Biochemical Characterization of the Extracellular Domain of the 75-Kilodalton Tumor Necrosis Factor Receptor

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ABSTRACT: An expression plasmid encoding the extracellular domain of the 75-kDa human tumor necrosis factor (TNF) type 2 receptor (TNF-R2) was constructed and used to generate a stable cell line secreting soluble TNF-R2 (sTNF-R2). Purified sTNF-R2 was resolved by SDS–PAGE into one band of approximate M_r 43 000, consistent with a molecular weight of 36 000 ± 4800 obtained by sedimentation equilibrium analysis. The apparent molecular weight observed by gel filtration chromatography was approximately 136 000. Glycosylation analysis revealed that Asn-149 is fully glycosylated, while Asn-171 is incompletely glycosylated (~50%), and that a proline-, serine-, and threonine-rich region (residues 175–234) contains O-linked carbohydrate structures. Scatchard analysis of [^{125}I]TNF-α and [^{125}I]TNF-β binding to sTNF-R2 gave dissociation constants (K_d) of 0.3 and 0.75 nM, respectively, comparable to those observed for intact cell-surface TNF-R2. The sTNF-R2 was found to block the cytotoxicity of both TNF-α and TNF-β in a murine L-M cell assay. The sizes of the sTNF-R2·TNF-α and sTNF-R2·TNF-β complexes determined by gel filtration chromatography were approximately 322 and 204 kDa, respectively. The stoichiometry of the sTNF-R2·TNF-α and sTNF-R2·TNF-β complexes was examined by size-exclusion chromatography, sedimentation equilibrium, and cross-linking. The data from these studies suggest that at least two molecules of sTNF-R2 can bind to a single TNF-α or TNF-β trimer.

TNF-α and TNF-β are two related cytokines that share approximately 30% amino acid sequence identity and are involved in mediating a wide spectrum of biological activities including cytotoxicity, proliferation, and antiviral responses [see Camussi et al. (1991), Fiers (1991), Jäättaelä (1991), and Vilček and Lee (1991) for recent reviews]. The mechanism(s) by which TNF-α and TNF-β induce their diverse cellular responses is (are) initiated by their binding to specific cell-surface receptors. Two distinct TNF receptors designated TNF-R1 (55 kDa) and TNF-R2 (75 kDa) have been recently identified [Hohmann et al., 1989; Brockhaus et al., 1990; also see Loetscher et al. (1991b), Mallett and Barclay (1991), and Sprang (1990) for reviews] and their cDNAs cloned and expressed (Loetscher et al., 1990a; Schall et al., 1990; Smith et al., 1990). Both receptors are present on the majority of cell types examined (Lewis et al., 1991) and have been found to bind both TNF-α and TNF-β with high affinity (Rubin et al., 1985; Kull et al., 1985; Baglioni et al., 1985; Aggarwal et al., 1985a; Tsujimoto et al., 1985; Yoshie et al., 1986). Although both TNF-α and TNF-β have been shown to exist as trimers in solution (Wingfield et al., 1987; Arakawa & Yphantis, 1987; Lewit-Bentley et al., 1988; Eck et al., 1988, 1992; Browning & Ribolini, 1989; Jones et al., 1989; Schoenfield et al., 1991), there is still some question whether monomers, dimers, or trimers are the biologically active form (Smith & Baglioni, 1987; Peterson et al., 1989; Heller et al., 1990).

Two distinct soluble TNF-binding proteins have been isolated and characterized from both urine and serum (Kohno et al., 1990; Peetre et al., 1988; Seckinger et al., 1989; Novick et al., 1989; Engelmann et al., 1990a; Gatanaga et al., 1990; Lantz et al., 1990). These TNF-binding proteins have been found to correspond to the extracellular domains of TNF-R1 and TNF-R2, and are generated proteolytically from the full-length cell-surface receptors. Whether the TNF-binding proteins play a physiological role by inhibiting TNF activities or affecting the stability of TNF is not yet known.

Recent studies using a recombinant extracellular domain of TNF-R1 have provided evidence that two to three molecules of sTNF-R1 can bind to a single TNF-α or TNF-β trimer (Loetscher et al., 1991a; Pennica et al., 1992a). It has also been shown that clustering of TNF-R1 on the cell surface by cross-linking with specific agonist antibodies is sufficient to induce a variety of TNF activities (Engelman et al., 1990b; Espevik et al., 1990; Tartaglia et al., 1991). These findings suggest that the initiation of signaling by TNF-R1 involves TNF-induced receptor oligomerization. Activation of TNF-R2 may occur by a similar mechanism since TNF-α and TNF-β can dimerize intact TNF-R2 on cells (Pennica et al., 1992b) and antibodies to this receptor can induce T-cell proliferation (Tartaglia et al., 1991). Here we have produced a recombinant extracellular domain of the human TNF-R2 (sTNF-R2) using a mammalian cell expression system, characterized its glycosylation, and studied its interaction with TNF-α and TNF-β. These data suggest that two to three molecules of sTNF-R2 bind to one TNF-α or TNF-β trimer.

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MATERIALS AND METHODS

Reagents. Recombinant human TNF-α (4.7×10^7 units/mg) and TNF-β (2.1×10^8 units/mg) were provided by the Genentech, Inc., manufacturing group. [^{125}I]TNF-α (specific activity, 25.8 μCi/μg) and [^{125}I]TNF-β (specific activity, 47.8 μCi/μg) were prepared using the lactoperoxidase method (Miyata et al., 1985). [^{125}I]TNF-α was also purchased from Amersham (specific activity, 46.1 μCi/μg). Monoclonal antibodies 1H9, 9B7, 2E8, and B (IgG2a, IgG2b, IgG2b, and IgG1 isotypes, respectively) were provided by B. M. Fendly, Genentech, Inc., and produced as described previously (Pennica et al., 1992a). Pansorbin was purchased from Calbiochem. Dithiothreitol (DTT) and iodoacetic acid (IAA) were purchased from Sigma. HPLC/Spectro Grade trifluoroacetic acid (TFA) was purchased from Pierce Chemical Co., and acetonitrile UV from Burdick & Jackson. Milli Q water (Millipore) was used for reversed-phase HPLC. Endoproteinase Asp-N ("sequencing grade") was purchased from Boehringer Mannheim and PGNase F (*N*-glycanase) from Genzyme. Bis[2-(succinimidooxycarbonyloxy)ethyl]sulfone (BSOCOES) was purchased from Pierce.

Expression of sTNF-R2 in Mammalian Cells. The mammalian expression plasmid pRK5-sTNF-R2 consisted of an 862 bp fragment of TNF-R2 cDNA inserted between the cytomegalovirus immediate early promoter and the SV40 termination and polyadenylation signals of the vector pRK5 (Schall et al., 1990). The fragment contains 81 bp of 5′ untranslated DNA and 771 bp encoding the 22 amino acid signal peptide and the 235 amino acid extracellular domain of TNF-R2 followed by a stop codon that was inserted using synthetic DNA. The plasmid pRSVneo, containing a neomycin resistance gene, was cotransfected with pRK5-sTNF-R2 into the human embryonic kidney cell line 293 (Graham et al., 1977) by the calcium phosphate precipitation method (Gorman, 1985). Approximately 20 G418-resistant clones were isolated and tested for sTNF-R2 secretion by dot blot analysis (Heller et al., 1990) of the media with [^{125}I]TNF-α. The clone, sTNF-R2-15, that secreted a high level of sTNF-R2 was selected for further experiments. sTNF-R2 was purified using the same protocol described previously for the purification of sTNF-R1 (Pennica et al., 1992a).

PNGase F and Endoproteinase Asp-N Treatment of sTNF-R2. PNGase F (1.15 manufacturer's units) was added to 32 μg of reduced and carboxymethylated (RCM) sTNF-R2 in 0.11 mL of 0.1 M ammonium bicarbonate and incubated at 37 °C for 15 h. At the end of the incubation, 0.36 μg of endoproteinase Asp-N (3.2 μg/mL final concentration) was added to a sample of untreated sTNF-R2 (32 μg) or PNGase F treated sTNF-R2 (32 μg), and the samples were incubated for an additional 15 h at 37 °C. The enzyme digests were fractionated by reversed-phase HPLC using a Hewlett-Packard Model 1090M HPLC system with a Vydac C_{18} narrow-bore column (2.1 mm × 25 cm). Buffer A was 0.1% aqueous TFA, and buffer B was 0.08% TFA in acetonitrile. The column was preequilibrated with 100% buffer A at a flow rate of 0.25 mL/min. After a 5-min hold, elution was carried out with a linear gradient from 0 to 40% buffer B in 120 min.

Peptide and Carbohydrate Analysis. The procedures used for reduction and S-carboxymethylation, amino acid analysis, Edman degradation, electrospray mass spectrometry, and carbohydrate composition analysis were as described previously (Harris et al., 1991).

Binding Assays. Saturation isotherm experiments were performed by incubating increasing concentrations of [^{125}I]-TNF-α or [^{125}I]TNF-β with a fixed concentration (2.7×10^{-10} M) of sTNF-R2 in 0.15 mL of binding buffer (PBS/0.5% BSA) at room temperature. Following a 2-h incubation period, 440 ng (1.9×10^{-8} M final concentration) of 9B7 nonblocking monoclonal antibody against sTNF-R2 was added to each reaction and incubated for an additional 2 h at room temperature. The samples were processed and the data analyzed as described previously (Pennica et al., 1992a). Nonspecific binding was determined by adding a 100-fold molar excess of unlabeled TNF-α or TNF-β to an identical set of reactions.

In competitive binding experiments, 5.3×10^{-10} M sTNF-R2 was incubated with 5.3×10^{-11} M [^{125}I]TNF-α trimer in a final volume of 0.15 mL for 2 h at room temperature, alone or in the presence of increasing concentrations of unlabeled human TNF-α or TNF-β. The reactions were processed as described above for the saturation isotherm experiments, except that monoclonal antibody 1H9 was used instead of mAb 9B7. Nonspecific binding was determined by omitting either the sTNF-R2 or the monoclonal antibody. All experiments were performed in duplicate. K_i values were determined by the computer program Scatplot.

Cytotoxicity Assay. Twofold serial dilutions of sTNF-R2 were mixed with 0.1 ng/mL either TNF-α (5.2 units/mL; 2.1 pM) or TNF-β (20.3 units/mL; 1.9 pM) in the presence of 10 μg/mL cycloheximide. Samples were assayed for cytolytic activity on mouse L-M fibroblasts as described previously (Kramer & Carver, 1986).

Gel Filtration. Gel filtration was performed at room temperature by FPLC using a Superose 6 column (10 × 300 mm, Pharmacia) equilibrated and eluted with PBS. Constant amounts of recombinant TNF-α or TNF-β (190 mM) were mixed with sTNF-R2 at varying ratios in a final volume of 0.1 mL, and processed as described previously (Pennica et al., 1992a).

Analytical Ultracentrifugation. One hundred microliter samples of TNF-α, TNF-β, sTNF-R2, and sTNF-R2·TNF-α or ·TNF-β complex in PBS were analyzed as described previously (Pennica et al., 1992a). The sTNF-R2·TNF complexes were prepared at either 2:1 or 3:1 final molar ratios. The samples were centrifuged at 15 000 rpm at 20 °C for 18–24 h in a Beckman Optima XLA analytical ultracentrifuge. The concentration gradient in the cell was determined by the UV absorption at 235 nm, and equilibrium was verified by comparing repetitive scans taken at 12-h intervals. The partial specific volumes for TNF-α and TNF-β (0.74 mL/g) and for nonglycosylated sTNF-R2 (0.71 mL/g) were computed as described previously (Pennica et al., 1992a). An average value of 0.63 mL/g was used for the partial specific volume of the carbohydrate component. The buoyant molecular weights, $M(1 - \bar{\nu}\rho)$, were determined by analyzing the absorbance gradient as a single ideal species for TNF-α, TNF-β, and sTNF-R2, and as two ideal species for the mixtures of TNF and sTNF-R2. These data were analyzed using the software package Kaleidagraph.

Cross-Linking of [^{125}I]TNF-α to sTNF-R2. [^{125}I]TNF-α (0.7 nM) was incubated alone or with purified sTNF-R2 (1.5×10^{-7} M) in PBS, pH 7.4, for 2 h at room temperature. The homobifunctional cross-linking reagent BSOCOES was added at a final concentration of 1 mM for 30 min. The reaction was stopped by the addition of one-tenth volume of 1 M glycine. Immunoprecipitation of the cross-linked products was performed by adding monoclonal antibody B to the reaction with [^{125}I]TNF-α alone or monoclonal antibody 2E8 to the reaction containing sTNF-R2, incubating for 2 h at 4 °C, and processing as described above for the binding assays. The pellets were

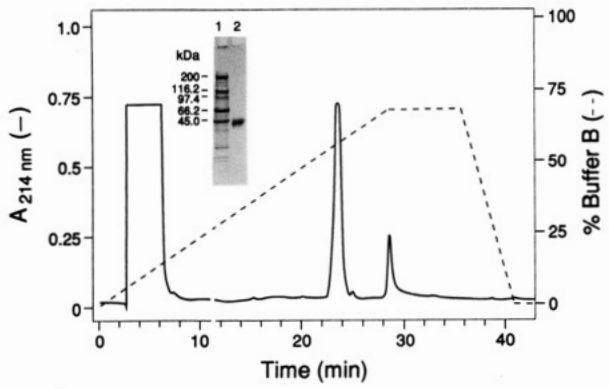

FIGURE 1: Reversed-phase HPLC purification and SDS–PAGE analysis of sTNF-R2. Fractions from the TNF-α affinity column containing sTNF-R2 were pooled and fractionated on a C_4 reversed-phase column with a linear acetonitrile gradient (dashed line) in a solvent system containing 0.1% TFA. The absorption at 214 nm was monitored, and 0.5-mL fractions were collected in 0.3 mL of PBS. The peak fractions, (retention time = 22–25 min) were pooled, lyophilized, and used for subsequent studies. The inset shows the HPLC-purified sTNF-R2 resolved on a 4–12% Tris/glycine/polyacrylamide gel and silver-stained. Lane 1, high molecular mass standards, Bio-Rad; lane 2, sTNF-R2.

washed once with 200 μL of cold PBS/0.5% BSA, resuspended in SDS–PAGE sample buffer, and heated at 100 °C for 10 min. The reactions were then centrifuged for 2 min at 15800g and the supernatants analyzed on a 4–12% Tris/glycine/polyacrylamide gel (Novex).

RESULTS

Expression and Purification of sTNF-R2. An expression vector encoding the extracellular domain of human TNF-R2 (sTNF-R2) was transfected into 293 human embryonic kidney cells. A stable cell line, 293/sTNF-R2-15, was isolated that secretes sTNF-R2 into the media. Conditioned, serum-free medium from 293/sTNF-R2-15 was collected from confluent cultures and concentrated, and the sTNF-R2 was purified by TNF-α affinity chromatography followed by reversed-phase HPLC to remove TNF-α and any other minor contaminating proteins (Figure 1). The minor peak seen at a retention time of ~28 min was shown to be TNF-α by amino acid composition analysis. The N-terminal sequence (Leu-Pro-Ala-Gln-Val) determined for the major peak (retention time = 22–25 min) corresponds to that previously reported for the intact 75-kDa TNF receptor purified from HL60 cells (Loetscher et al., 1990). The N-terminal sequence of our sTNF-R2, however, is longer than any of the heterogeneous forms reported for the urinary TNF binding proteins (Engelman et al., 1990). The sTNF-R2 peak was analyzed by SDS–PAGE and found to migrate at ~43 kDa (Figure 1, inset). The yield of purified sTNF-R2 was approximately 0.2–0.5 μg/mL of culture media.

Glycosylation Sites of sTNF-R2. The amino acid sequence of sTNF-R2 (Figure 2) includes 2 potential sites for N-linked glycosylation (Asn-149 and Asn-171), as well as a proline-rich region containing a total of 19 serine and threonine residues that could serve as attachment positions for O-linked carbohydrate (Wilson et al., 1991). The utilization of potential glycosylation sites in sTNF-R2 was examined by analysis of peptides generated by endoproteinase Asp-N digestion of the protein.

Asp-N digestion of reduced and S-carboxymethylated sTNF-R2 is predicted to yield a total of nine peptides (Figure 2), of which peptide A7 contains Asp-149, peptide A8 contains Asn-171, and peptide A9 contains the region rich in proline, serine, and threonine residues. The reversed-phase HPLC

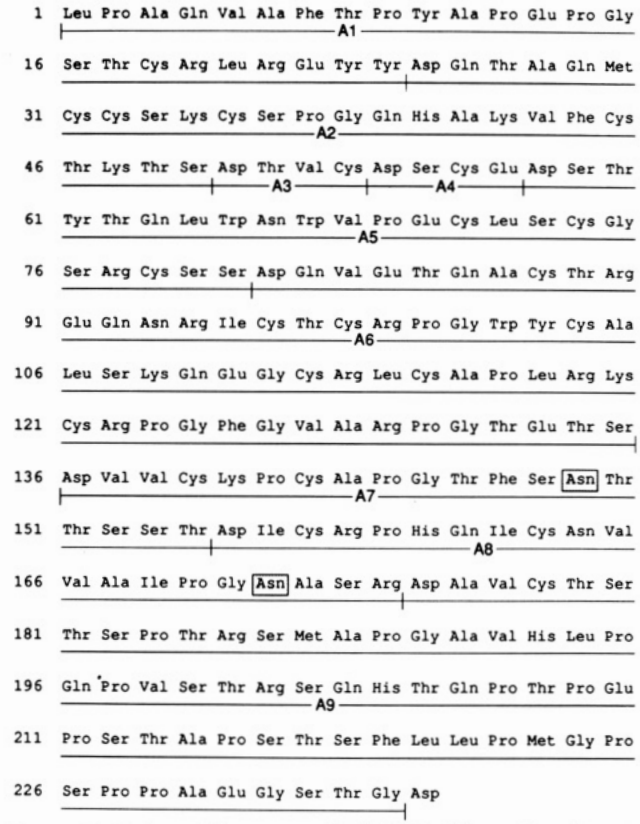

FIGURE 2: Amino acid sequence of sTNF-R2. The predicted Asp-N cleavage sites of sTNF-R2 are indicated, and the resulting peptides are ordered sequentially starting at the N-terminus of the molecule and are labeled A1–A9. The potential N-linked glycosylation sites are enclosed in boxes.

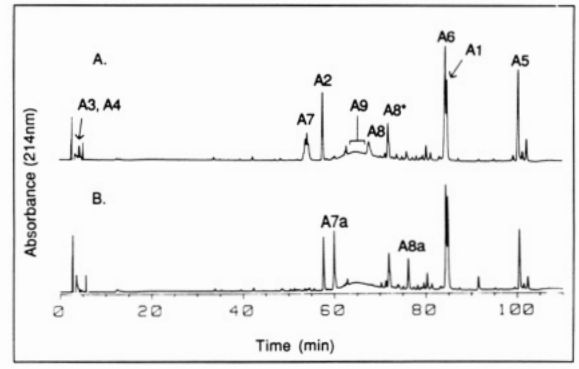

FIGURE 3: Reversed-phase HPLC Asp-N maps of RCM sTNF-R2. Each chromatogram was generated with 1.3 nmol of Asp-N-digested RCM sTNF-R2. (A) Endoproteinase Asp-N digest of sTNF-R2. (B) PNGase F followed by endoproteinase Asp-N digestion of sTNF-R2. Chromatographic conditions were as described under Materials and Methods. The labeled peaks were collected and identified by amino acid analysis, by N-terminal sequence analysis, or by mass spectrometry.

map of Asp-N-treated sTNF-R2 is shown in Figure 3A. The peptide identifications in Figure 3 were based on a combination of amino acid analysis, Edman degradation, and electrospray mass spectrometry of the collected peptides. The A7 peptide, which contains Asn-149, was identified as a broad peak eluting at 55 min. Glycosylation of this peptide was confirmed by Edman degradation and by electrospray mass spectrometry. The A8 peptide, which contains Asn-171, was observed as two species eluting at 68 min and at 72 min. The first of these peaks (A8) was determined by the above methods to be glycosylated, while the second peak (A8*) was determined to be a nonglycosylated form of the same peptide. The relative peak areas of the A8 and A8* peaks indicate that Asn-171

is glycosylated in ~50% of the sTNF-R2 molecules.

The glycosylation pattern of Asn-149 and Asn-171 was confirmed by Asp-N digestion of PNGase F-treated sTNF-R2 (Figure 3B). PNGase F treatment removes N-linked oligosaccharides with concomitant conversion of the attachment Asn residue to Asp, thereby generating a new cleavage site for endoproteinase Asp-N. In the Asp-N map of PNGase F-treated sTNF-R2, only peptides A7 and A8 were affected. The new peaks eluting at 60 and 76 min were identified as peptides A7a (residues 136–148) and A8a (residues 155–170), respectively (Figure 2). Peptide A8* was not affected by PNGase F treatment.

Peptide A9, which includes the region rich in Pro, Ser, and Thr residues, was identified as a broad peak between 60 and 70 min (Figure 3). The broadness of the peak is probably due to the presence of cis and trans proline isomers in the peptide (Melander et al., 1982). O-linked glycosylation of this peptide was confirmed by monosaccharide composition analysis; 7.5 mol of *N*-acetylgalactosamine and 14.4 mol of galactose were found per mole of peptide, suggesting that several of the Ser and/or Thr residues carry O-linked carbohydrate.

Binding of TNF-α and TNF-β to sTNF-R2. The functional integrity of the ligand-binding domain of sTNF-R2 was examined by [^{125}I]TNF-α and [^{125}I]TNF-β binding analysis. Saturation isotherms for the binding of radiolabeled TNF-α and TNF-β to sTNF-R2 were performed by incubating increasing concentrations of iodinated ligand with a fixed concentration of sTNF-R2. The binding of both [^{125}I]TNF-α and [^{125}I]TNF-β was specific and saturable (Figure 4A,B). Scatchard analysis of the data (Figure 4A,B, insets) indicates a single class of binding sites for both ligands with K_d values of 0.3 nM for TNF-α and 0.75 nM for TNF-β. These values are consistent with those previously reported for the full-length membrane-associated TNF-R2 transiently or stably expressed in mammalian cells (Dembic et al., 1990; Smith et al., 1991; Pennica et al., 1992b). Competitive binding experiments were performed by adding increasing concentrations of cold TNF-α or TNF-β to fixed amounts of [^{125}I]TNF-α and sTNF-R2. Both TNF-α and TNF-β caused a dose-dependent decrease in specific [^{125}I]TNF-α binding to sTNF-R2 (Figure 4C). Consistent with the results of the Scatchard analyses, the competition data indicate that sTNF-R2 has about a 4-fold lower affinity for TNF-β than for TNF-α. K_i values of 0.33 and 1.3 nM were obtained for TNF-α and TNF-β, respectively.

Neutralization of TNF-α and TNF-β Bioactivity by sTNF-R2. Soluble TNF-R2 was tested for its ability to inhibit the cytotoxicity of TNF-α and TNF-β on the mouse L-M fibroblast cell line (Figure 5). Consistent with the binding analysis, TNF-α was more efficiently neutralized than TNF-β. However, relatively high concentrations of sTNF-R2 were needed to inhibit the cytotoxic activity of TNF-α or TNF-β. EC_{50} values of approximately 30 and 500 nM were obtained for TNF-α and TNF-β, respectively.

Gel Filtration of the sTNF-R2·TNF-α and sTNF-R2·TNF-β Complexes. Complex formation of sTNF-R2 with TNF-α or TNF-β was examined by size-exclusion chromatography. TNF-α, TNF-β, and sTNF-R2 were first chromatographed separately on Superose 6 under nondenaturing conditions to determine their respective elution positions. The molecular masses of TNF-α and TNF-β determined by this method are 29–35 and 16–19 kDa, respectively, instead of the actual sizes of 52 kDa for TNF-α and 50 kDa for TNF-β. Using gel filtration, other investigators have observed sizes ranging from 33 to 46 kDa for TNF-α (Aggarwal et al., 1985b; Arakawa & Yphantis, 1987; Schoenfeld et al., 1991)

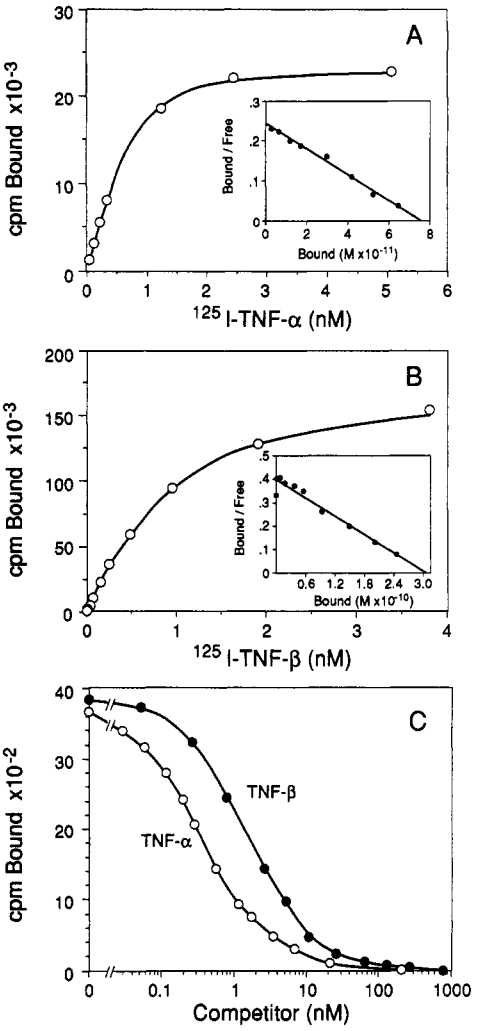

FIGURE 4: Binding of [^{125}I]TNF-α and [^{125}I]TNF-β to sTNF-R2. Saturation isotherms of the specific binding of [^{125}I]TNF-α and [^{125}I]-TNF-β to sTNF-R2. Duplicate samples of sTNF-R2 (2.7×10^{-10} M) were incubated with increasing concentrations of [^{125}I]TNF-α (A) or [^{125}I]TNF-β (B) alone or in the presence of a 100-fold molar excess of unlabeled TNF-α or TNF-β, respectively, and the specific binding at each concentration was determined. The insets present the data transformed by Scatchard analysis. (C) Displacement curves showing the inhibition of specific binding of [^{125}I]TNF-α by unlabeled TNF-α (○) or TNF-β (●). Increasing concentrations of either TNF-α (30 pM–211 nM) or TNF-β (53 pM–790 nM) were added to a fixed amount of [^{125}I]TNF-α (5.3×10^{-11} M) and sTNF-R2 (5.3×10^{-10} M) and processed as described under Materials and Methods. All binding experiments were performed 2–3 times with similar results. The results from a single representative experiment are shown.

and a size of 19 kDa for TNF-β (Schoenfeld et al., 1991). The lower than predicted values determined by gel filtration are due to interaction with the matrix (Fiers, 1992). Analysis of sTNF-R2 by gel filtration showed an elution position corresponding to a molecular mass of approximately 136 000 Da, suggesting that either it is aggregated or it is an elongated protein. The sedimentation equilibrium experiments described below support the latter interpretation.

Complex formation between sTNF-R2 (monomer) and TNF-α (trimer) was analyzed by gel filtration at molar ratios of 0.5:1, 1:1, 2:1, 3:1, and 4:1 (Figure 6A). At the 0.5:1 and 1:1 ratios, two peaks of apparent sizes 280 and 29 kDa are seen corresponding to complex and free TNF-α, respectively. At a 2:1 ratio, the complex shifts to a slightly higher molecular mass of about 300 kDa. At 3:1 and 4:1 ratios, the complex migrates at approximately 322 kDa. However, at the 4:1 ratio, a distinct peak of free sTNF-R2 begins to accumulate,

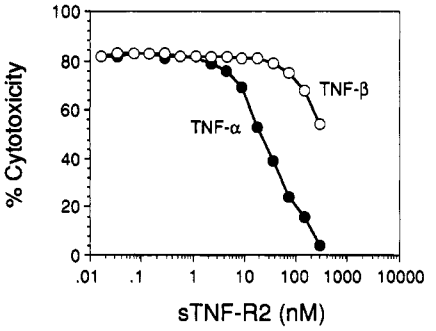

FIGURE 5: Inhibition of TNF-α and TNF-β activity by sTNF-R2. TNF-α or TNF-β were added at a final concentration of 0.12 ng/mL to the wells of a 96-well microtiter plate seeded 48 h earlier with 2 $\times 10^4$ mouse L-M cells/well. Twofold serial dilutions of sTNF-R2 [7.2 μg/mL (2.8×10^{-7} M) initial concentration] were made. All wells contained cycloheximide at a final concentration of 10 μg/mL. Triplicate plates were then assayed for viability as previously described (Kramer & Carver, 1986). The mean residual cytotoxic activity of TNF-α (●) and TNF-β (○) is plotted as a function of the concentration of sTNF-R2.

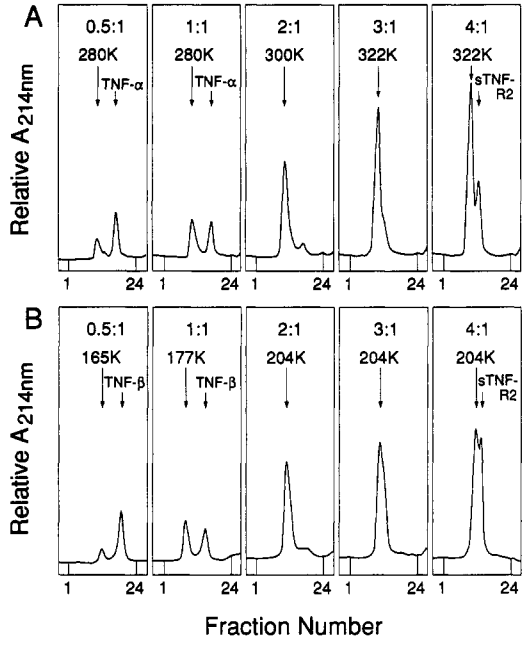

FIGURE 6: Gel filtration chromatography of various ratios of TNF-α or TNF-β and sTNF-R2. Constant amounts (190 nM) of TNF-α (panel A) or TNF-β (panel B) were mixed with sTNF-R2 at the indicated molar ratios, assuming that TNF-α and TNF-β are trimers and sTNF-R2 is a monomer. The concentrations of TNF-α, TNF-β, and sTNF-R2 were determined by amino acid composition analysis. The mixtures were incubated for >2 h at room temperature in a volume of 0.1 mL of PBS. The mixtures were chromatographed on an FPLC system using a Superose 6 column eluted with PBS at 0.35 mL/min. Peaks were monitored for the absorbance at 214 nm.

whereas at the 2:1 and 3:1 ratios most of the sTNF-R2 and TNF-α are in the complex. These results suggest that at least two molecules of sTNF-R2 can bind to a single TNF-α trimer.

The complex between sTNF-R2 and TNF-β was analyzed similarly (Figure 6B). At a ratio of 0.5:1 (sTNF-R2 to TNF-β), two peaks of apparent size 165 and 16 kDa are seen corresponding to complex and free TNF-β, respectively. At a 1:1 ratio, the complex peak shifts to a slightly higher apparent molecular mass of about 177 kDa, but free TNF-β is still apparent. At a 2:1 ratio, the size of the complex is 204 kDa, and most of the sTNF-R2 and TNF-β are bound in a complex. At 3:1 and 4:1 ratios, the complex size remains at 204 kDa; however, free soluble receptor begins to accumulate and is

Table I: Molecular Weight Analysis by Sedimentation Equilibrium

sample	$M(1-\bar{\nu}\rho)^a$	$\bar{\nu}$	M_r ($\times 10^{-3}$)^a	stoichiometry^b
TNF-α	13200	0.739	51.4	
TNF-β	12200 ± 500	0.739	47.3 ± 2	
sTNF-R2	11200 ± 1500	0.685	36.0 ± 4.8	
sTNF-R2·TNF-α				
2:1	31760 ± 2000	0.707^c	109.9 ± 7	1.6 ± 0.5
		0.702^d	108.0 ± 7	1.6 ± 0.4
3:1	41890 ± 2500	0.707^c	145.0 ± 8	2.6 ± 0.7
		0.702^d	142.5 ± 8	2.5 ± 0.7
sTNF-R2·TNF-β				
2:1	37470 ± 560	0.706^c	129.2 ± 2	2.3 ± 0.4
		0.701^d	127.0 ± 2	2.2 ± 0.4
3:1	36980 ± 2060	0.706^c	127.5 ± 7	2.2 ± 0.6
		0.701^d	125.4 ± 7	2.2 ± 0.5

^{a} Molecular weights determined in duplicate experiments except for TNF-α. Values are the mean ± average deviation. ^{b} Calculated stoichiometry of the complex expressed as moles of sTNF-R2 per mole of TNF-α or TNF-β trimer. ^{c} Partial specific volume calculated assuming the sTNF-R2·TNF complex exists at a stoichiometry of 2:1. ^{d} Partial specific volume calculated assuming the sTNF-R2·TNF complex exists at a stoichiometry of 3:1.

seen as a shoulder in the 3:1 ratio and a distinct peak in the 4:1 ratio. The data from these experiments suggest that two molecules of sTNF-R2 can bind to a single TNF-β trimer.

Analytical Ultracentrifugation. TNF-α, TNF-β, and the sTNF-R2·TNF complexes were also analyzed by sedimentation equilibrium (Table I). Analysis of TNF-α and TNF-β alone gave molecular masses of 51 and 47 kDa, respectively. These values are close to the values expected for these known trimeric molecules (Eck & Sprang, 1989; Eck et al., 1992).

The value of the molecular weight calculated for sTNF-R2 is dependent on the partial specific volume for the glycoprotein. The partial specific volume, calculated assuming that the mass of sTNF-R2 is 30% carbohydrate, gave a molecular weight value of 36 000 ± 4800, and is consistent with the known molecular weight of the protein (estimated from the protein sequence determined from the cDNA sequence). Therefore, sTNF-R2 exists as a monomer in solution rather than as an aggregate as suggested by gel filtration experiments.

The sTNF-R2·TNF complexes were prepared by mixing sTNF-R2 and either TNF-α or TNF-β at 2:1 or 3:1 ratios before analysis by sedimentation equilibrium. The partial specific volumes for the complexes were estimated for both 2:1 and 3:1 ratios, and for 30% glycosylation of sTNF-R2. As shown previously (Pennica et al., 1992a), the values determined for the complex molecular weight (Table I) are not highly dependent on the assumption of stoichiometry used to compute the partial specific volume. The values determined suggest that two to three molecules of receptor are bound per trimer of TNF-α or TNF-β. Interestingly, the molecular weight of the sTNF-R2·TNF-α complex is greater at a 3:1 than at a 2:1 ratio, whereas the sTNF-R2·TNF-β complex molecular weight is the same at both ratios. This suggests that there may be differences in how these two cytokines bind to sTNF-R2.

Cross-Linking of TNF-α with sTNF-R2. To visualize the receptor–ligand complexes, [^{125}I]TNF-α was incubated alone or with sTNF-R2 at room temperature and cross-linked by the addition of BSOCOES. The cross-linked ligand–receptor products were then immunoprecipitated with a monoclonal antibody to sTNF-R2 and analyzed by SDS–PAGE. Cross-linking of [^{125}I]TNF-α to sTNF-R2 yielded two cross-linked products with apparent sizes of 135 and 191 kDa (Figure 7). It is possible that these products represent one TNF-α trimer complexed with two and three sTNF-R2 molecules, respectively. The smaller complex observed at a molecular mass of approximately 59 kDa is the size expected for one sTNF-R2

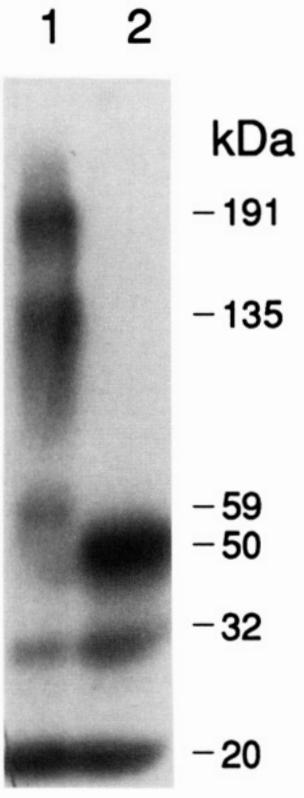

FIGURE 7: Cross-linking of [^{125}I]TNF-α to sTNF-R2. [^{125}I]TNF-α (0.7 nM) was incubated either alone or with sTNF-R2 (1.5×10^{-7} M) and cross-linked as described under Materials and Methods. Following immunoprecipitation, the products were resolved on a 4–12% Tris/glycine/polyacrylamide gel followed by autoradiography. Lane 1, [^{125}I]TNF-α + sTNF-R2 immunoprecipitated with the sTNF-R2 specific monoclonal antibody 2E8. Lane 2, [^{125}I]TNF-α only, immunoprecipitated with the TNF-α specific monoclonal antibody B. The approximate molecular masses of the protein bands in both lane 1 and lane 2 are indicated to the right of the figure.

molecule cross-linked to one TNF-α monomer (43 kDa + 17 kDa). The minor products seen migrating at approximately 17 and 32 kDa are monomer and dimer forms of TNF-α that are coimmunoprecipitated with receptor. As shown in lane 2, cross-linking of [^{125}I]TNF-α alone yields predominantly trimer migrating at a molecular weight of about 50 000 with minor amounts of dimer and monomer.

DISCUSSION

In this report, we describe the generation and characterization of the recombinant extracellular domain of the 75-kDa human TNF receptor. Purified sTNF-R2 shares several properties with the full-length TNF-R2 expressed on whole cells. Most importantly, it binds both TNF-α and TNF-β with high affinity. Also, it has the same N-terminal sequence, Leu-Pro-Ala-Gln-, determined for native TNF-R2 on HL60 cells (Loetscher et al., 1990b) and contains both N- and O-linked glycosylation.

The extracellular domain of TNF-R2 contains potential N-linked glycosylation sites at Asn-149 and Asn-171 and a C-terminal region that is rich in serine, threonine, and proline residues, suggestive of O-linked glycosylation (Russell, 1984; Wilson et al., 1991). Characterization of the two potential N-linked sites was performed by Asp-N peptide mapping of the glycosylated and deglycosylated sTNF-R2 in conjunction with Edman degradation and mass spectrometry. Asn-149 is fully glycosylated while Asn-171 is about 50% glycosylated. Monosaccharide composition analysis of the peptide rich in Pro, Ser, and Thr residues (peptide A9, residues 175–234) demonstrated that this peptide contains O-linked carbohydrate attached to several of the Ser and/or Thr residues. The apparent molecular mass of sTNF-R2 determined by sedimentation equilibrium (36.0 ± 4.8 kDa) implies the presence of 11 ± 5 kDa of glycosylation (predicted molecular mass of nonglycosylated sTNF-R2 ~25 kDa). This is consistent with a previous report that N- and O-linked glycosylations contribute 2–4 and 6–10 kDa, respectively, to the molecular mass of the 75-kDa receptor (Hohmann et al., 1989).

Gel filtration analysis of sTNF-R2 yielded a significantly higher size estimate (~136 kDa) than did SDS–PAGE analysis (~43 kDa) or analytical ultracentrifugation (36 ± 4.8 kDa). The data from the sedimentation equilibrium analysis demonstrate that sTNF-R2 exists predominantly as a monomer in solution. Anomalous behavior on gel filtration has been previously observed for the extracellular domains of the related molecules TNF-R1 (Loetscher et al., 1991a; Pennica et al., 1992a) and the p75 NGF receptor (Vissavajjhala & Ross, 1990), something that has been attributed to the cysteine-rich repeats forming an elongated rodlike structure. It is also likely that the presence of O-glycosylation in sTNF-R2 contributes to the extremely rapid migration of sTNF-R2 on gel filtration. Recent studies have demonstrated that even relatively short O-glycosylated sequences have a significant effect on the size and shape of a glycoprotein since steric interactions between the carbohydrate and adjacent amino acids induce the peptide core to form a stiff and extended conformation (Jentoft, 1990). In fact, gel filtration analysis of a variant of sTNF-R2 lacking this O-linked region yielded a much smaller molecular size (unpublished observations), consistent with that previously observed for sTNF-R1 (Pennica et al., 1992a).

The purified sTNF-R2 was found to be functional as determined by its ability to form high-affinity complexes with TNF-α (K_d ~0.3 nM) and TNF-β (K_d ~0.75 nM) in solution. sTNF-R2 was also found to neutralize the cytotoxic activity of TNF-α (EC_{50} ~30 nM) more efficiently than TNF-β (EC_{50} ~500 nM). However, these EC_{50} values are about 75–250-fold higher than the K_i values determined from the binding displacement studies. Possible explanations for this discrepancy are the extreme sensitivity (detection limit ~1 pg/mL TNF-α) and long incubation time (48 h) of the L-M cell cytotoxicity assay. Another possibility is that the monoclonal antibody used in the binding assay (but not included in the bioassay) increases the affinity of sTNF-R2 via dimerization of the extracellular domain. Consistent with this interpretation, we have found that the inclusion of anti-TNF-R2 monoclonal antibodies increases the ability of sTNF-R2 to neutralize TNF-α (data not shown).

Size-exclusion chromatography, sedimentation equilibrium, and cross-linking were performed to determine the stoichiometry of binding between sTNF-R2 and TNF-α or TNF-β. These studies indicate at least two and possibly three molecules of sTNF-R2 can bind to one TNF-α or TNF-β trimer and are consistent with what we have observed by cross-linking TNF-α and TNF-β to 293 cells overexpressing full-length TNF-R2 (Pennica et al., 1992b). In those studies, we found high molecular mass complexes (220 and 280 kDa) with TNF-β which could be consistent with two and three TNF-R2 molecules cross-linked with one TNF-β trimer, respectively. Cross-linking with TNF-α, under similar conditions, showed only a 220-kDa product presumably consisting of two TNF-R2 molecules complexed with one TNF-α trimer.

Receptor aggregation is a widespread phenomenon and is known to play an important role in transmembrane signaling,

ligand-induced internalization, and down-regulation (Kahn et al., 1978; Schlessinger et al., 1978; Yarden & Schlessinger, 1987). In this report, we show that TNF-α and TNF-β can simultaneously bind two or three sTNF-R2 molecules. It is interesting to note that the majority of monoclonal antibodies (mAbs) to TNF-R2 have direct agonist activity (unpublished observations) while agonist activity by the majority of mAbs to TNF-R1 requires either cross-linking with a second antibody or a combination of at least two mAbs (Engelmann et al., 1990b; Wong et al., 1992). This may be because TNF-R2 is more readily activated by aggregation of only two receptors per complex even though the trimeric structure of TNF would be more consistent with a 3:1 binding ratio. Although the mechanism by which TNF-R2 initiates signal transduction is not yet clearly understood, the data presented here and elsewhere (Tartaglia & Goeddel, 1992; Tartaglia et al., 1991; Pennica et al., 1992b) provide evidence that TNF-R2 oligomerization by TNF-α, TNF-β, or receptor specific antibodies is an important first step in the activation of this receptor.

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