Advances in Brief

Cysteine 230 Modulates Tumor Necrosis Factor-related Apoptosis-inducing Ligand Activity

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Abstract

Biologically active tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) protein is known to form a heterotrimer in solution. Unexpectedly, the recombinant active human TRAIL protein purified from bacteria produced two bands (a Mr 21,000 monomer derived from the disruption of the trimer in SDS gels and a Mr 42,000 dimer) on nonreducing SDS gels. The treatment of this TRAIL protein with DTT, a reducing agent, abolished formation of the Mr 42,000 band, suggesting that the Mr 42,000 band was the result of intermolecular disulfide bridge formation. Inspection of the amino acid sequence of human TRAIL protein identified a unique cysteine residue at position 230, and subsequent site-directed mutagenesis revealed that this amino acid residue is responsible for the appearance of the Mr 42,000 dimer. The binding analysis using the TRAIL protein and a TRAIL receptor (death receptor 5) revealed that both the dimer and the trimer bind to death receptor 5 with similar affinity. Interestingly, mutation of cysteine 230 to glycine completely abolished the apoptotic activity of TRAIL protein. The disruption of the dimer in the mixture of TRAIL dimer and trimer increased the apoptotic activity slightly, suggesting that the dimer has less apoptotic activity than the trimer. Therefore, our data indicate that cysteine 230 is not only required for TRAIL function but also modulates the apoptotic activity of TRAIL by forming an intermolecular disulfide bridge.

Introduction

Apoptosis is a genetically regulated biological process that plays an important role in the development and homeostasis of multicellular organisms (1–3). Thus, aberrations of this process can be detrimental to organisms. For example, excessive apoptosis causes damage to normal tissues in certain autoimmune disorders, whereas a failure of apoptosis allows cells to grow unlimitedly, resulting in cancers.

Members of the TNF family such as TNF-α, FasL, and TRAIL have been identified as apoptosis inducers. On binding to cognate receptors, TNF-α and FasL recruit cellular components including Fas-associated death domain-containing protein (4–6) and procaspase-8 to signal apoptotic activity (7–9). This initial signaling event is followed by serial activation of downstream executioner caspases leading to cleavage of cytosolic, cytoskeletal, and nuclear proteins as well as DNA, resulting in apoptotic cell death.

TRAIL, a recently identified TNF family member, is a type II transmembrane protein (10, 11) and a potent inducer of apoptosis. A recent study (12) on the crystal structure of TRAIL protein revealed that soluble TRAIL forms a homotrimer similar to other TNF family members. Currently, relatively little is known about the signaling events activated by TRAIL, including the adaptor molecule(s) and initial caspase(s). Four different TRAIL-Rs have been identified thus far: (a) DR4/TRAIL-R1 (13); (b) DR5/TRAIL-R2 (14–16); and (c) two DcRs [DcR1 and DcR2 (13, 15–17)]. DR4 and DR5 are intact functional TRAIL-Rs through which the apoptosis-inducing activity of TRAIL is transmitted into the cytoplasm. DcR1 and DcR2 are truncated TRAIL-Rs in which the cytoplasmic regions containing the death domains are deleted. Thus, overexpression of DcR1 and DcR2 blocks the function of DR4 and DR5 (13, 15–17), probably by competing with DR4 or DR5 for TRAIL.

Although TRAIL is a TNF family member (10, 11), it has some notable differences compared with TNF-α and FasL. First, TRAIL exhibits much stronger apoptotic activity than other TNF family members and is able to kill many cell types without potentiating agents such as actinomycin D or cycloheximide (10, 18, 19). Second, TRAIL kills tumor cells more effectively than normal cells by virtue of differential expression of DcRs and DR4 and DR5 (14, 15, 17). TRAIL has also been shown to kill HIV-1-infected T cells more effectively than uninfected T cells (20). Third, unlike the restricted expression of Fas, TRAIL-Rs are constitutively expressed in almost all tissues (10, 11). Therefore, it seems likely that TRAIL may have broader cell and tissue targets than the Fas-FasL system. Fourth, TRAIL activates nuclear factor κB only very weakly, suggesting that TRAIL is unlikely to initiate inflammatory cascades on systemic administration. Thus, unlike FasL or agonistic Fas antibody that induces fulminant massive liver damage (21, 22) when introduced systemically, TRAIL exhibited no detectable cytotoxicity in vivo (19, 23). Finally, TRAIL-induced apoptosis does not depend on p53 status, which is considered to be a critical factor in cancer therapies using chemotherapeutic agents or radiation. These features have focused considerable attention on TRAIL as a potential therapeutic to treat human cancers and AIDS.

Despite the considerable roles of TRAIL in cellular physiology, little is known about the functional domains and functionally critical amino acid residues of TRAIL protein. Here we report that cysteine 230 is a required amino acid residue for apoptotic function of the TRAIL protein.

Materials and Methods

Production of TRAIL Protein. Previously, we described the purification of human TRAIL protein [amino acids 114–281 (18)]. TRAIL (C230G) protein in which cysteine 230 was mutated to glycine was purified in essentially the same manner as described for TRAIL protein (18). To disrupt dimeric TRAIL, purified TRAIL protein was incubated with DTT (10 mM, final concentration) for 3 h at 4°C and dialyzed against a dialysis buffer [10 mM Tris-CI (pH 7.5), 20 mM NaCl, and 10 mM ZnCl2].

Western Blotting. The samples were fractionated on 15% SDS gels, transferred onto nitrocellulose membrane, and subjected to Western blotting using an antihistidine antibody (Santa Cruz Biotechnology) as described previously (18).

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3 The abbreviations used are: TNF, tumor necrosis factor; FasL, Fas ligand; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL receptor; DR, death receptor; DcR, decoy receptor.
Pull-down Assay. Ten μl of agarose beads (protein A+G; Santa Cruz Biotechnology) were preincubated with buffer A (PBS and 100 μg/ml BSA) for 30 min at room temperature. The combination of purified TRAIL protein (2 μg), purified DR5-Fc protein (2 μg; Alexis, CA) and purified monoclonal anti-FLAG-tag antibody (2 μg; Stratagene) was added to the preincubated agarose beads and incubated for 1 additional h at room temperature. The agarose beads were collected and washed six times at room temperature with buffer B [25 mM HEPES, 0.1% NP40, 100 mM NaCl, 1 mM EDTA, 5 mM MgCl2, 0.1 mM DTT, 100 μg/ml phenylmethylsulfonyl fluoride, 2 μg/ml protease inhibitor mixture, and 5% glycerol (adjusted to pH 7.4)]. The input (10% of total protein used in each reaction) and bound proteins were resolved on a 15% SDS gel and subjected to Western blotting using antihistidine antibody. Arrows and arrowheads, dimeric and monomeric forms of TRAIL protein, respectively.

Site-directed Mutagenesis. To mutate cysteine 230 to glycine in the reported TRAIL expression plasmid (18), the QuikChange site-directed mutagenesis kit (Stratagene) was used. All mutagenesis procedures were performed as described in the manufacturer’s instructions.

Apoptosis Assays. HeLa cells maintained in F-12K culture medium supplemented with 10% (v/v) calf serum and antibiotics (100 μg/ml gentamicin and 100 μg/ml penicillin-streptomycin) were incubated with purified TRAIL or TRAIL (C230G) protein for 6 h. Viable cells were stained with crystal violet followed by spectrophotometric analysis as described previously (18).

Results and Discussion

Identification of Dimeric TRAIL Protein. Biologically active soluble human TRAIL protein (amino acids 114–281) is known to form a homotrimer (12, 23). Previously, we purified recombinant human TRAIL protein (amino acids 114–281) from bacteria and showed that this TRAIL protein is a potent inducer of apoptosis (18). Biochemical analysis revealed that the major form of this recombinant TRAIL protein is trimer (data not shown). Nevertheless, this TRAIL protein generated two bands (M, 21,000 and M, 42,000) on nonreducing SDS gels (Fig. 1). Because the estimated molecular weight of amino acids 114–281 is M, 21,000, the appearance of a M, 42,000 band suggested the formation of a dimer. The M, 42,000 band disappeared after treatment with DTT, a reducing agent. However, boiling had no effect. These observations suggested that the dimeric TRAIL may be generated by an intermolecular chemical bond formation.

Binding of Dimeric TRAIL Protein to Its Cognate Receptor. To examine whether the dimer form of TRAIL protein binds to TRAIL-Rs, TRAIL protein was incubated with TRAIL-R DR5 or a negative control antibody (FLAG-Ab) and pulled-down with protein (A+G)-agarose bead. As shown in Fig. 2, the monomer TRAIL protein (this monomer resulted from trimer disruption by SDS) efficiently bound to DR5 (Lanes 1 and 2) but did not bind to control FLAG-Ab (Lanes 1 and 2). The dimer TRAIL protein also bound to DR5 (Lanes 1 and 2). The affinity of trimer and dimer TRAIL proteins to DR5 was similar (Lanes 1 and 2).
Cysteine 230 Is Required for Both Dimer Formation and Apoptotic Function of TRAIL. The capacity of the reducing agent DTT to abolish the dimer form of TRAIL (Fig. 1) suggested that an intermolecular disulfide bridge might account for dimer formation. Inspection of the amino acid sequence of human TRAIL protein identified a unique cysteine residue at position 230 (Fig. 3A). This cysteine residue was conserved in mouse TRAIL protein (10), pointing to a key functional role for this amino acid residue in the biological function of TRAIL protein. To examine the contribution of this cysteine residue, the cysteine was mutated to glycine (Fig. 3A), and TRAIL (C230G) protein was purified (Fig. 3B). The mutant TRAIL (C230G) protein appeared only as a monomer on SDS gel (Fig. 3B), indicating that cysteine 230 is responsible for forming dimeric TRAIL protein.

Next, to examine the role of cysteine 230 in the apoptotic function of TRAIL, HeLa cells were treated with TRAIL or mutant TRAIL (C230G) protein. Interestingly, TRAIL (C230G) protein exhibited no apoptotic activity in a time frame in which wild-type TRAIL induced massive apoptosis (Fig. 3C). The crystal structure of TRAIL protein revealed that cysteine 230 is positioned at the upper portion of the TRAIL trimer, which may form a receptor contact surface (12). If cysteine 230 is involved in ligand-receptor interaction, mutation of cysteine 230 to glycine may impair the binding of TRAIL protein to receptor. Another possibility is that cysteine 230 may function to promote intermolecular interactions for trimer formation because cysteine 230 appears to face the interface of trimeric TRAIL protein (12). Failure of TRAIL protein to form a correct trimer may result in loss of apoptotic activity.

TRAIL Dimer Has Decreased Apoptotic Activity. To assess the functional role of dimeric TRAIL, the TRAIL protein was treated with DTT to disrupt the dimer form of TRAIL. The DTT was then dialyzed off to allow the protein to form a trimer. This conversion process slightly increased the apoptotic activity of TRAIL protein (Fig. 4), indicating that cysteine 230 affects the apoptotic activity of TRAIL protein through oxidation-reduction and that oxidized dimeric TRAIL protein has lower (or null) apoptotic activity than the trimeric TRAIL protein.

Here, we demonstrate that cysteine 230 is required for the apoptotic function of TRAIL protein. Further characterization of the functional role(s) of cysteine 230 and the consequences of modifications of this residue in tumorigenesis and other disease models is required.

Note Added in Proof

When this manuscript was under review, studies describing a functional role of cysteine 230 were published (24, 25). Hymowitz et al. (24, 25) have demonstrated that this cysteine is required for trimer formation of TRAIL protein.

References

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