immunodominant region of the autoantigen MBP. This may provide insight into the molecular mechanisms of MS and help in the design of new specific therapeutic approaches.


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5. Sequences of MBP peptides: MBP(84–102): DENPVVHFFKNIVTPRTPP MBP(143–168): FKGVDAQGTLSKIFKLGGRD. Single-letter abbreviations for the amino acid residues are: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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11. Myelin basic protein–specific T cell lines were grown from peripheral blood mononuclear cells at 200,000 cells per well in the presence of human MBP (10 μg/ml). Under these conditions 1 to 20% of the wells were positive for MBP; therefore, most lines are likely to have been generated from a single MBP-reactive T cell. Cells were stimulated two times with MBP and tested for their peptide specificity by use of a panel of 13 overlapping synthetic MBP peptides. All cell lines analyzed reacted specifically with one of the 13 synthetic MBP peptides (*4*). After a third stimulation with the specific MBP peptide, RNA was extracted from cell culture pellets (20,000 to 50,000 cells) by extraction with guanidinium isothiocyanate/phenol chloroform and isopropranol precipitation in the presence of carrier tRNA. Single-stranded cDNAs were synthesized with oligo-dT and avian myeloblastosis virus reverse transcriptase. PCR amplification was done with a panel of 19 oligonucleotides corresponding to the CDR2 region of the TCR β chain and a $C_\beta$ primer. Amplifications were done for 30 cycles (94°C, 1 min; 55°C, 2 min; and 72°C, 3 min) with 1 μg of each primer in 50-μl reactions. Amplified products were separated in 1% agarose gels, transferred to nitrocellulose, and hybridized with an internal oligonucleotide probe. Probes were end-labeled with [γ-$^{32}$P]ATP (adenosine triphosphate) and T4 polynucleotide kinase to a specific activity of $10^8$ cpm/μg and hybridized. Blots were washed at a final stringency of 6× SSC (saline sodium citrate) at 70°C and autoradiographed for 2 to 18 hours. T cell lines that were positive for more than two $V_\beta$ segments were considered not to be derived from a single MBP-reactive T cell and were therefore excluded from analysis. For sequencing, amplification was performed with a $V_\beta$17 primer specific for the leader segment, which contained an internal Pst I restriction site. Amplified DNA was treated with proteinase K, extracted with phenol chloroform, precipitated with ethanol, and digested with restriction endonucleases Bgl II and Pst I. Gel-purified DNA was ligated into M13mp19, and single-stranded DNA was sequenced by the dideoxy method. Negative controls were included during the procedure to test for possible contamination of RNA samples or reagents used for cDNA synthesis and amplification.
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# A Receptor for Tumor Necrosis Factor Defines an Unusual Family of Cellular and Viral Proteins


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**Tumor necrosis factor α and β (TNF-α and TNF-β) bind surface receptors on a variety of cell types to mediate a wide range of immunological responses, inflammatory reactions, and anti-tumor effects. A cDNA clone encoding an integral membrane protein of 461 amino acids was isolated from a human lung fibroblast library by direct expression screening with radiolabeled TNF-α. The encoded receptor was also able to bind TNF-β. The predicted cysteine-rich extracellular domain has extensive sequence similarity with five proteins, including nerve growth factor receptor and a transcriptionally active open reading frame from Shope fibroma virus, and thus defines a family of receptors.**


TUMOR NECROSIS FACTOR α (TNF-α, cachectin) and β (TNF-β, lymphotoxin) are structurally and functionally homologous proteins secreted by activated macrophages and lymphocytes, respectively (*1*). These cytokines have pleiotropic activities in vitro and in vivo, including cytotoxic effects against tumors and virus-infected cells, stimulation of interleukin-1 secretion, stimulation of prostaglandin E2 and collagen production, inhibition of lipogenic gene expression in adipocytes, and stimulation of various immune effector cells (*2*). Clinical interest has focused on TNF because it appears to be a common mediator of inflammation, endotoxin-induced shock (*1*), and the wasting syndrome commonly observed in chronic infections and neoplastic disease (*3*). TNF receptors appear on virtually all somatic cells (*1*), and generally the ligands cross-compete for binding (*4*), suggesting they share a common receptor. As an aid to studying the TNF system in molecular detail, we isolated a cDNA clone of the receptor.

The SV40-transformed human lung fibroblast cell line WI26-VA4 was used as a source of mRNA for construction of a cDNA library. This cell line binds both TNF-α and -β and displays multiple affinity classes; approximately 23,000 binding sites per cell (*N*) were detected with $^{125}$I–TNF-α that could be fit to two affinity classes, low ($K_{a1}$ = 0.16 ± 0.10 $nM^{-1}$, $N_1$ = 19,700 ±


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4,800) and high ($K_{a2}$ = 6.2 ± 3.9 $nM^{-1}$, $N_2$ = 3,000 ± 1,400) (Fig. 1A). TNF-β binds with lower affinity than TNF-α and the ligands cross-compete for binding (Fig. 1B). Double-stranded cDNA was synthesized by standard procedures, inserted into the mammalian expression vector pDC302 (*5*) and a TNF receptor clone isolated by a direct expression approach. Plasmid DNA from about 1000 *Escherichia coli* (DH5α) transformants were pooled, transfected into COS cells, and screened by contact autoradiography (*6*), which detects positive pools by the ability of those COS cells expressing TNF receptor inserts to bind $^{125}$I-labeled TNF-α. After screening 175,000 clones, one positive pool (#737) was obtained, subdivided, and converged to a single clone in two cycles of this procedure. By autoradiographic plate binding (*6*), the pure clone when transfected into COS cells expressed a receptor that bound both $^{125}$I–TNF-α and -β; binding of either ligand was completely inhibited by a 200-fold excess of the same or homologous unlabeled cytokine (*7*). Quantitative in situ binding studies of the COS-expressed receptor with $^{125}$I–TNF-α agreed with these results and showed the binding to be complex (Fig. 1C). As with the native WI26-VA4 receptor, the recombinant COS receptor displayed both low ($K_{a1}$ = 0.18 ± 0.06 $nM^{-1}$) and high ($K_{a2}$ = 10.1 ± 1.0 $nM^{-1}$) affinity classes for $^{125}$I–TNF-α. TNF-β bound with lower affinity and competitively inhibited $^{125}$I–TNF-α binding (Fig. 1D). Thus, ligand binding properties of both the native and recombinant receptor appear similar. The origin of the multiple affinity classes for TNF-α is unclear. Indeed, most workers (*1, 4, 8, 9*), but not all (*10*), have reported monophasic Scatchard plots for TNF-α. However, TNF-α is predominantly a homotrimer (*11*) and therefore intrinsically capable of multivalent binding. In one report (*12*), differential biological effects could be related to biphasic binding of TNF-α. While not necessarily sharing a common origin, multiple affinity classes are a common feature of many receptor systems (*13*).

The isolated TNF receptor cDNA was used as a probe to analyze the mRNA expressed in a variety of cell lines and tissues (Fig. 2). A single size class of transcripts of ~4.5 kb was detected in WI26-VA4, Raji cells (a B lymphoblastoid line), LPS-stimulated peripheral blood monocytes (PBM), induced peripheral blood T cells (PBL), and placental tissue. A transcript of slightly larger size (~5.0 kb) was detected in thymic tissue, and splenic tissue contained transcripts of both size classes. The origin of these differences is not clear, but the presence of TNF receptor transcripts in these different cells is consistent with the near ubiquitous distribution of the receptor.

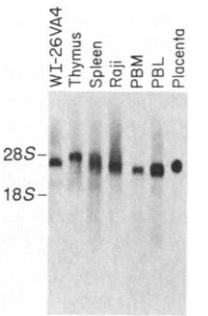


**Fig. 2.** RNA blot analysis of TNF receptor mRNA. Polyadenylated RNA (3.5 μg) was used from each source, except placental tissue (5 μg total RNA). PBL were cultured for 6 days in IL-2 and OKT3 monoclonal antibody, then restimulated for 8 hours with conconavalin A (Con A) and PMA (*6*). RNA was fractionated on a 1.1% agarose-formaldehyde gel, blotted onto Hybond-N (Amersham), and hybridized with a labeled antisense RNA probe prepared from the 630-bp Not I–Bgl II fragment of the TNF receptor cDNA that had been subcloned into a Bluescript plasmid (Stratagene). Filter hybridization and washing conditions were as described (*5*). Variable exposure times were used in preparing the figure.

The 3.7-kb insert of clone 737 was subcloned and sequenced (*5*) (Fig. 3). The cDNA contains a string of adenines at the 3′ end and an upstream consensus polyadenylation signal. The discrepancy between the size of the isolated cDNA and that of the transcripts estimated from Northern analysis may be due to a deficiency of 5′ sequences in this clone. It is also possible that alternative polyadenylation signals are utilized. Upstream of the polyadenylation site is a 299-bp segment that has homology to the Alu family of repetitive sequences (*14*). The sequence contains a single large open reading frame encoding 461 amino acids with features typical of an integral membrane protein (*15*). The initiating methionine precedes 22 hydrophobic residues characteristic of a leader sequence; the most probable cleavage site (*16*) predicts Leu$^{23}$ as the mature $NH_2$-terminus. Another hydrophobic region of 30 amino acids is located between residues 258 and 287, bordered by charged residues at either end (Asp$^{257}$ and Lys$^{288-290}$), consistent with a transmembrane segment that makes a single helical span. Immediately upstream of this element is a region of 57 amino acids rich in threonine, serine, and proline residues. Such a composition is indicative of O-linked glycosylation sites containing sialic acid and is found in similar extracellular regions of several receptors, including those for nerve growth factor (NGF) (*17*) and low density lipoprotein (LDL) (*18*). The $NH_2$-terminal 162 amino acids (positions 39 to 200) are rich in

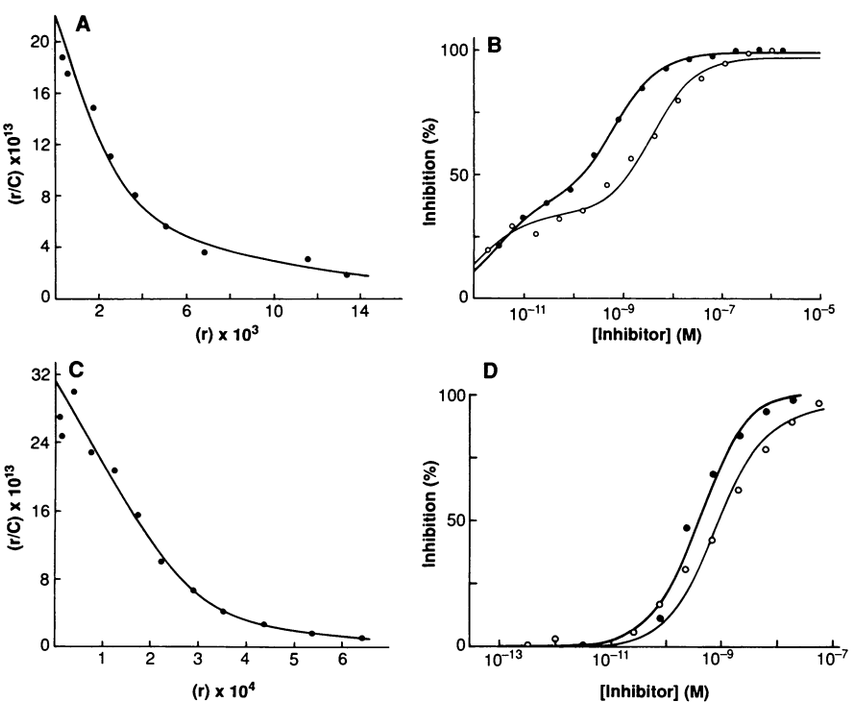


**Fig. 1.** TNF binding characteristics of native and recombinant TNF receptors (*31*). (**A**) Direct binding of $^{125}$I–TNF-α to WI26-VA4 cells (Scatchard coordinate system). (**B**) Inhibition of $^{125}$I–TNF-α binding to WI26-VA4 cells by unlabeled TNF-α (●) and TNF-β (○). TNF-α inhibition: $K_{I,1}$ (low affinity) = 1.6 ± 0.2 nM; $K_{I,2}$ (high affinity) = 0.8 ± 0.1 pM. TNF-β inhibition: $K_{I,1}$ (low affinity) = 0.29 ± 0.06 nM; $K_{I,2}$ (high affinity) = 1.3 ± 0.6 pM. (**C**) Direct binding of $^{125}$I–TNF-α to recombinant (COS) TNF receptor. (**D**) High affinity site inhibition of $^{125}$I–TNF-α binding to recombinant (COS) TNF receptor by unlabeled TNF-α (●) or -β (○). $K_I$ (α) = 6.7 ± 2.9 nM; $K_I$ (β) = 3.3 ± 0.8 nM. C, free concentration of TNF (molar); r, molecules of TNF bound per cell. All parameter values are ± standard error. Data fit to one or two site models as described (*32*).

cysteines (22 residues) and also contain two potential N-linked glycosylation sites. The receptor terminates in a cytoplasmic domain of 174 amino acids, rich in serines (18%), six of which are contiguous. Five cysteines and one potential N-linked glycosylation site are also present in this domain.

A computer search of several sequence databases (*19*) queried with the entire 439-residue sequence of the mature TNF receptor revealed five proteins with striking similarity: human and rat NGF receptor, CD40, cDNA clone 4-1BB, and T2 (Fig. 4). Four of these are transmembrane proteins, two of which are known receptors (for human and rat NGF). CD40 is a B cell–localized surface antigen, found also on neoplastic cells of epithelial origin, that becomes phosphorylated in the cytoplasmic domain after binding the CD40-specific monoclonal antibody G28-5 (*20*). Clone 4-1BB was identified as a murine cDNA from induced helper and cytolytic T cell clones (*21*). Both molecules have been suggested to be cytokine receptors for unidentified ligands. All identity between these four proteins is localized to the cysteine-rich regions of the extracellular domains; no homology was detected between the TNF receptor cytoplasmic domain and any proteins in the database. T2 is a transcriptionally active open reading frame from the Shope fibroma virus (SFV), a poxvirus that produces invasive malignancies in newborn rabbits (*22*). Although dominated by 22 conserved cysteines, the alignment is also reinforced by other conserved amino acids, particularly tyrosine, glycine, and proline. Thus, the extracellular domains of these molecules, presumably heavily disulfide bonded, probably share a common structural motif. Central to this motif would appear to be repeating homologous domains. Several groups have shown that the cysteine-rich regions of NGF receptor and CD40 can be resolved into either pseudo twofold repeats of about 80 amino acids or pseudo fourfold repeats of about 40 residues (*17, 20*). Similar repeats can be shown with the TNF receptor and T2, consistent with all these genes having arisen by duplication and divergence from a common gene. Since both NGF and TNF are oligomeric, repeating substructures in their receptors may aid in binding and predicts that the putative ligands for CD40 and 4-1BB may also be oligomers. The net charge associated with the cysteine-rich domains of these family members varies (−19 for NGF receptor; +1 for TNF receptor), which may be related to ligand specificity. Presumably, it is this $NH_2$-terminal region that contains the TNF binding site. Multiple lines of evidence have localized the (apoprotein B) ligand binding site of the LDL receptor to the $NH_2$-terminal (60-kD), cysteine-rich

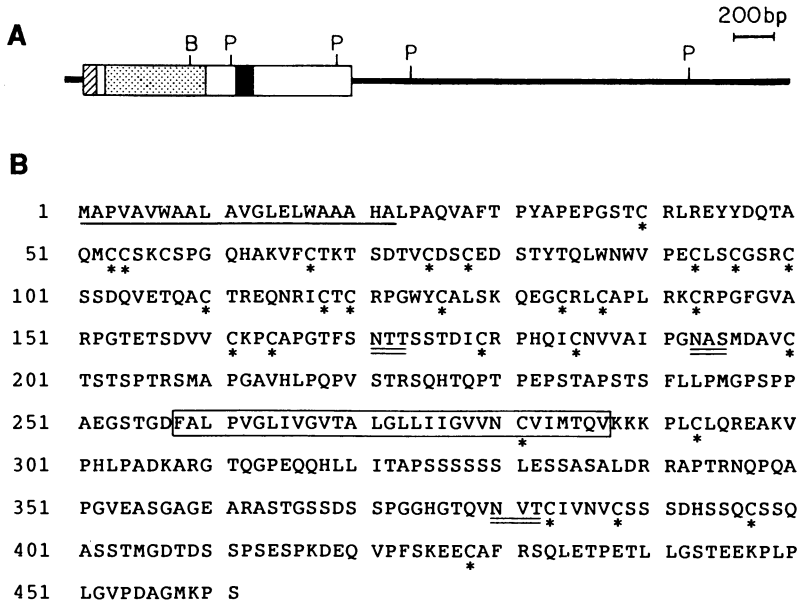


**Fig. 3.** Sequence of the human TNF receptor cDNA clone. (**A**) Schematic representation and restriction map of the cDNA. The entire coding region is boxed. The leader is hatched, the cysteine-rich region is shown stippled, and the transmembrane segment is solid. B = Bgl II; P = Pvu II. (**B**) The deduced amino acid sequence of cDNA coding region. The leader region is singly underlined, the transmembrane domain is shown boxed, potential N-linked glycosylation sites are doubly underlined, and cysteines are identified by an asterisk. The entire nucleotide sequence is available upon request and has been deposited at GenBank, accession number M32315.

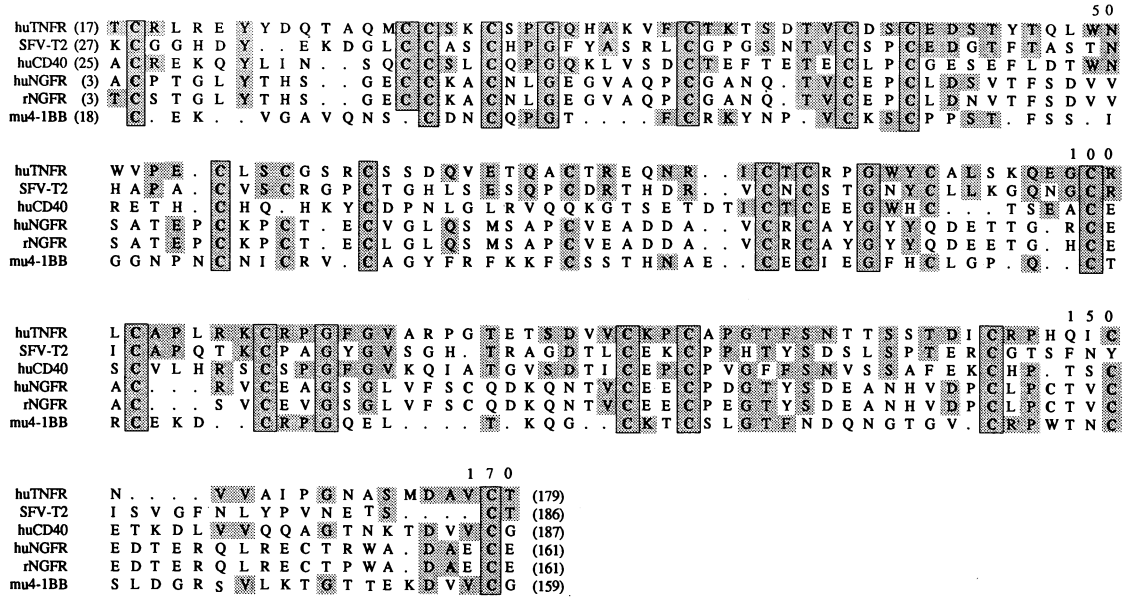


**Fig. 4.** Sequence similarities among the TNF receptor superfamily. Consensus alignment of residues from the cysteine-rich regions of human TNF receptor (huTNFR), T2 open reading frame of Shope fibroma virus (SFV-T2), human CD40 (huCD40), human and rat nerve growth factor receptor (huNGFR and rNGFR), and murine cDNA clone 4-1BB (mu4-1BB). Numbers at $NH_2$- and COOH-termini refer to residues as cited in publications describing cDNA cloning (*17, 20, 21, 22*); numbers at top right of each block mark residues from $NH_2$-terminus at top left. Shaded residues reflect those common to huTNF receptor and at least one other protein. Cysteines are in bold, and boxed residues are invariant.

domain (*18*).

Sequences containing cysteine-rich repeats are present in a number of proteins, including the CD18 adhesion molecules (*23*), epidermal growth factor (EGF) precursor, *Drosophila notch* protein, the *neu* oncogene, and the external domains of receptors for LDL, EGF, and insulin (*18, 24*). Although many of these proteins show homology to each other, we detect little similarity to the TNF receptor. Optimal alignments of family members using the National Biomedical Research Foundation (NBRF) ALIGN program (*19*) show the strongest similarity is between the TNF receptor and T2, with a score of 19 standard deviations (SD) above the mean score for an ensemble of randomly permuted molecules of the same lengths and amino acid composition. ALIGN scores greater than 3.0 are considered significant and indicate common ancestry. Almost 40% of the residues are identical, approaching the conservation level between many murine and human cytokines and their receptors (*25*). Slight variants of T2 may also exist in other poxvirus family members, and some of these viruses are strongly immunosuppressive (*22*). Although T2 possesses a signal peptide sequence, the molecule appears to lack a hydrophobic segment typical of transmembrane regions, suggesting that T2 may be a soluble entity secreted from virally infected cells. Thus, perhaps T2 may bind TNF, or another cytokine, serving to locally dampen the host immune response. The protective effects of such a "soluble receptor" would no doubt confer a selective advantage to the pathogen. CD40, however, is also similar to this TNF receptor (38.5% amino acid identity; 15.2 SD), yet does not bind TNF-α when expressed in COS cells at high levels in an immunoreactive form (*26*). TNF receptor is more distantly related to 4-1BB and NGF receptor (9.0 and 12.3 SD, respectively).

The signal transduction mechanism of TNF is unclear. The receptor cytoplasmic domain, as with other family members, shows no similarity with known proteins, including the cytoplasmic domain of the human T cell interleukin-1 (IL-1) receptor (*6*), despite the fact that TNF and IL-1 mediate many common biological activities (*1*). The TNF receptor expressed in COS cells does not bind radiolabeled human IL-1α or -β, nor does the recombinant human IL-1 receptor bind TNF (*7*). No sequences present are typical of tyrosine kinases, protein kinase C, or phosphorylation sites corresponding to substrates for these kinases (*27*). The cytolytic activity of TNF, however, appears to depend on the presence of a 200-kD protein distinct from the receptor, and with which it comodulates (*28*).

Several groups have characterized TNF binding proteins from urine. Uromodulin is a renal glycoprotein that binds IL-1, IL-2, and TNF-α with high affinity, but does not inhibit ligand binding to their respective receptors and shows no sequence similarity to the TNF receptor reported here (*29*). Two groups have recently reported purification and sequencing of soluble TNF-α binding proteins from urine with molecular weights of 27 to 30 kD (*30*). However, the $NH_2$-terminal sequence of these proteins is not found in the predicted sequence of clone 737. TNF-α receptors on myeloid cells are probably different from those on cells of epithelial origin (*8*). An 80-kD form of the receptor contains O- and N-linked carbohydrate; a 60-kD form lacks O-linked carbohydrate, possesses a different form of N-linked carbohydrate, and displays different tryptic peptide maps. Monoclonal antibodies to these two receptors also do not cross-react. The receptor we have described may correspond to the 80-kD form. Affinity cross-linking of the recombinant receptor using either $^{125}$I–TNF-α or -β shows a single species of 80 kD (*7*). Because the calculated protein is 46 kD, carbohydrate appears to be attached, and both O- and N-linked glycosylation sites are present in the sequence.

The availability of a full-length cDNA clone for a human TNF receptor will now permit detailed studies into the molecular mechanisms by which ligand-receptor interactions produce the pleiotropic effects of this important cytokine. Soluble, recombinant forms of this receptor may also be produced to explore the clinical value of TNF inhibition in pathological settings.

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26. The CD40 and human TNF receptor clones were separately transfected into COS cells (CD40-COS, TNFr-COS) and expression was monitored by radiolabeled ligand binding using contact autoradiography (*6*). CD40-COS clearly and specifically bound $^{125}$I–G28 antibody, but did not bind $^{125}$I–TNF-α (0.2 nM). $^{125}$I-G28 binding was not inhibited by a 500-fold molar excess of unlabeled TNF-α. Similarly, TNFr-COS specifically bound $^{125}$I–TNF-α and -β but not $^{125}$I-G28 (0.2 nM).
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31. COS cells were transfected with the vector pDC302 containing the TNF receptor cDNA insert (clone 737) or control vector lacking insert as described (*5, 6*). For quantitative in situ binding studies, transfected COS cells were replated (24 hours after transfection) into six well trays (CoStar) and analyzed 48 hours later at near confluence ($6 \times 10^5$ cells per well). COS monolayers were washed once with phosphate-buffered saline (PBS), then incubated with $^{125}$I–TNF-α at various concentrations in binding media [RPMI 1640, bovine serum albumen (10%), $NaN_3$ (0.1%), 20 mM Hepes, pH 7.4] at 4°C for 2 hours. Free $^{125}$I–TNF-α was determined by counting gamma emissions in the supernatant. Monolayers were then washed once with ice-cold RPMI, detached with 0.1% trypsin in PBS, and counted to determine bound ligand. Nonspecific ligand binding was determined by inclusion of a 200-fold molar excess of unlabeled ligand. Inhibition assays used $^{125}$I–TNF-α at 0.2 nM. Data were analyzed and theoretical curves plotted as described (*6, 32*). TNF-α and TNF-β (R&D Sciences) were radiolabeled using Iodogen (Pierce) to a specific activity of $2 \times 10^{15}$ cpm/mmol (*4*). Radiolabeled TNF-α gel filtered as a single peak with an apparent molecular weight of 55 kD (*7*), consistent with a trimeric status (*11*).
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***"The good news is we have the human genome. The bad news is the computer alphabetized it."***