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A second tumor necrosis factor receptor gene product can shed a naturally occurring tumor necrosis factor inhibitor

(phorbol 12-myristate 13-acetate/receptor shedding/cDNA cloning/gene expression/lymphotoxin)

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ABSTRACT **An inhibitor of tumor necrosis factor (TNF) has been isolated from the human histiocytic lymphoma cell line U-937 that is capable of inhibiting both TNF-α and TNF-β. Protein sequencing has verified that it is distinct from a previously described TNF inhibitor that is a soluble fragment of a TNF receptor molecule (TNFrI). The cDNA sequence of this second TNF inhibitor clone suggests that it is also a soluble fragment of a TNF receptor. Expression of this cDNA sequence in COS-7 cells verified that it encodes a receptor for TNF-α (TNFrII) that can give rise to a soluble inhibitor of TNF-α, presumably through proteolytic cleavage. The extracellular domain of TNFrII has significant homology with that of TNFrI and these two receptors share a striking conservation of cysteine residue alignment with the extracellular domain of the nerve growth factor receptor. These three receptor molecules are therefore members of a family of polypeptide hormone receptors.**

Tumor necrosis factor α (TNF-α) is a cytokine that is released, primarily by monocytes/macrophages in response to infection or injury. It is one of the principal mediators of the inflammatory process in infected or injured animals (1). Excess TNF-α can have profoundly deleterious effects on animals, for example in septic shock (2). The ways in which TNF-α action is controlled in animals are of great interest because medical intervention in situations where TNF-α is overproduced could be of benefit to affected individuals. Several recent reports have described how TNF-α action may be controlled by an inhibitory protein that can bind and prevent receptor binding of the cytokine (3–7).

The previously described TNF-α inhibitor (molecular mass = 30 kDa) can be found in the culture medium of the human histiocytic lymphoma cell line U-937 when treated with phytohemagglutinin P (PHA) and the phorbol ester PMA (phorbol 12-myristate 13-acetate). The cDNA sequence (refs. 8 and 9 and our unpublished results) suggested that the inhibitor is a soluble form of the extracellular domain of a membrane-associated TNF-α receptor (which we call TNFrI). The implication of this finding is that TNF-α action is controlled in two ways: by the presence of the soluble inhibitor and by desensitization of cells through a loss of their cell-surface receptors.

In the course of those studies, we detected a second TNF-α inhibitor of 40 kDa in U-937 cell culture medium. We describe here the purification, cloning, sequencing,‡ and expression of this second human TNF-α inhibitor and show that it is a soluble fragment of a second TNF-α receptor expressed in U-937 cells. We designate this second TNF receptor TNFrII, and it appears to be related to TNFrI. Following the initial submission of this work, Smith *et al.* (10) reported the characteristics of this second TNF-α receptor.

MATERIALS AND METHODS

Cells. Both U-937 cells (ATTC CRL 1593) and NCTC clone 929 of mouse L cells (L929; ATCC CCL 1) were grown in RMPI 1640 medium supplemented with 10% (vol/vol) fetal calf serum. COS-7 cells (ATCC CRL 1651) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum.

Cytokines. Recombinant human TNF-α (2×10^7 units/ml) and human TNF-β (2×10^7 units/ml) were purchased from Amgen Biologicals and Endogen (Boston), respectively.

TNF Inhibitor Assay. TNF inhibitor activity was measured in an assay of cytotoxicity using the TNF-susceptible cell line L929 as described (6).

Binding Assay for ^{125}I-Labeled TNF-α (^{125}I-TNF-α). ^{125}I-TNF-α was purchased from Amersham (specific activity, 600–1000 Ci/mmol; 1 Ci = 37 GBq). Aliquots of 3×10^5 COS-7 cells were incubated at 4°C for 2–4 hr in a final 100-μl volume of culture medium containing 0.5 ng of ^{125}I-TNF-α. Assays were performed as described (11).

Preparation of the 40-kDa TNF Inhibitor. U-937 cells were seeded at a cell density of 10^5 cells per ml in 20 liters of medium. When the culture reached a density of 10^6 cells per ml, the cultures were harvested by centrifugation at $1500 \times g$ for 7 min and resuspended with serum-free RPMI 1640 medium at a cell density of 2×10^6 per ml. Cells were grown in the presence of 5 μg of PHA and 10 ng of PMA per ml. After 20–24 hr, cells were removed by centrifugation at $1500 \times g$ for 7 min, and 2 mM phenylmethylsulfonyl fluoride and 0.1% sodium azide were added to the supernatant. This supernatant was concentrated by using an Amicon spiral cartridge (S1Y10) to about 100 ml and was used for subsequent purification and characterization of TNF inhibitors.

Purification of the 40-kDa TNF Inhibitor. The concentrated U-937 culture medium above was applied at a flow rate of 0.1 ml/min to an Affi-Gel 1-ml column (Bio-Rad) to which 13 mg of recombinant human TNF-α was coupled. The column was washed with 50 mM Tris·HCl (pH 7.5), and the bound proteins were eluted with 50 mM sodium phosphate adjusted with HCl to pH 2.5. The eluate from this column was applied to a reverse-phase HPLC C_8 column (2×200 mm; Supelco)

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Abbreviations: TNF-β, tumor necrosis factor β/lymphotoxin; TNF-α, tumor necrosis factor α; PMA, phorbol 12-myristate 13-acetate; PHA, phytohemagglutinin P; TNFrI, TNF receptor I; TNFrII, TNF receptor II; L929, NCTC clone 929 of mouse L cells; NGFr, nerve growth factor receptor; PCR, polymerase chain reaction.

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‡The sequence reported in this paper has been deposited in the GenBank data base (accession no. M38549).

equilibrated with 0.1% trifluoroacetic acid. Bound proteins were eluted at a flow rate of 1 ml/min with a 0–60% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Fractions of 1.0 ml were collected and assayed. Protein concentrations were determined as described (12), and SDS/PAGE and silver staining were done as described (13, 14).

Isolation and Sequencing of Proteolytic Fragments. About 100 μg of reduced and carboxymethylated 40-kDa TNF inhibitor (performed as described in ref. 15) was dissolved at 2 mg/ml in 0.2 M ammonium bicarbonate/1 M guanidine hydrochloride. To this was added either endoproteinase Glu-C (protease V8; 1:50, wt/wt) or Arg-C (1:50, wt/wt) (Boehringer Mannheim). After 24 hr of incubation at 25°C, 1% trifluoroacetic acid was added and filtered through a 0.2-μm filter. The clarified peptide mixtures were separated on an Aquapore RP-300 column (Applied Biosystems; microbore HPLC) by using a linear gradient of acetonitrile in 0.1% trifluoroacetic acid at a flow rate of 0.1 ml/min. Six and seven peptides were purified from endoproteinases Glu-C- and Arg-C-digested mixtures, respectively, and 10–100 pmol of each were sequenced by using an Applied Biosystems sequencer (model 447A sequencer/120A analyzer).

cDNA Cloning and Polymerase Chain Reaction (PCR). Total cellular RNA was prepared from U-937 cells (16) that had been exposed to PHA/PMA for 9 hr. mRNA was purified by oligo(dT) chromatography (17), and 5 μg was used to synthesize cDNA (18); 100 ng of this cDNA was used as template for PCR (19) primed by two degenerate hexanucleotide probes, probe 1 (sense, Ala-3 through Thr-8) and probe 7 (antisense, Pro-100 through Ala-105). The 339-base-pair (bp) product of this reaction hybridized with a third degenerate hexanucleotide, probe 6 (Asp-25 through Cys-31). This DNA fragment was subcloned into M13mp19, and the sequence was determined by using the chain-termination method (20). A cDNA library was constructed in phage λgt10 (21) with the cDNA described, and 1.2×10^6 members of the U-937 cDNA library were plated at a density of 50,000 clones per plate. These clones were screened (22) with a ^{32}P-labeled oligonucleotide probe complementary to base pairs 321–347 of the cDNA sequence (Fig. 3). Positive clones were confirmed by hybridizing to ^{32}P-labeled probes 6 and 7. One clone was sequenced entirely on both strands (20).

Expression Vectors and Transfections. Expression vectors were made by using the plasmid pCMVβXVPL, which contains the simian virus 40 (SV40) origin of replication, the cytomegalovirus immediate early promoter and enhancer, the rabbit β-globin second intron, and the SV40 early polydenylylation signal. The entire 2.4-kilobase (kb) *Eco*RI insert from the phage λgt10 clone was inserted into the polylinker of this vector. pTNFrIIXVA and pTNFrIIXVB contain the cDNA in the sense and antisense orientations, respectively. pTNFrIISVstop is like TNFrIIXVA except that cDNA sequences 3′ of Asp-235 are deleted and a stop codon is inserted immediately after that codon. Analogous plasmids containing

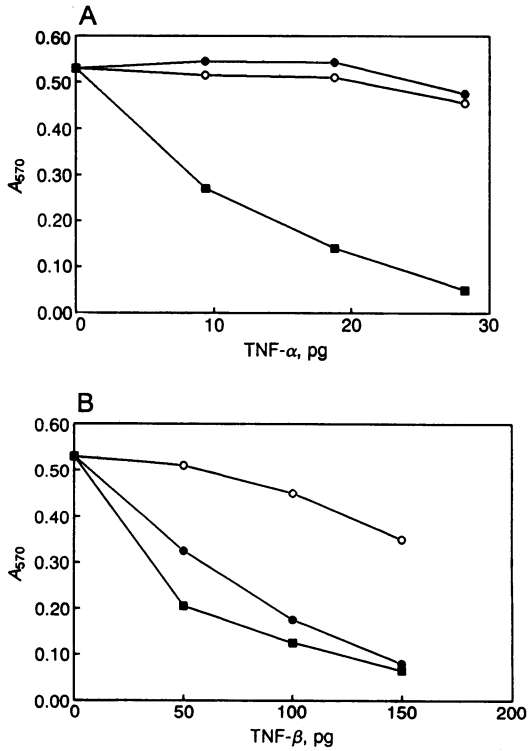

FIG. 2. Activity of the two TNF inhibitors against TNF-α and TNF-β in the L929 cytotoxicity assay. Ten nanograms of the 30-kDa TNF inhibitor or 10 ng of the 40-kDa TNF inhibitor was incubated with various concentrations of recombinant human TNF-α (*A*) or human TNF-β (*B*) for 1 hr at room temperature, and then the materials were tested on an L929 cytotoxicity assay system (6). Assays were done in the presence of the 30-kDa inhibitor (●), the 40-kDa inhibitor (○), or no inhibitor (■).

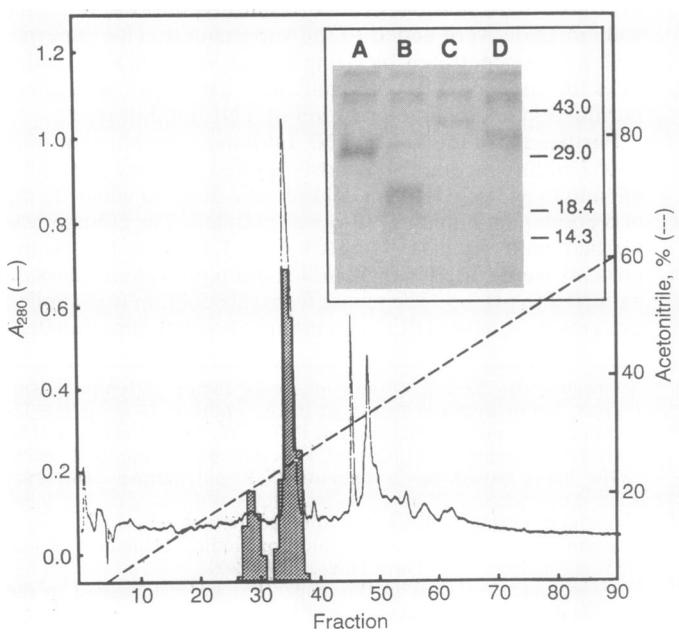

FIG. 1. Reverse-phase HPLC pattern of the U-937-derived TNF inhibitors. Twenty liters of U-937 culture medium was isolated and processed as described. Bioassays are indicated as shaded areas and were performed as described (6). (*Inset*) Molecular mass analysis of U-937-derived TNF inhibitors and their deglycosylated forms by SDS/PAGE. About 200 ng of fractions 28 and 35 was dried and treated with or without *N*-Glycanase. *N*-Glycanase treatment was carried out as described by the manufacturer (Genzyme). Lanes: A, fraction 28; B, fraction 28 treated with *N*-Glycanase; C, fraction 35; D, fraction 35 treated with *N*-Glycanase. Molecular mass markers (in kDa; BRL) were ovalbumin (43.0), carbonic anhydrase (29.0), α-lactoglobulin (18.4), and lysozyme (14.3).

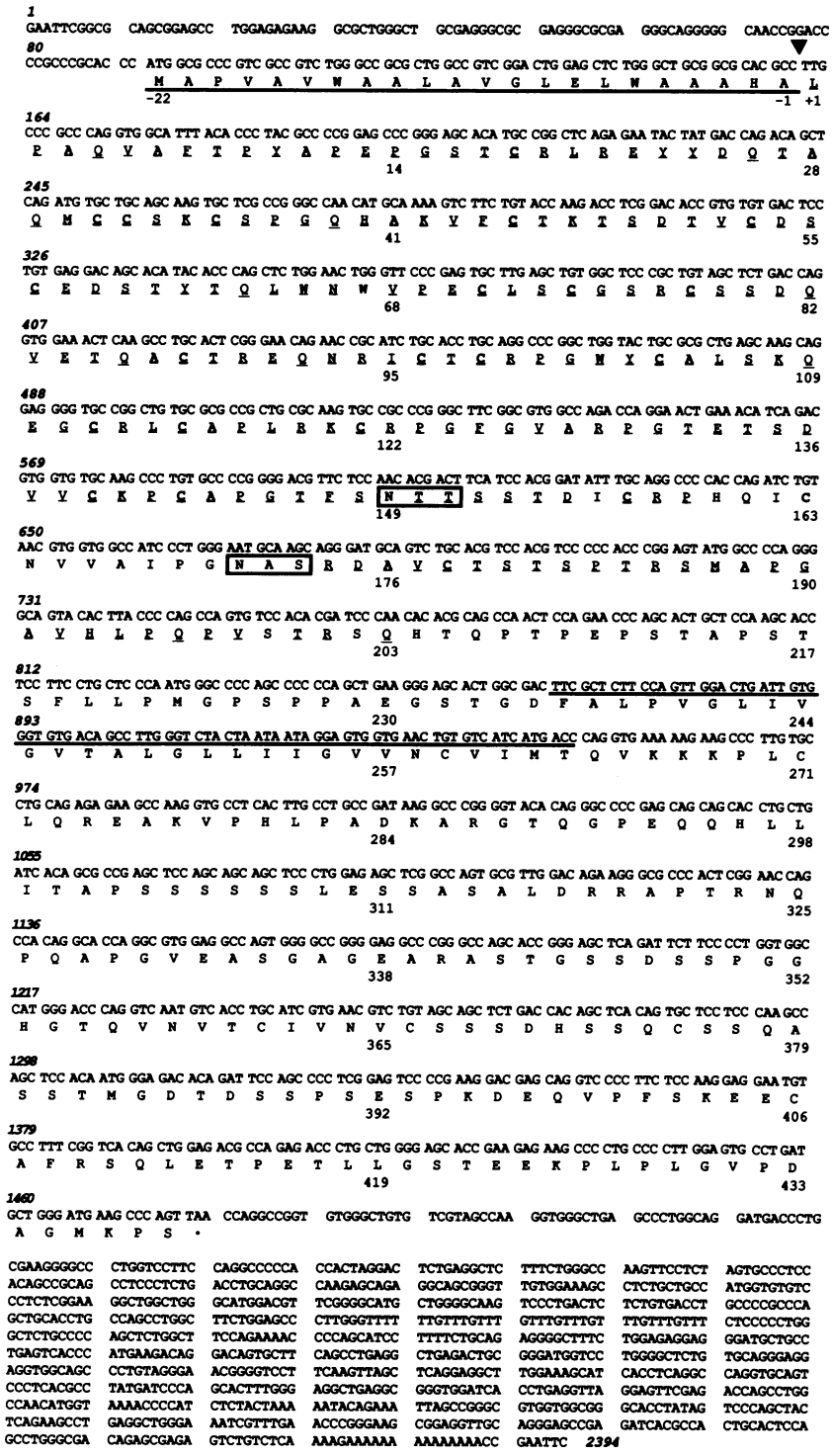

FIG. 3. DNA and deduced amino acid sequence of human TNFrII. DNA sequence is numbered in boldface above the sequence. The amino acid sequence is numbered below the sequence. Residues identified by amino acid sequencing are indicated by individual underlining; the putative signal peptide is indicated by solid continuous underlining; consensus glycosylation sites are boxed; cysteines are in bold print; and the putative transmembrane domain is overlined. The arrow between amino acids −1 and +1 represents the predicted signal peptidase cleavage site and the first identified amino acid in the purified U-937-derived protein.

the TNFrI cDNA are named pTNFrIXVA, pTNFrIXVB, and PTNFrIXVstop, respectively. These vectors were transfected into COS-7 cells by using the "lipofection" technique (23).

RESULTS

Isolating and Sequencing the Second TNF-α Inhibitor Protein. U-937 cell-conditioned medium was obtained and processed as described. Fig. 1 shows the A_{215} profile of a C_8 reverse-phase HPLC column and the ability of fractions from this column to counteract the cytotoxicity of TNF-α on murine L929 cells. Two peaks of activity were seen, one peak corresponds to the TNF-α inhibitor previously described (3–7). The second peak of activity migrates at ≈40 kDa on SDS/PAGE, distinctly different from the 30-kDa inhibitor described previously (both are shown in Fig. 1 *Inset*). Like the first inhibitor, the second protein is glycosylated, since

the enzyme *N*-Glycanase decreases its molecular weight by ≈4 kDa (Fig. 1 *Inset*). Both of these proteins inhibit TNF-α cytotoxicity on L929 cells. However, only the second inhibitor significantly inhibits the cytotoxicity of TNF-β on these cells (Fig. 2).

Amino acid sequencing showed that this 40-kDa TNF-α inhibitor is unrelated to the 30-kDa inhibitor. We purified 70 μg of the 40-kDa TNF inhibitor from 20 liters of induced U-937 cell-conditioned medium and determined the amino-terminal sequence of the purified protein as well as the amino acid sequence of a large number of peptides as described. We identified ≈180 amino acid residues, most of which were contiguous (Fig. 3) and are not found in the 30-kDa inhibitor characterized previously (3–7).

Isolating and Sequencing cDNA Encoding the 40-kDa Protein. cDNA from 9-hr treatment (PMA/PHA)-induced U-937 mRNA was used as a template in a PCR primed by the degenerate oligonucleotides corresponding to two regions of the TNF inhibitor protein sequence. The sequence of the PCR product was determined and shown to encode an open reading frame whose peptide sequence matched that of the 40-kDa TNF inhibitor (Fig. 3).

A λgt10 cDNA library (21) was screened, and DNA was prepared (24) from a clone hybridizing to three different oligonucleotide probes. This clone contained a single *Eco*RI fragment insert of 2.4 kb. The insert was cloned into M13mp19, and its sequence was determined from both strands (Fig. 3). Sequence analysis revealed a single long open reading frame beginning at base pair 93 and ending at base pair 1478, encoding a polypeptide of 48.3 kDa. The predicted structure of the protein encoded by the clone suggests that it is a second TNF receptor and that the protein we purified and sequenced is a soluble form of a portion of this receptor. The predicted structure includes: (*i*) a 22-amino acid hydrophobic region at the amino terminus that could act as a signal sequence; (*ii*) a large, cysteine-rich region of 235 amino acids that could be an extracellular domain containing a sequence identical with all of the peptide sequence obtained from the 40-kDa TNF inhibitor; (*iii*) a 28-amino acid hydrophobic sequence that could be a transmembrane domain; and (*iv*) 176 amino acids that could be a cytoplasmic domain. There are two consensus N-linked glycosylation sites located in the putative extracellular domain of this protein. One of these, Asn-149, is glycosylated in the inhibitor because it appears as a blank in the amino acid sequence analysis of peptides spanning this region. Following the hydrophobic transmembrane domain lie three consecutive lysine residues. Multiple basic residues are often found as the cytoplasmic extreme of receptors (25).

Northern blot analysis of mRNA isolated from U-937 cells revealed a single major band of 3.7 kb that hybridized to ^{32}P-labeled cDNA; this mRNA was found only in the mRNA from cells treated with PMA/PHA (data not shown). Although we isolated a total of 29 cDNA clones, we did not find any that contained inserts larger than 2.4 kb. Primer extension analysis indicated that our cDNA clones probably lack sequence in the 5′ end of the message (data not shown). However, this cDNA clone encodes what appears to be the entire open reading frame of this message (see Fig. 3).

The 2.4-kbp cDNA Encodes a Second TNF-α Receptor. We tested the hypothesis that this cDNA encodes a receptor for TNF-α by expressing it in COS-7 cells. Expression vectors were constructed with the plasmid pCMVβXVPL. The number of TNF-α-binding sites on those cells increased substantially when a vector (pTNFrIIXVA) containing the 2.4-kbp cDNA was transfected into COS-7 cells (Table 1). No increase in TNF-α binding was observed in cells when the cDNA was located in the expression vector in the antisense orientation (not shown). No TNF inhibitor was produced by cells transfected with pTNFrIIXVA. Because PMA/PHA is required to find the TNF inhibitors in the medium of U-937 cells (our unpublished results), the transfected COS-7 cells were treated with PMA/PHA. TNF inhibitory activity was only seen when these cells were treated under such conditions (Table 1). These results may be contrasted with the behavior of cells transfected with the gene for the TNFrI, which produce a TNF inhibitor even in the absence of PMA/PHA (Table 1).

Table 1. Expression of TNF-α receptor cDNA in COS-7 cells

Mammalian expression vectors	Cytotoxicity assay, % survival		Receptor-binding assay, cpm of ^{125}I-TNF-α bound specifically
	Without PMA/PHA	With PMA/PHA	
No TNF-α	100	—	—
TNF-α alone	32	—	—
Mock	35	31	477
TNFrIIXVA	32	48	4886
TNFrIIXVstop	49	—	—
TNFrIXVA	47	60	2246
TNFrIXVstop	59	—	—
U-937 cells	—	—	1154

COS-7 cells were transfected with the mammalian expression vectors listed. Cell culture medium from these cells (incubated for 48 hr in serum-free conditions) was assayed for the presence of an inhibitor of the TNF-α-induced cytotoxicity of L929 cells (6). The percentage of cells surviving after treatment with or without TNF-α (1 nM) in the presence of cell culture medium from cells transfected with these plasmids is shown. PMA was used at a concentration of 10 ng/ml, and PHA, at 5 μg/ml. Cells were grown in serum-containing medium for 2 days after transfection, and receptor binding assays were done as described. Specific binding of ^{125}I-TNF-α represents the number of cpm bound by 10^6 cells minus the cpm bound in the presence of 500-fold excess of TNF-α.

DISCUSSION

The cDNA sequence presented here encodes a second TNF receptor that is capable of generating a soluble TNF inhibitor. Structural features of the predicted amino acid sequence, such as a signal sequence and putative extracellular, transmembrane, and cytoplasmic domains provide evidence that this molecule is a receptor. Expression of this cDNA in COS-7 cells verifies that this cDNA clone encodes a receptor. The cDNA sequence of the extracellular domain of this cloned receptor matches all of the amino acid sequence obtained from the peptides of the purified 40-kDa inhibitor of TNF-α isolated from U-937 cells, indicating that this receptor molecule, like the one described previously (refs. 8 and 9; Table 1), is capable of generating a soluble inhibitor of TNF-α. Again, expression in COS-7 cells verifies this conclusion.

The 40-kDa TNF inhibitor that we isolated from U-937 cells is similar to the TNF-binding protein (termed TBPII) isolated from human urine by Englemann *et al.* (7). Their sequence indicates Val-Ala-Phe-Thr-Pro as the amino terminus of the protein. However, the U-937 protein has two additional amino acids and a molecular weight of ≈40 kDa, while the protein isolated by Englemann *et al.* (7) is only 30 kDa. The relationship between these two proteins needs clarification. The cDNA sequence of the extracellular domain of the receptor from which our protein is derived predicts that our inhibitor should have a molecular weight of ≈27 kDa. This is far different from the apparent size of the purified protein as determined by its migration on SDS/PAGE, even after *N*-Glycanase treatment. This discrepancy may be explained either by incomplete deglycosylation of the protein or by an eccentric tertiary structure in the presence of SDS and a reducing agent. Experiments with *in vitro*

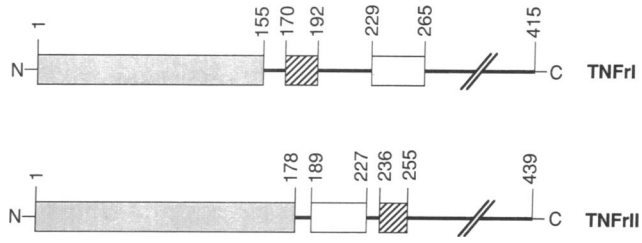

FIG. 4. Schematic representation of the TNFrI and TNFrII structures. Extracellular cysteine-rich domains are stippled; proline-rich regions are open boxes; and putative transmembrane domains are hatched.

transcription and translation of this cDNA suggest that the full-length receptor protein migrates at an aberrantly high apparent molecular mass on SDS/PAGE, which tends to support the latter explanation (data not shown).

Comparison of the amino acid sequence of TNFrI (refs. 8 and 9 and our unpublished results) with that of TNFrII reveals regions of homology. They both contain proline-rich regions. Amino acids 229–265 in TNFrI (8, 9) contain 9 proline residues, and amino acids 189–228 in TNFrII (Fig. 3) contain 11 proline residues, and 7 of these can be aligned by the method of Needleman and Wunsch (26). Ironically, these two proline-rich regions reside on opposite sides of the presumed transmembrane region of the two receptor molecules (Fig. 4). In addition, they both possess large regions in their extracellular domains that are rich in cysteine residues. TNFrI contains 24 cysteines in its first 155 amino acids and TNFrII has 22 cysteines in its first 178 amino acids. The cysteines in these two molecules can be aligned quite well with each other as well as with the nerve growth factor receptor (NGFr) (25). Fig. 5 shows this alignment schematically along with the repeated domain structure first described for the NGFr (25). This domain structure is also evident in the two TNF receptors, occurring three times in TNFrI and twice in TNFrII.

The cDNA described here, in conjunction with the cDNA for TNFrI (our unpublished results), indicates that U-937 cells are capable of expressing at least two distinct TNF-α receptors. This apparently conflicts with both the Scatchard analysis findings of Stauber *et al.* (11) and immunoreactivity findings of Wallach and his coworkers (7) that there is a single TNF receptor species present on U-937 cells.

The phenomenon of receptor shedding (i.e., loss of ligand binding sites on cell surfaces and appearance of extracellular domains of receptors in culture medium) is becoming a common theme. Many receptors such as human growth hormone receptor (27), the NGFr (28), the Mel^{14} gene product in neutrophils (29), and the two TNF receptors (refs. 8 and 9; Fig. 1) undergo this process. Receptor shedding appears to depend on proteolysis, but whether a common proteolytic process is involved in the shedding of these different receptor fragments remains to be seen. Receptor shedding could regulate hormone action in two ways: (*i*) the release of the extracellular domains of receptors should desensitize cells to particular ligands, and (*ii*) the soluble receptor can bind and inhibit the action of the ligand. Regardless of the physiological significance of receptor shedding, naturally occurring receptor fragments may provide the basis for a rational therapeutic approach to preventing disorders where hormones are present at excessively high levels—for example, the toxic levels of TNF-α that lead to septic shock.

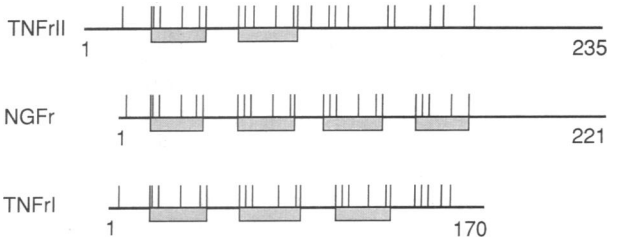

FIG. 5. Schematic representation of the extracellular domains of TNFrII, NGFr, and TNFrI. The locations of cysteines are indicated by vertical lines. Stippled boxes below the line represent the multiple domain nature of the extracellular regions of these three receptors accented by the recurring pattern of cysteine residues.

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