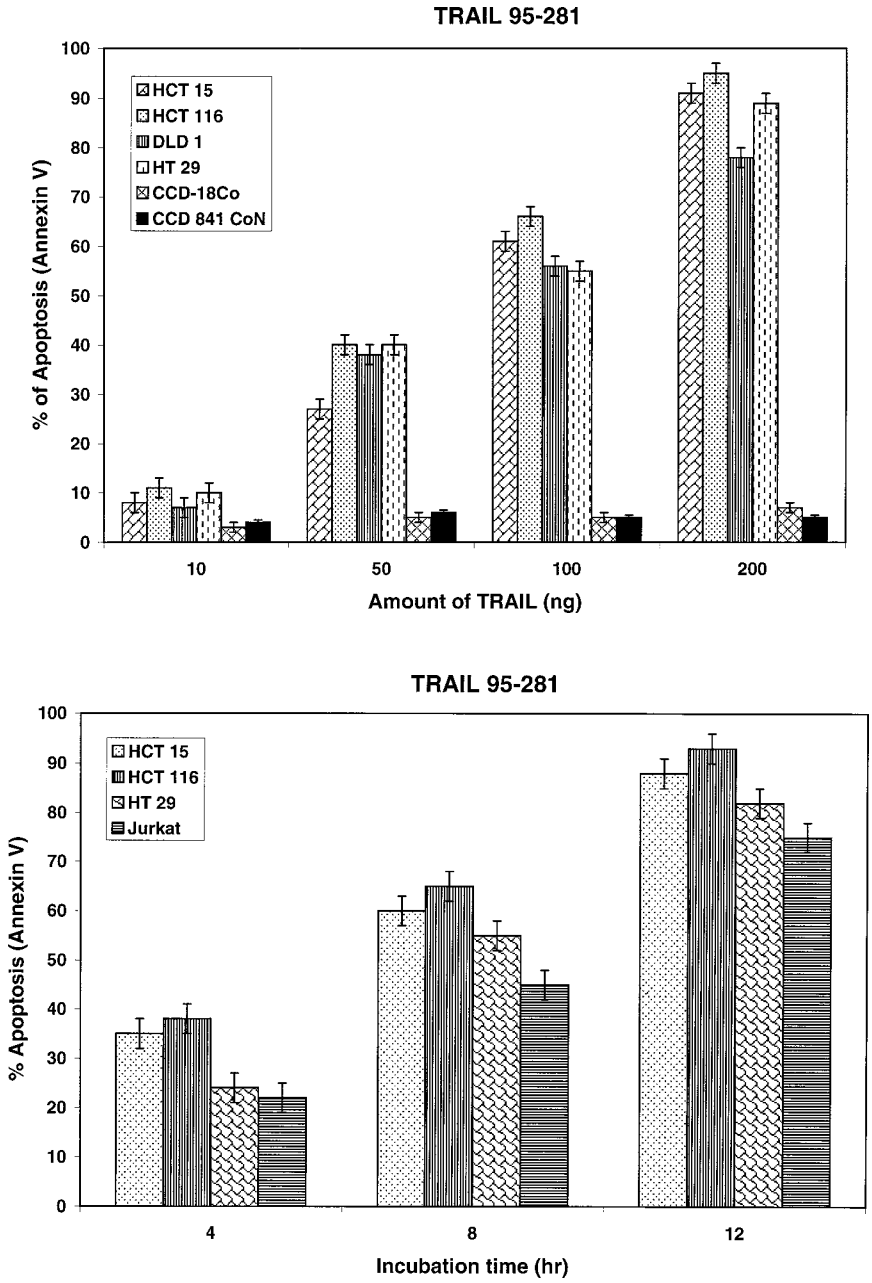

Figure 3 Evaluation of apoptosis in colon cancer cells triggered by sTRAIL (amino acids 95–281)

(Top panel) The indicated colon cancer and normal colon cells were treated with varying amounts of the trimeric form of TRAIL (amino acids 95–281). The percentage of apoptotic cells was measured after 12 h of treatment. (Bottom panel) To rule out the possibility that TRAIL triggers necrosis of the cell lines, the indicated colon cancer and Jurkat cell lines were incubated with 200 ng/ml of the trimeric form of sTRAIL for 4, 8 and 12 h. Each treatment was performed in duplicate and the plotted values represent the means ± S.E.M. of four independent experiments.

cytokine still forms homotrimers. Soluble recombinant TRAIL (amino acids 114–281) as well as the GST-tagged TRAIL (both amino acids 95–281 and 114–281) were also eluted as trimers under the same non-denaturing conditions (results not shown). The trimer form of TRAIL gets converted into dimeric and monomeric forms upon prolonged storage that is accelerated by dilution (results not shown).

Having established the conditions suitable for the purification of different forms of TRAIL we decided to check if the pro-apoptotic activity of TRAIL resides exclusively in its trimeric form. The apoptotic activities of trimeric, dimeric and monomeric sTRAIL (amino acids 95–281) were compared by incubating colon carcinoma cells with equal amounts of each of these forms. As is shown by Figures 2(a) and 2(b), only the trimeric form of sTRAIL was able to induce significant apoptosis of colon carcinoma cell lines after 8 h of incubation, whereas the dimeric and monomeric forms were unable to do so. Regardless of the molecular structure, however, sTRAIL was unable to induce apoptosis in normal colon cells (Figures 2 and 3, top panel). More than 80% of cells underwent apoptosis in the 12-h treatment (Figure 3, top panel). To rule out the possibility that TRAIL induced necrosis, the indicated cell lines were incubated

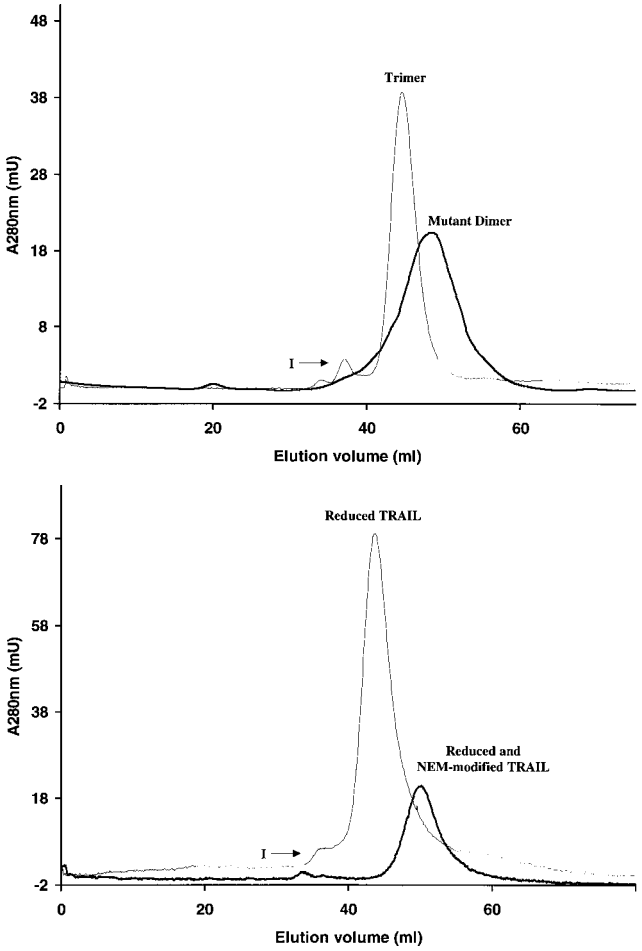

Figure 4 Size-exclusion chromatography of different forms of 6-His-tagged sTRAIL (amino acids 95–281)

(Top panel) Elution profiles of wild-type sTRAIL and its single-site mutant (cysteine-230 to glycine). (Bottom panel) Elution profiles of reduced and NEM-modified wild-type sTRAIL. I indicates the presence of a minor protein contaminant of bacterial origin.

with TRAIL for 4, 8 and 12 h. Only those cells were scored as apoptotic that stained positive for annexin V but negative for propidium iodide. We observed that TRAIL induces apoptosis but not necrosis within 12 h (Figure 3, bottom panel). We conclude that the apoptotic activity of TRAIL is linked to its ability to form homotrimers.

Cysteine-230 plays a crucial role in trimerization of TRAIL

The recent study on the crystal structure of recombinant sTRAIL [12] identified the residues of the insertion loop (amino acids 137–152) and an extended contact surface (amino acids 205–237) [16] as important for the interaction of TRAIL with its cognate receptor. Based on the structural alignment data and the crystal structure of TRAIL we selected cysteine-230 as a candidate amino acid that could be involved in the intermolecular interactions leading to trimer formation. Moreover, it has to be emphasized that only this member of the TNF family possesses an unpaired cysteine. To test our prediction we conducted a series of site-directed mutations, where cysteine was substituted by glycine or serine. Ensuing mutants were then expressed, as explained in the Materials and methods section. All mutations ablated the homotrimer-formation property of TRAIL. Surprisingly enough, a preponderance of dimers rather than monomers was observed (Figure 4, top panel) and the cleavage of tags did not prevent dimer formation (Table 1), as indicated by the size-exclusion chromatography. The apparent native molecular-mass and Stokes-radius values of these new dimers were different from those estimated for the non-mutated dimers (Table 1). A possible explanation is that in addition to multiple weak interactions, the stability of non-mutated dimers was further augmented by the formation of disulphide bonds [16]. Hence, it is conceivable that all these interactions change the shape of the dimer and therefore the exclusion size of the protein. It has to be noted that the monomeric form of mutated TRAIL also behaves abnormally during size-exclusion chromatography (Table 1). In addition to that, mutation of cysteine to serine and cysteine to glycine resulted in the expression of a less-soluble protein. These observations strongly indicate that a single substitution of cysteine-230 results in a significant structural distortion. Mutated monomers still bind to each other but the result is a structurally incompetent dimer that is not able to accommodate the third monomer. Mutated dimers could also be converted into monomers by dilution, and none of the TRAIL mutants, either in dimeric or monomeric form, showed any pro-apoptotic activity, even at a higher concentration of 500 ng/ml (results not shown).

Another crystallographic study of TRAIL complexed with DR5 revealed that in the trimeric form two cysteine residues form a disulphide bridge [17]. Surprisingly, the formation of this bridge seemed not to be required for TRAIL binding to its cognate receptor. However, the pro-apoptotic activity of the modified TRAIL was not investigated by these authors. It has also been proposed that zinc ions bound to the TRAIL molecule may help to stabilize the trimer [18]. Indeed, the very recent report [16] has shown that Zn^{2+} is co-ordinated by the cysteine residues of each monomer and is necessary for the biological activity of TRAIL. Therefore, we aimed to check whether Zn^{2+} is also involved in the maintenance of the trimeric structure of TRAIL. To that end TRAIL was isolated in the presence of a high concentration (50 mM) of DTT and eluted from the affinity column with the buffer containing 100 mM EDTA. The resulting preparation was then analysed by size-exclusion chromatography. Reduced TRAIL still maintained a trimeric configuration (Figure 4, bottom panel, and Table 1). However, the FPLC-purified reduced TRAIL showed a drastically diminished pro-apoptotic activity, even at a concentration of 500 ng/ml. Thus, we conclude that (i) the treatment including both reducing and chelating agents removed Zn^{2+} ions from the protein (see also [16]) and (ii) Zn^{2+}-depleted TRAIL was still able to maintain its tertiary conformation. To acknowledge further the role of cysteine-230 in the formation of trimers the FPLC-purified reduced TRAIL was modified by a thiol-alkylating reagent, NEM. Size-exclusion-chromatography analysis showed that the chemical modification of cysteine residues, present in the trimer, results in the loss of the proper structure and appearance of dimers (Figure 4, bottom panel, and Table 1). Interestingly enough this new structure is similar to that formed by mutated TRAIL (Table 1). It is, therefore, obvious that cysteine-230 plays a critical role in the final steps of TRAIL homotrimerization. As revealed by the crystal structure, cysteine-230 is located on the top surface that is formed by the outer β-sheet [12]. Furthermore, this residue is oriented towards the trimerization interface [16] and hence may be involved in both discrete intersubunit interactions [12] and Zn^{2+} co-ordination [16]. We postulate that cysteine-230 contributes substantially to the overall structural arrangement of the TRAIL molecules in solution and is in-

dispensable for the proper intracellular signalling by the ligand–receptor complex. Since the single mutation at cysteine-230 causes such a drastic change in the biological properties of TRAIL, it is tempting to speculate that such a mutation *in vivo* may contribute to its inability to eliminate tumours.

We are grateful to Dr Sultan T. Al-Sedairy, the Executive Director of the Research Center, for providing excellent research facilities and constant encouragement. The assistance of Kelly Dobler in the growth and maintenance of colon cancer cell lines is gratefully acknowledged. We thank Shabina Nazir for her help in the preparation of the Figures.

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Received 12 April 2000; accepted 17 May 2000