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Immunology

Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein

(cytokine/soluble receptor/receptor family/lymphotoxin)

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ABSTRACT **The cDNA for one of the receptors for human tumor necrosis factor (TNF) has been isolated. This cDNA encodes a protein of 455 amino acids that is divided into an extracellular domain of 171 residues and a cytoplasmic domain of 221 residues. The extracellular domain has been engineered for expression in mammalian cells, and this recombinant derivative binds TNFα with high affinity and inhibits its cytotoxic activity *in vitro*. The TNF receptor exhibits similarity with a family of cell surface proteins that includes the nerve growth factor receptor, the human B-cell surface antigen CD40, and the rat T-cell surface antigen OX40. The TNF receptor contains four cysteine-rich subdomains in the extracellular portion. Mammalian cells transfected with the entire TNF receptor cDNA bind radiolabeled TNFα with an affinity of 2.5×10^{-9} M. This binding can be competitively inhibited with unlabeled TNFα or lymphotoxin (TNFβ).**

Tumor necrosis factor α (TNFα) is a potent cytokine that elicits a broad spectrum of biological responses. TNFα causes the cytolysis or cytostasis of many tumor cell lines *in vitro*, induces the hemorrhagic necrosis of transplanted tumors in mice, enhances the phagocytosis and cytotoxicity of polymorphonuclear neutrophils, and modulates the expression of many proteins, including lipoprotein lipase, class I antigens of the major histocompatibility complex, and cytokines such as interleukin 1 and interleukin 6 (1–4). TNFα appears to be necessary for a normal immune response (5, 6), but large quantities produce dramatic pathogenic effects (7–9). TNFα has been termed "cachectin" since it is the predominant factor responsible for the wasting syndrome (cachexia) associated with neoplastic disease and parasitemia (1, 3). TNF is also a major contributor to toxicity in Gram-negative sepsis, since antibodies against TNF can protect infected animals (7, 10).

The many activities of TNFα are mediated by binding to a cell surface receptor. Radioligand binding studies have confirmed the presence of TNF receptors on a wide variety of cell types (11–14). Although these receptors are expressed in limited numbers (1000–10,000 per cell), they bind TNFα with high affinity ($K_a = 10^9$ M^{-1} at 4°C). The TNF receptor has been characterized as a 55- to 80-kDa glycoprotein that binds both TNFα and the structurally related lymphotoxin (TNFβ). Lymphotoxin has biological activities that are similar, if not identical, to those of TNFα, presumably because both are recognized by the same receptor (4). Recently, several laboratories have detected heterogeneity in TNF receptor preparations (15, 16) and have proposed that at least two distinct cell surface molecules bind TNFα. In addition, both of these receptors appear to be released from cells in soluble form, as TNF-binding proteins of 30 kDa have been isolated from urine and serum (16–18). This soluble extracellular domain retains the capacity to bind ligand with high affinity and therefore may be important in regulating concentrations of TNFα *in vivo*.

To further elaborate the structure of the TNF receptor, we have identified a cDNA for one of the receptor forms.* COS cells transfected with this cDNA bind TNFα with high affinity and this binding can be inhibited by unlabeled TNFα or lymphotoxin. A derivative of the TNF receptor, the extracellular domain, has also been expressed in COS cells. This results in the secretion of a soluble recombinant receptor domain with characteristics similar to those of the TNF-binding protein.

MATERIALS AND METHODS

Reagents. Recombinant human TNFα and TNFβ were generously supplied by Genentech as highly purified proteins derived from *Escherichia coli*. The specific activities of these preparations were approximately 10^7 units/mg, as measured in the murine L929 cell cytotoxicity assay. The synthetic oligonucleotides were prepared by Oswel DNA Service (University of Edinburgh).

Isolation of TNF Receptor cDNA Clones. Purification and partial amino acid sequence analysis of the TNF-binding protein have been described (16, 17). The sequence of a peptide fragment (Glu-Met-Gly-Gln-Val-Glu-Ile-Ser-Ser-Thr-Val-Asp-Arg-Asp-Thr-Val-Cys-Gly) was used to design a synthetic oligodeoxynucleotide probe (5′-AAG-GAG-ATG-GGC-CAG-GTT-GAG-ATC-TCT-TCT-ACT-GTT-GAC-AAT-GAC-ACT-GTG-TGT-GGC-3′). The 57-mer DNA probe was labeled with ^{32}P by T4 polynucleotide kinase (New England Biolabs) and used to screen a placental cDNA library in λgt10 (19, 20). Approximately 800,000 phage were transferred to nitrocellulose filters and screened at reduced stringency (21). Filters were incubated for 2 hr at 42°C in 0.05 M sodium phosphate, pH 6.5/20% formamide/0.75 M sodium chloride/0.075 M sodium citrate/1% polyvinylpyrrolidone (Sigma)/1% Ficoll/1% bovine serum albumin (Sigma), and sonicated salmon sperm DNA (Sigma) at 50 ng/ml. The radiolabeled probe was then added to the filters (10^6 cpm/ml), which were hybridized for 16 hr. Filters were washed extensively in 0.06 M sodium chloride/0.006 M sodium citrate/1% SDS at 37°C and positive clones were identified by autoradiography. Ten hybridizing clones were plaque-purified (19) and cDNA insert size was determined by polyacrylamide gel electrophoresis of *Eco*RI-digested phage DNA. The inserts of two cDNA clones were sequenced by the dideoxy chain-termination technique (22).

Southern and Northern Blot Analysis. DNA was isolated from human lymphocytes by the method of Blin and Stafford (23) and used for Southern blot analysis (24). DNA was

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Abbreviations: TNF, tumor necrosis factor; PCR, polymerase chain reaction.
*The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37764).

digested with restriction endonucleases (New England Biolabs), fractionated in a 1% agarose gel, and transferred to nitrocellulose. Hybridization and washing were conducted under stringent conditions (20) using a ^{32}P-labeled preparation of a 600-base-pair (bp) fragment of the TNF receptor cDNA. Northern blot analysis was performed (25) on oligo(dT)-selected RNA isolated from human placenta, spleen (generously provided by the Cooperative Human Tissue Network, Birmingham, AL), and fibroblast cell line 293. Following electrophoresis in a formaldehyde/1.2% agarose gel, the RNA was transferred to nitrocellulose and hybridized with the TNF receptor DNA probe under stringent conditions.

Mammalian Cell Expression of the Human TNF Receptor and Derivatives. The coding region of the majority of the human TNF receptor was isolated as an *Eco*RI fragment and cloned into a mammalian cell expression vector (26), resulting in plasmid prTNFR. The *Eco*RI fragment encodes 374 amino acids of the TNF receptor; 81 carboxyl-terminal residues of the cytoplasmic domain are therefore missing from this plasmid construction and 23 unrelated residues are added. A derivative of the TNF receptor was produced by engineering a termination codon just prior to the transmembrane domain (following Ile-159). The polymerase chain reaction (PCR) technique (27) was used to generate a 300-bp restriction fragment containing Bgl II site at the 5′ end and a *Hin*dIII site preceded by a TAG stop codon at the 3′ end. The PCR primers were 5′-GCTGCTCCAAATGCCGAAAG-3′ and 5′-AGTTCAAGCTTTTACAGTGCCCTTAACATTCTAA-3′. The PCR product was purified by gel electrophoresis and cloned into the TNF receptor expression plasmid (described above) digested with *Bgl* II and *Hin*dIII. DNA sequencing confirmed that the resulting plasmid (pTNFRecd) contained the designed DNA sequence.

The TNF receptor expression plasmids were transfected into monkey COS-7 cells by using Lipofectin (GIBCO/BRL) according to the manufacturer's instructions. Cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum.

Analysis of Recombinant TNF Receptor Derivatives. TNFα was radioiodinated with the Iodo-Gen method (Pierce) according to the manufacturer's directions. The specific activity of the ^{125}I-labeled TNFα was 10–30 μCi/μg (1 μCi = 37 kBq). COS cells transfected with the TNF receptor cDNA (prTNFR, 1300-bp *Eco*RI fragment) were incubated for 24 hr and then seeded into six-well tissue culture plates (Nunc) at 4.5×10^5 cells per well. The cells were incubated for a further 48 hr and then receptor expression was quantitated by radioligand binding for 2 hr at 4°C. Nonspecific binding of ^{125}I-TNFα was determined in the presence of a 1000-fold molar excess of unlabeled TNFα. Binding data were analyzed by the method of Scatchard (28).

The TNF receptor derivative was analyzed for inhibition of ^{125}I-TNFα binding to the natural receptor on human U-937 monocytic cells. Culture supernatant was harvested 72 hr after COS cells were transfected with pTNFRecd. U-937 cells (2×10^6 cells in 200 μl) were incubated with 1 nM ^{125}I-TNFα and dilutions of COS cell medium for 2 hr at 4°C. Cells were then centrifuged through 20% sucrose to remove unbound TNFα. Nonspecific binding was determined in the presence of 1 μM unlabeled TNFα.

The TNF receptor derivative was also analyzed for inhibition of TNFα cytotoxic effects *in vitro*. The cytotoxicity assay was performed as described on the TNF-sensitive cell line WEHI 164 clone 13 (29). Serial dilutions of culture supernatant from COS cells transfected with pTNFRecd or from mock-transfected controls were incubated with a constant amount of TNFα (1 ng/ml) for 1 hr at 37°C before addition to the assay.

RESULTS

Isolation and Characterization of the TNF Receptor cDNA. Partial amino acid sequence of the TNF-binding protein (16, 17) was used to design a synthetic oligonucleotide probe. The radiolabeled probe was used to screen a human placental cDNA library in λgt10, and 10 hybridizing phage were isolated. The nucleotide and deduced amino acid sequences of the longest cDNA clone are depicted in Fig. 1. The third potential ATG initiation codon occurs at position 156 of the nucleotide sequence; the first two ATG codons are closely followed by termination codons, and the third ATG is preceded by the best translation initiation consensus nucleotides (30). The cDNA encodes an open reading frame of 1365 bases that codes for a polypeptide of 455 residues. The peptide sequence determined by amino acid sequencing was identified in the encoded cDNA (18 of 19 matching residues). The amino-terminal end identified for the TNF-binding protein corresponds to the cDNA-encoded sequence (17 of 19 matching residues) beginning at residue 41. The first 35 amino acids are generally quite hydrophobic and probably represent a signal sequence. Residues 36–40 (−5 to −1 in Fig. 1) are highly charged (Asp-Arg-Glu-Lys-Arg) and such a sequence is not typically found in secretory signal sequences (31); perhaps the natural receptor is processed by proteolysis after residues 39 and 40, which comprise a dibasic cleavage site (Lys-Arg). Hydropathy analysis of the protein sequence predicts a signal transmembrane domain of 23 amino acids. This hydrophobic sequence divides the protein into an extracellular domain of 171 residues and a cytoplasmic domain of 221 residues. The amino acid composition determined for the TNF-binding protein (17) corresponds well with the predicted composition of the extracellular domain encoded by the cDNA (results not shown). The discrepancy between the predicted receptor size (49 kDa) and the size determined by SDS/polyacrylamide gel electrophoresis (55–60 kDa, refs. 12–14) is probably due to glycosylation; there are four potential N-linked glycosylation sites in the sequence, three of which are in the extracellular domain. The sequence contains a large number (i.e., 30) of cysteine residues, 24 of which are in the extracellular domain. The arrangement of these cysteines is similar to that of several other cell surface proteins (see *Discussion*), suggesting that the TNF receptor is structurally related to a family of receptors.

A Northern blot analysis is presented in Fig. 2*A*. The ^{32}P-labeled 600-bp cDNA fragment hybridized to a single predominant band of oligo(dT)-selected RNA from human placenta or spleen. A minor larger transcript was also observed in RNA from a fibroblast cell line. The size of the hybridizing species is 2400 bases, in good agreement with the size of isolated cDNA. Fig. 2*B* shows a Southern blot of human genomic DNA hybridized with the same 600-bp probe from the cDNA. In each of the three different restriction digests, only a single hybridization signal was observed. Thus the TNF receptor that we have isolated appears to be encoded by a single gene.

Expression of Recombinant TNF Receptor Sequences in Mammalian Cells. To confirm that the cDNA shown in Fig. 1 indeed encodes the TNF receptor, the cDNA was engineered for expression in mammalian cells. The cDNA contains an *Eco*RI site at position 1270 of Fig. 1. The receptor coding sequence was isolated as a 1300-bp *Eco*RI fragment (containing all but the last 81 codons of the cytoplasmic domain) and inserted into the mammalian cell expression vector containing a cytomegalovirus promoter and simian virus 40 transcription termination sequences (26). The resulting plasmid was transfected into COS cells, which were analyzed for TNF receptor expression after 3 days. As shown in Fig. 3*A*, the transfected cells specifically bound radioiodinated TNFα in a saturable and dose-dependent fashion. The

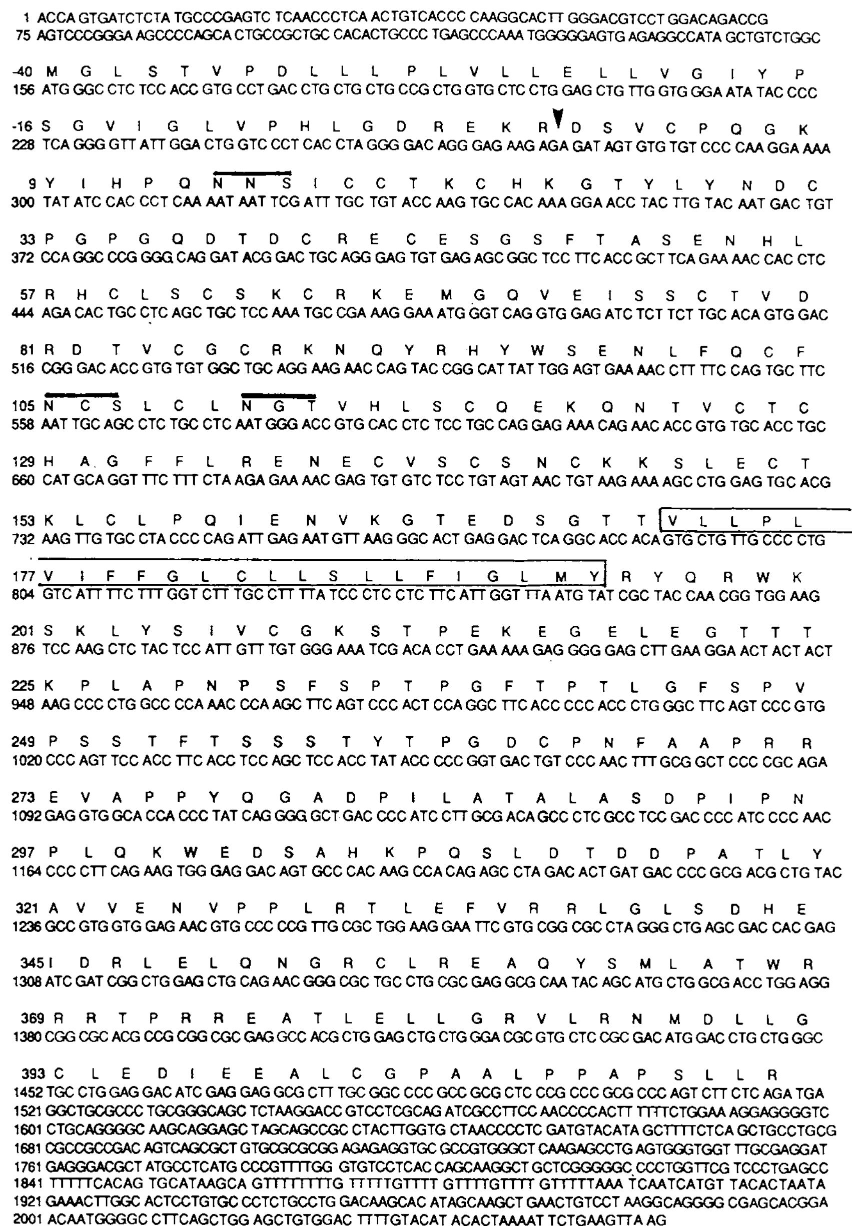

FIG. 1. Nucleotide sequence of the human TNF receptor cDNA and encoded amino acid sequence. The predicted signal sequence is numbered −40 to −1. The first residue of the TNF-binding protein (16, 17) is preceded by an arrowhead, the transmembrane domain is boxed, and potential N-linked glycosylation sites in the extracellular domain are overlined. The sequence homologous with the designed oligonucleotide probe is found at nucleotide positions 477–533.

population of COS cells expressed ≈1×10^5 receptors per cell. The measured binding affinity of recombinant receptors was 2.5×10^9 M^{-1} at 4°C, which is in close agreement with the value for the natural receptor on human cells (13, 14). The binding of ^{125}I-TNFα (1 nM) to these cells could be inhibited by the addition of unlabeled TNFα or lymphotoxin (Fig. 3*B*). COS cells transfected with just the expression vector did not significantly bind ^{125}I-TNFα (<2% of the binding seen with the cDNA transfection).

The extracellular domain of the TNF receptor is naturally shed from cells (16–18). To produce a similar recombinant derivative, at stop codon preceding the transmembrane domain was engineered into the cDNA by PCR mutagenesis. The modified DNA was inserted into the expression plasmid and subsequently transfected into COS cells. After 3 days, the COS cell medium was tested for inhibition of TNFα binding to human U-937 cells. As shown in Fig. 4*A*, the transfected-cell medium inhibited ≈70% of the binding of TNFα. The recombinant TNF receptor derivative was next tested for inhibition of TNFα biological activity. A sensitive bioassay for TNFα is measurement of cytolysis of mouse WEHI 164 (clone 13) cells. The transfected-cell medium inhibited 60% of TNFα cytotoxicity on this cell line (Fig. 4*B*). Medium from mock-transfected COS cells did not inhibit TNFα cytotoxicity or binding. These experiments demonstrate that the recombinant extracellular domain of the TNF receptor is capable of binding TNF and inhibiting its biological activity.

DISCUSSION

This paper describes the cDNA cloning of a human TNF receptor. The cDNA was isolated with a synthetic oligonucleotide probe based on partial amino acid sequence. The two peptide sequences determined for the purified TNF-binding protein correspond to sequences encoded in the cDNA. Confirmation that this cDNA encodes the TNF receptor was established by mammalian cell expression studies: this cDNA directs the expression of a cell surface protein that specifically binds TNFα with high affinity.

Southern hybridization suggests that the cDNA is encoded by a single gene. Several other laboratories have provided strong evidence for at least two structurally distinct human TNF receptors (15, 16). Consequently, other forms of the TNF receptor must differ significantly from that isolated here, since only single hybridization signals are observed in

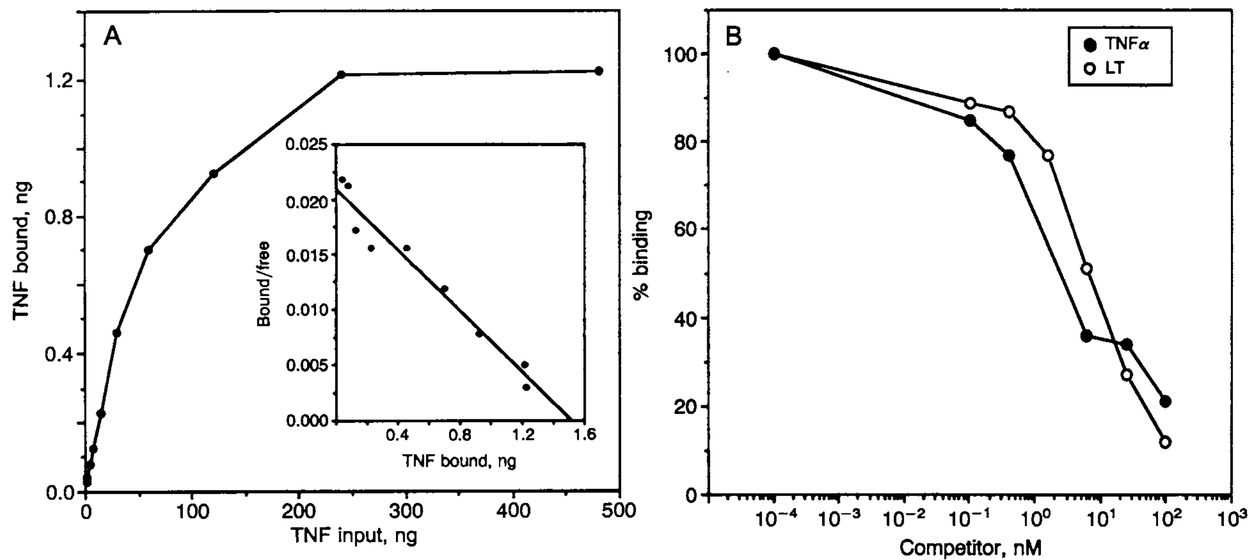

FIG. 3. Binding characteristics of recombinant human TNF receptor expressed in COS-7 cells. (*A*) Direct binding of recombinant ^{125}I-TNFα to COS-7 cells transfected with prTNFR. (*Inset*) Scatchard analysis derived from these data. (*B*) Competition analysis. Monolayers of COS-7 cells transfected with the TNF receptor cDNA were incubated with 1 nM ^{125}I-TNF in the presence of various concentrations of unlabeled TNFα (●) or TNFβ (lymphotoxin, LT) (○).

the Southern analysis (Fig. 2*B*). Similarly, the ligands for this receptor, TNFα and lymphotoxin, share 35% amino acid similarity but their coding sequences do not cross-hybridize (32).

The TNF receptor exhibits significant amino acid sequence homology to three other cell surface proteins: the low-affinity nerve growth factor receptor (28% similarity; ref. 33), human CD40 (a B-cell surface marker; 25% similarity, ref. 34), and rat OX40 (found on activated T cells that are CD4-positive; 24% identity; ref. 35). The similarity among these proteins is confined to the extracellular domain. As shown in Fig. 5, each of these contain four (three in OX40) cysteine-rich subdomains that probably evolved from a common motif. Consequently, these proteins are members of a family of cell surface molecules that are structurally related. The distinct functions of each of these molecules may be a result of their dissimilar cytoplasmic domains. The ligands for OX40 and CD40 have not been identified, but antibodies against them can augment T-cell and B-cell responses, respectively (34, 35). TNFα and nerve growth factor do not share sequence similarity, but both ligands affect the growth and differentiation of target cells. Perhaps this family of cell surface molecules has evolved to recognize structurally distinct ligands but retained a common scaffold that is generally useful for recognition of polypeptide ligands. While the dissimilar cytoplasmic domains suggest independent modes of signal transduction, each receptor appears to function in the growth or differentiation of the cell.

The natural production of a soluble receptor domain has been observed for other cytokine receptors, including those for interleukins 2, 4, and 6 and interferon γ (36). The formation of a soluble extracellular domain can arise by several mechanisms—for example, by degradation of the receptor (as is the case for the interleukin 2 receptor) or by synthesis of an independent transcript that does not encode a transmembrane domain [as seen for the interleukin 4 receptor (37) and the soluble or cell-bound forms of immu-

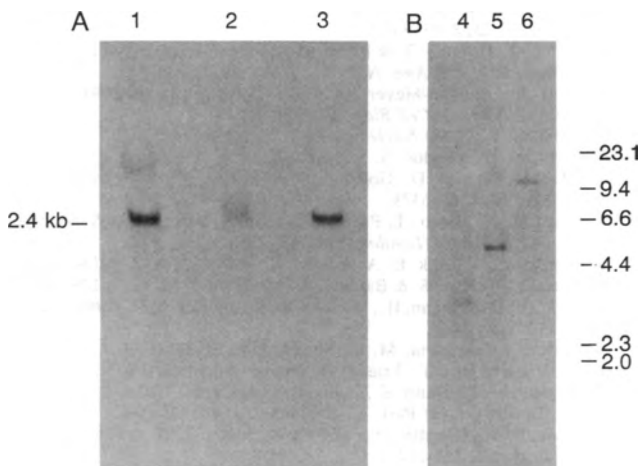

FIG. 2. (*A*) Northern blot of oligo(dT)-selected RNA (10 μg per lane) from human 293 cells (fibroblast cell line) (lane 1), placenta (lane 2), and spleen (lane 3) hybridized with the TNF receptor cDNA (*Sma* I–*Eco*RI fragment). kb, Kilobases. (*B*) Southern blot of human genomic DNA (5 μg per lane) digested with *Pst* I (lane 4), *Hin*dIII (lane 5), or *Eco*RI (lane 6) and hybridized with the same probe as used for the Northern blot.

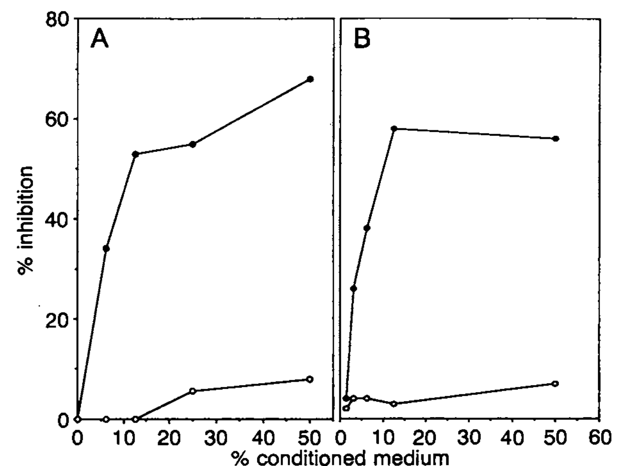

FIG. 4. Effects of soluble TNF receptor on TNF binding and biological activity. (*A*) Effect of culture supernatants from COS-7 cells transfected with a cDNA encoding a soluble form of the TNF receptor (pTNFRecd; ●) or mock-transfected (○) on ^{125}I-TNF binding to U-937 cells. (*B*) Effect of these supernatants on TNF-mediated killing of WEHI 164 (clone 13) line.

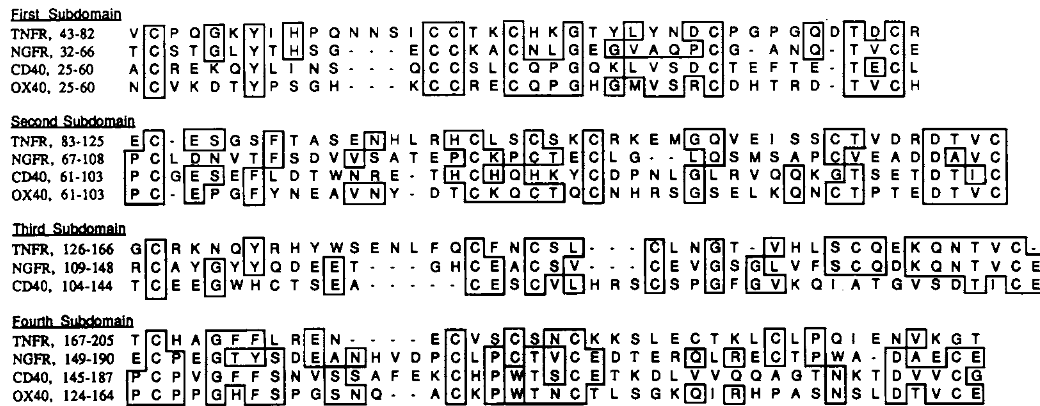

FIG. 5. Alignment of the cysteine-rich subdomains of the extracellular portions of the human TNF receptor (TNFR), rat nerve growth factor receptor (NGFR; ref. 33), human CD40 (34), and rat OX40 (35). Common residues are boxed. OX40 contains only three subdomains and lacks the third. Residue numbers refer to the precursor form and begin with the initiating methionine.

noglobulin]. We observed a single predominant signal in a Northern blot of human spleen and placenta mRNA, suggesting that one size of transcript produces this form of the TNF receptor. Consequently, the formation of the TNF-binding protein is most likely a result of proteolytic cleavage from the membrane-bound receptor. Presumably the extracellular domain is cleaved by proteolysis near the transmembrane junction, resulting in the release of a soluble receptor fragment.

By introducing a termination codon prior to the transmembrane domain, we have expressed a soluble form of the extracellular domain. This recombinant product mimics the natural TNF-binding protein in its sequence, amino acid composition, and ability to inhibit TNF biological activity (16, 17). The natural TNF-binding protein may play an important role in the regulation of TNF-mediated responses by binding and sequestering the cytokine. The recombinant extracellular domain may similarly provide therapeutic benefit in disorders such as cachexia, sepsis, and autoimmune diseases where TNF has been shown to play a significant causative role, such as rheumatoid arthritis (38). Attempts to demonstrate the therapeutic potential of the recombinant soluble TNF receptor can now be instigated.

Note. After submission of this manuscript for review, two papers appeared (39, 40) describing the sequence of the human TNF receptor cDNA, which is identical to that reported here.

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