Eur. J. Biochem. 223, 831–840 (1994)

Characterization of ligand binding by the human p55 tumour-necrosis-factor receptor

Involvement of individual cysteine-rich repeats

Anne E. CORCORAN[1], Kathy BARRETT[1], Martin TURNER[1,3], Alex BROWN[1,3], Anna-Maija KISSONERGHIS[1], Margaretha GADNELL[2], Patrick W. GRAY[1,4], Yuti CHERNAJOVSKY[1] and Marc FELDMANN[1]

[1] The Mathilda and Terence Kennedy Institute of Rheumatology, Sunley Division, London, England
[2] Pharmacia Biosensor AB, Uppsala, Sweden
[3] NIMR, Mill Hill, London, England
[4] ICOS Corporation, Bothell, Washington, USA

(Received March 1/May 18, 1994) – EJB 94 0294/3

Two soluble tumour-necrosis-factor-α(TNF)-binding proteins are derived from the extracellular domains of the p55 and p75 TNF receptors. They are considered to play a pivotal regulatory role in TNF-mediated inflammatory processes, including diseases such as rheumatoid arthritis, by competing with the cell surface receptors for TNF and lymphotoxin (LT, tumour-necrosis factor β). The extracellular domains of the two receptors each contain four similar cysteine-rich repeats of about 40 amino acids, in common with several other cell surface proteins including the p75 nerve-growth-factor receptor and the CD40 and Fas antigens. The aim of this study was to characterize the involvement of the four cysteine-rich repeats of the human p55 TNF receptor in TNF and LT binding by both membrane-bound and soluble forms of the receptor. Individual repeats were systematically deleted by PCR mutagenesis and the variants transiently expressed in COS cells. Immunoprecipitated receptor variants exhibited the expected sizes on SDS/PAGE gels, and bound a panel of conformation-dependent anti-(TNF receptor) antibodies. Binding of TNF by the four soluble derivatives was compared with binding by the wild-type soluble receptor using a TNF-affinity column and a BIAcore™ Biosensor, by measurement of their ability to inhibit TNF cytotoxicity on WEHI cells, and ^{125}I-TNF binding to U937 cells. Δ4, which lacks the fourth cysteine-rich repeat, bound TNF comparably with the full-length soluble receptor. TNF-binding affinity was unaltered by deletion of the fourth membrane-proximal cysteine-rich repeat, as determined by Scatchard analysis of the transmembrane derivatives. We conclude that the fourth cysteine-rich repeat is not required for TNF binding. In contrast, both the soluble and the transmembrane derivatives lacking any one of the first, second or third repeats failed to bind TNF. Although we cannot entirely exclude the possibility that this may be due to indirect conformational change, rather than the removal of essential epitopes, our results suggest that the first three repeats are each required for TNF binding by both the soluble and the cell-surface receptor.

Tumour-necrosis factor α (TNF), a pleiotropic cytokine produced primarily by mononuclear phagocytes, plays a pivotal role in a wide variety of immune and inflammatory responses. These include septic shock, cachexia [1], cerebral malaria [2] and rheumatoid arthritis (RA) [3, 4]. TNF exerts its biological effects by interaction with high-affinity cell surface receptors, which also bind the structurally related cytokine, lymphotoxin (LT) [5]. Two distinct human TNF receptors (TNF-R) have been cloned and characterized, each of which binds TNF and LT. These are a 55-kDa species designated p55 TNF-R [6–8] and a 75-kDa species designated p75 TNF-R [9]. The ligand-binding region resides in the extracellular domain of each of these receptors. Previously two soluble TNF-binding proteins, termed TBP I and TBP II, were observed in human serum and urine. These were shown to be homologous to the extracellular domains of the p55 and p75 TNF-R, respectively [10, 11]. They are considered to be generated by proteolytic cleavage of the mature cell surface receptor [12]. Their levels are elevated in a number of disease states such as RA [13] and cancer [14], and thus they may function as physiological regulators of TNF-mediated inflammatory processes [13].

The two TNF receptors exhibit 28% similarity at the amino acid level. This is confined to the extracellular domain (ECD) and consists of four repeating cysteine-rich motifs, each of approximately 40 amino acids. Each motif contains four to six cysteines in conserved positions, indicating a high degree of structural similarity within and between the receptors. Dayhoff analysis [8] shows greatest intersubunit similarity among the first three repeats in each receptor. This characteristic structure is shared with a number of other receptors and cell surface molecules, which together comprise the TNF-R/nerve-growth-factor(NGF)-receptor superfamily.

Correspondence to A. E. Corcoran, The Mathilda and Terence Kennedy Institute of Rheumatology, Sunley Division, Lurgan Avenue, London, England W6 8LW
Fax: +44 81 563 0399.

Abbreviations. TNF, tumour-necrosis factor α; LT, lymphotoxin, tumour-necrosis factor β; TNF-R, TNF receptor; ECD, extracellular domain; RU, resonance units; RA, rheumatoid arthritis; NGF, nerve growth factor; DMEM, Dulbecco's modified Eagle's medium; CAT, chloramphenicol acetyltransferase.

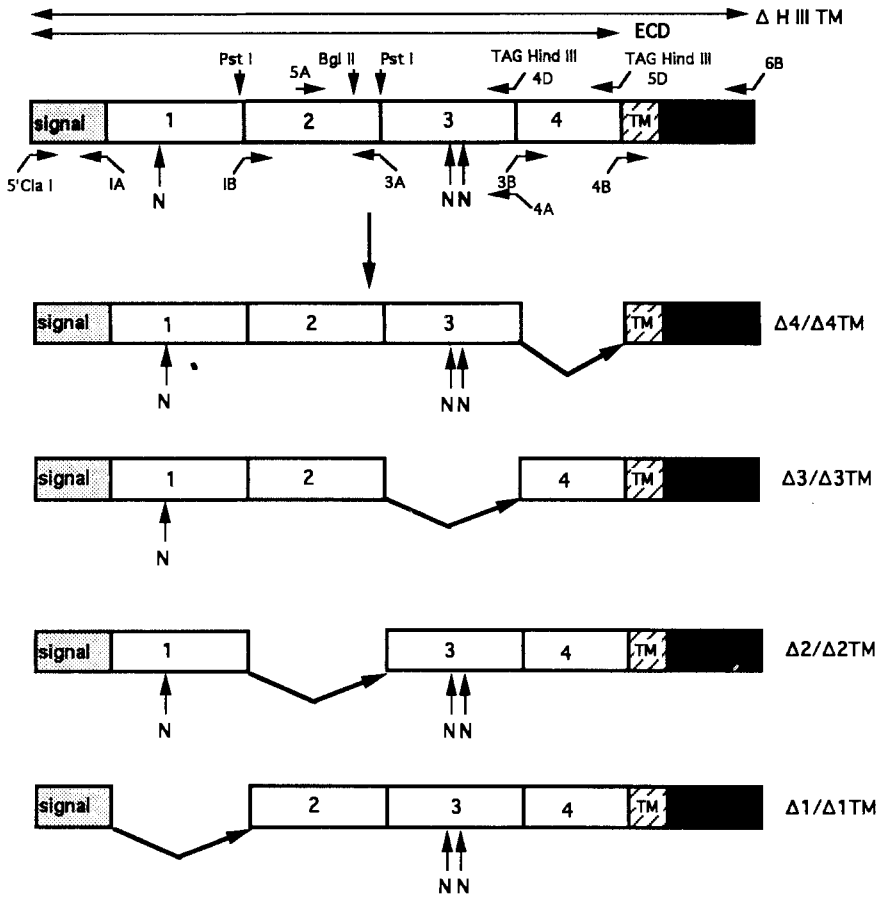

Fig. 1. PCR mutagenesis of the p55 TNF-R. A schematic diagram of the strategies used to derive four soluble and four membrane-bound derivatives of the p55 TNF-R. Stippled box, signal peptide, residues −41 to −1; white boxes, cysteine-rich repeats; TM, transmembrane domain; hatched boxes, extra region included in the membrane-bound constructs. The positions of primers 5′ Cla I, IA, IB, 3A, 3B, 4A, 4B, 4D, 5A, 5D and 6A are indicated by horizontal arrows. TAG indicates an engineered stop codon. N represents the three putative N-linked glycosylation sites at positions 14, 105 and 111. Important restriction sites are shown with vertical arrows.

These include the low-affinity NGF receptor [15], the CD40 B cell surface antigen [16], the Shope fibroma virus T2 open reading frame [17] and Fas antigen [18]. Furthermore, the ligands for a number of these molecules have similar structures and comprise the TNF superfamily. These include TNF, LT, LTβ [19] and the CD40 ligand [20]

Crystallographic studies of TNF [21] and LT [22] have shown that both cytokines exist as homotrimers, with subunits packed edge to edge in a threefold symmetry. This appears to be the active form of TNF [23] which initiates signal transduction by cross-linking two or more cell surface TNF-R [24]. Structurally neither TNF nor LT reflect the repeating pattern of their receptors. Each monomer is cone shaped and contains two hydrophilic loops on opposite sides of the base of the cone. A restricted locus within this region is necessary for receptor interaction [25, 26]. Recent crystallization of a p55 soluble TNF-R/LT complex [27], which depicts the receptor as a non-overlapping end to end assembly of the four cysteine-rich repeats, has confirmed the hypothesis that loops from adjacent monomers join together to form a groove between monomers, and that TNF-R bind in these three intersubunit grooves [21].

The aim of our study was to define functionally the regions of the p55 TNF-R involved in its interaction with TNF and LT, both to increase our understanding of the binding mechanism, and to define a minimal TNF-binding moiety which could be useful for therapeutic purposes. Given the small area of TNF and LT putatively involved in binding, these regions might similarly constitute only a proportion of the extracellular domain of the receptor. We focused on the cysteine-rich repeats, reasoning that their repeating pattern may reflect functional redundancy, whereby only some of the repeats participate in TNF-binding. In the structurally related NGF receptor, only the third and fourth cysteine-rich repeats are involved in NGF binding [28]. They might also confer flexibility on the receptor, whereby any one of thc repeats would be sufficient for TNF-binding.

We chose an approach of systematic deletion of single repeats from the cell surface and soluble receptors, both to analyse the individual repeats and further to investigate whether these repeats could substitute for one another. Our results show that for both the cell surface and soluble receptor, removal of the fourth cysteine-rich repeat does not alter TNF binding, whereas removal of any one of the first three cysteine-rich repeats results in complete loss of ligand binding.

MATERIALS AND METHODS

Reagents

Recombinant human TNF and LT, purified from *Escherichia coli*, were generously supplied by Genentech Inc. Specific activities were 5.6×10^7 U/mg and 2.1×10^8 U/mg, re-

spectively, as measured in the murine L929 cell cytotoxicity assay. TNF and LT were radioiodinated with $Na^{125}I$ (IMS30, Amersham) and Iodogen (Pierce) to specific activities of 40–70 mCi/mg, according to the manufacturer's instructions. Rabbit polyclonal anti-TBP1 serum [11], raised against TBP 1 purified from human urine, and biotinylated rabbit polyclonal anti-(p55 TNF-R) serum, were kind gifts of Dr D. Wallach (Weizmann Institute, Rehovot, Israel) and Dr W. Buurman (University of Limburg, The Netherlands), respectively. Anti-(p55 TNF-R) mAb were generously provided as follows: TBP-1 [29], Dr G. Adolf (Bender & Co., Ges mBH, Austria); H398 [30], Dr K. Pfizenmaier (Max-Planck Society, Gottingen, Germany); 1H7, Dr W. Buurman (University of Limburg, The Netherlands); 982, 984 and 986 [31], Dr D. Goeddel (Genentech Inc., South San Francisco CA); htr9 [32], Dr M. Brockhaus (Hoffman-La Roche AG, Basel, Switzerland).

Cell lines

The monkey kidney fibroblast cell line COS-7 (ATCC, Rockville MD) and the WEHI 164 clone 13 mouse fibrosarcoma cell line (Dr T Meager, NIBS, South Mimms, England) were maintained in Dulbecco's modified Eagle's medium (DMEM; Flow Labs.), with 5% heat-inactivated foetal bovine serum, at 37°C, 10% CO_2. The human monocytic cell line U-937 (ICRF, London) was maintained in RPMI 1640 (Flow Labs.) with 10% foetal bovine serum, at 37°C, 5% CO_2.

Generation of TNF-R deletion mutants

Soluble receptor mutants

The plasmid pTNFRecd [6], which encodes the soluble p55 TNF-R in a mammalian expression vector under the transcriptional control of a cytomegalovirus immediate-early promoter was further modified to remove 5′ untranslated sequences to yield ECD. The resultant construct encodes the signal peptide, a two amino acid spacer, followed by the four cysteine-rich repeats, and terminating with the six succeeding amino acids adjacent to the transmembrane domain. The four variants were constructed as depicted in Fig. 1 and designated $\Delta 1-\Delta 4$ to denote the deleted cysteine repeat. To generate $\Delta 1$, the gel-purified products of two PCR using 5′Cla I/1A and 1B/5D primers were mixed and amplified using the external primers 5′Cla I and 5D. The fragment was digested with *Cla*I and *Bgl*II and cloned into *Cla*I/*Bgl*II digested ECD. Similarly, PCR using 5′Cla I/3A and 3B/5D primer pairs yielded $\Delta 3$. Digestion of ECD with *Pst*I and religation generated $\Delta 2$. A PCR fragment generated with oligonucleotides 5A and 4D, was restricted with *Bgl*II and *Hin*dIII and cloned into *Bgl*II/*Hin*dIII digested ECD to yield $\Delta 4$. The sequence integrity of the clones was ascertained by DNA sequencing using Sequenase™ (United States Biochemical Corporation).

Transmembrane mutants

The wild-type construct, ΔHindIII TM, was derived from p55 TNF-R cDNA [6] by excision of cDNA sequence 3′ of the unique *Hin*dIII site at position 967 and addition of a termination codon, to provide sequence coding for the signal peptide, the extracellular and transmembrane domains and 37 amino acids of the cytoplasmic domain. The four variants were constructed as shown in Fig. 1 and designated $\Delta 1$TM, $\Delta 2$TM, $\Delta 3$TM and $\Delta 4$TM. To generate $\Delta 1$TM, the *Cla*I–*Bgl*II fragment was excised from $\Delta 1$ and ligated into ΔHindIII TM. Digestion of ΔHindIII TM with *Pst*I, and religation, yielded $\Delta 2$TM. PCR amplification with 5′ *Cla*I/3A and 3B/6B primers yielded $\Delta 3$TM. Similarly, 5A/4A and 4B/6B primers yielded $\Delta 4$ TM. The sequences of the primers were as follows:

5′Cla I, 5′GTTCTATCGATAAGAGGCCATAGCTGTCTGGC-3′;

1A, 5′GCTCTCACACTCTCTCTTCTCCCTGTCCCCTAG-3′;

1B, 5′-AGGGAGAAGAGAGAGTGTGAGAGCGGCTCCTTC-3′;

3A, 5′-TGCATGGCAGGTACACACGGTGTCCCGGTCCAC-3′;

3B, 5′-GACACCGTGTGTACCTGCCATGCAGGTTTCTTT-3′;

4D, 5′-GGCCAAGCTTCAGGTGCACACGGTGTTCTG-3′;

5A, 5′-GCTGCTCCAAATGCCGAAAG-3′;

5D, 5′-AGTTCAAGCTTTACAGTGCCCTTAACATTCTAA-3′;

4A TM, 5′-GCCTGAGTCCTCGCAGGTGCACACGGTGTTCTG-3′;

4B TM, 5′-GTGTGCACCTGCGAGGACTCAGGCACCACAGTG-3′;

6A TM, 5′-CGGCGTCGACCTAGAAGCTTGGGTTTGGGGCCAG-3′.

The amino acids deleted were as follows: $\Delta 1/\Delta 1$TM, D1–R42; $\Delta 2/\Delta 2$TM, E43–R88; $\Delta 3/\Delta 3$TM, G86–C126; $\Delta 4/\Delta 4$TM, C128–T171.

Mammalian cell expression of mutant constructs

The constructs were transiently transfected into COS-7 cells using standard calcium phosphate techniques. 5×10^5 cells in 90-mm dishes were cotransfected with 20 μg wild-type or variant plasmid plus 1 μg PBLCAT9, a plasmid containing the chloramphenicol acetyltransferase (CAT) reporter gene [33]. After 24 h, the cells were glycerol shocked with 10% glycerol in DMEM for 4 min. After a further 48 h, cells expressing membrane-bound constructs or supernatants from cells expressing soluble derivatives were harvested and tested immediately, or stored at −70°C in 1 mM phenylmethlysulphonyl fluoride and 1 mM EDTA, 0.2 g/l BSA.

Metabolic ^{35}S-radiolabelling and immunoprecipitation

52 h after transfection, the COS-7 cells were washed with NaCl/P_i 137 mM NaCl, 20 mM sodium phosphate pH 7.4).

3 ml serum-free DMEM without cysteine, but containing L-[^{35}S]cysteine at 75 mCi/ml (NEN Dupont, specific activity 1210 Ci/mmol), was added to the cells for 8 h at 37°C. Labelled supernatants were harvested, supplemented with 1 mM phenylmethylsulphonyl fluoride, 1 mM EDTA, and 0.2 g/l BSA and frozen at −70°C. Adherent COS-7 cells were removed from the culture vessel with 0.02% EDTA in NaCl/P_i, using a rubber policeman. Cell pellets were incubated on ice in 1 ml lysis buffer (10 mM Tris pH 7.4, 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride) for 30 min, centrifuged to remove nuclei and frozen at −70°C.

^{35}S-labelled supernatants (900 µl) or cell lysates (500 µl) were cleared with normal rabbit serum, followed by three cycles of 100 µg of goat anti-rabbit immunoglobulin H and L chain Immunobeads™ (Bio-Rad Laboratories Ltd.). The supernatants were incubated with rabbit polyclonal anti-TBP 1 at 0.5 M NaCl for 5 h at 4°C, followed by overnight rotation with 100 µg goat anti-rabbit Immunobeads. The Immunobeads were washed in lysis buffer supplemented to 0.5 M NaCl, 0.1% SDS, and finally in 10 mM Tris, pH 7.4, 0.1% SDS. Equal amounts of trichloroacetic-acid-precipitable radioactivity (5×10^5 cpm) were resolved on 10% SDS/PAGE gels under reducing conditions and autoradiographed. The autoradiographs were scanned on a densitometer and integral values in a linear range were used to determine relative levels of protein expression.

mAb-binding analysis by ELISA

Soluble receptor derivatives were captured on microtitre plates by a previously coated panel of individual mAb raised to the soluble p55 TNF-R. Bound derivatives were detected by biotinylated anti-(p55 TNF-R) polyclonal serum, followed by streptavidin-biotin conjugated horseradish peroxidase, and a colour reaction with tetramethylbenzidine hydrochloride.

Affinity purification of soluble receptor derivatives

5-ml aliquots of ^{35}S-labelled supernatants were applied to a 0.3 ml TNF-affinity column (5 mg human TNF coupled to Affigel 15; Biorad). Bound protein was eluted with a pH gradient (0.1 M sodium phosphate, pH 7.5 to 0.1 M citric acid, pH 2.1). 500-µl fractions were neutralised with 1 M Tris, pH 8.0. Protein elution was followed by scintillation counting and peak fractions were autoradiographed on SDS/PAGE gels.

BIAcore™ biosensor analysis of TNF-TNFR interactions

BIAcore™ is a biosensor based system for real-time BIA (biospecific interaction analysis). Its detection principle is based on surface plasmon resonance, which measures the interactions of biomolecules close to a surface. Recombinant human TNF (50 µg/ml) was immobilized on a Pharmacia Biosensor CM5 dextran-coated sensor chip by amino-coupling at pH 4.2, using the Amine Coupling kit (Pharmacia Biosensor AB). COS cell supernatants containing soluble TNF-R derivatives were injected and their binding to TNF measured in real time by changes in optical properties near the sensor chip surface. The TNF sensor surface was regenerated with 10 mM HCl.

WEHI cytotoxicity assay

COS-7 cell supernatants were concentrated on Amicon™ concentrators (molecular-mass cut-off 10 kDa) and assayed for their inhibition of the cytotoxic effect of TNF on the TNF-sensitive murine cell line WEHI 164 clone 13 [34]. TNF (100 pg/ml) was incubated with twofold dilutions of concentrated supernatants at 37°C for 1 h and assayed as described [34].

Inhibition of binding of ^{125}I-labelled TNF to U937 cells

Increasing dilutions of COS cell supernatants were incubated with 1 nM ^{125}I-labelled TNF for 1 h at 37°C then added in triplicate to U937 cells (2×10^6 cells/well) in 96-well plates, for 2 h at 4°C. Non-specific binding was determined by prior incubation of cells with 250 nM unlabelled TNF. Cells were spun through 20% sucrose in NaCl/P_i to remove unbound TNF and analysed on a γ counter.

Plate binding assay on transmembrane derivatives

2.5×10^5 COS-7 cells were transiently transfected with 10 µg DNA encoding the transmembrane derivatives. The cells were washed with NaCl/P_i and incubated for 2 h at 4°C with 1 nM ^{125}I-labelled TNF or LT, either alone or with 250 nM unlabelled cytokine. Cells were washed with NaCl/P_i/1% BSA, lysed with 1 ml 1% Triton X100, 10% glycerol and 25 mM Hepes, pH 7.4, and analysed on a γ counter.

Binding assays and Scatchard analysis

COS-7 cells transfected with the transmembrane constructs were harvested with 0.02% EDTA in NaCl/P_i after 72 h. They were incubated in triplicate (1×10^6 cells/well) with increasing concentrations of ^{125}I-labelled TNF (0.1 to 50 nM) at 4°C for 3 h. Non-specific binding was determined with a 300-fold molar excess of unlabelled TNF. Cells were spun through 20% sucrose and pellets and supernatants analyzed on a γ counter. Binding affinities were calculated by Scatchard analysis.

RESULTS

Construction and expression of TNF-R derivatives

A soluble derivative of the p55 TNF-R encoding the ECD, and a membrane-bound receptor truncated at the 3′ end (ΔHindIIITM) were constructed. Four derivatives of each were engineered by PCR mutagenesis, each lacking one of the four extracellular cysteine-rich repeats, as depicted in Fig. 1. To verify that the TNF-R constructs had been correctly translated and the expected proteins expressed, ^{35}S-labelled COS-7 cell lysates and supernatants were immunoprecipitated with anti-TBP1 polyclonal serum and analysed on SDS/PAGE gels, as shown in Fig. 2. Each of the variants was successfully immunoprecipitated with the polyclonal serum. The different reduced relative molecular masses observed in comparison with the wild-type ECD or ΔHindIIITM are consistent with the deletion of one repeat from each variant and one or two glycosylation sites from Δ1 and Δ3, respectively. These three putative N-glycosylation sites (Fig. 1) contribute approximately 33% of the molecular mass of 32 kDa for the ECD [35]. The wild-type ECD (Fig. 2A) produced a major band of 32 kDa and a minor band of 28 kDa, probably indicating different glycosylation patterns, as previously observed [36]. The presence of major and minor bands was also observed in Δ2 and Δ1 (Fig. 2A). The relative molecular masses of Δ4, Δ3, Δ2 and Δ1 were 29, 21,

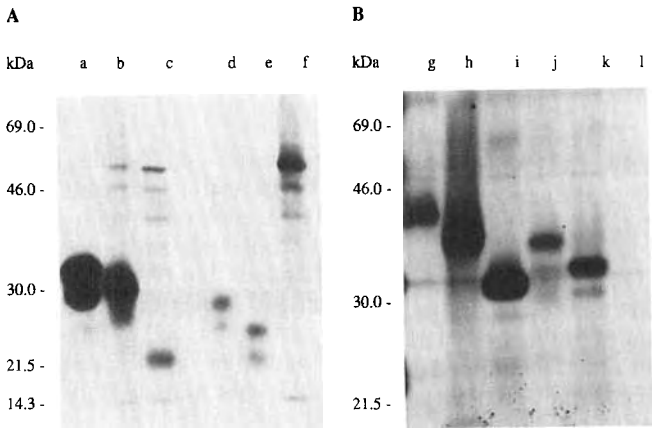

Fig. 2. Immunoprecipitation and SDS/PAGE analysis of TNF-R derivatives. COS-7 cells transfected with soluble and transmembrane TNF-R constructs were labelled for 8 h with [^{35}S]cysteine. The supernatants and cell lysates were immunoprecipitated with a polyclonal anti-(soluble p55 TNF-R) serum. The precipitated products were resolved on 10% polyacrylamide gels under reducing conditions and autoradiographed. (A) Soluble derivatives: lane a, wild-type ECD; lane b, Δ4; lane c, Δ3; lane d, Δ2; lane e, Δ1; lane f, supernatant from cells transfected with vector alone. The numbers on the left indicate the position of ^{14}C-labelled molecular-mass standards. (B) Transmembrane derivatives: lane g, wild-type ΔHindIII-TM; lane h, Δ4TM; lane i, Δ3TM; lane j, Δ2TM; lane k, Δ1TM; lane l, cells transfected with vector alone.

27 and 24 kDa, respectively. The transmembrane derivatives exhibited masses of 40.5, 37, 30, 35 and 32 kDa for ΔHindIII-TM, Δ4TM, Δ3TM, Δ2TM and Δ1TM, respectively.

The immunoprecipitations were performed with equal amounts of trichloroacetic-acid-precipitable radioactivity. However, the amounts of soluble receptor protein immunoprecipitated with the polyclonal serum varied considerably. A representative experiment is shown in Fig. 2A and B. Scanning densitometry showed the full-length ECD and Δ4 to be equivalent, while Δ1, Δ2 and Δ3 consistently expressed 10–15-fold less immunoprecipitable protein. This was not due to differences in transfection efficiency, as measurement of cotransfected chloramphenicol acetyl-transferase (CAT) activity indicated that the cells were transfected with similar efficiencies (data not shown). It is possible that deletion of repeats might have altered receptor conformation such that the polyclonal serum did not recognize some epitopes. We consider this unlikely since the polyclonal serum recognized the transmembrane derivatives comparably (with minor variations between experiments). Further, increasing antibody concentration did not alter the amounts immunoprecipitated. We conclude that the soluble derivatives Δ1, Δ2 and Δ3 were expressed or secreted at lower levels than Δ4 and ECD.

Conformation-dependent mAb recognition

To further investigate the possibility that changes in TNF-binding or polyclonal anti-TBP1 immunoprecipitation by the derivatives were due simply to a disruption of conformation, their recognition by a panel of conformation-dependent anti-(p55 soluble receptor) mAb was measured in an ELISA . Due to the lower levels of protein putatively expressed by Δ3, Δ2, and Δ1, freshly harvested supernatants were concentrated so that each sample contained equivalent amounts of immunoprecipitable soluble receptor protein for this and the following assays. The mock supernatant was concentrated to the same extent as the most dilute derivative. Antibodies 982 and 984 are conformation dependent [31]. The other five antibodies were also conformation dependent, since they bound the ECD on Western blots under non-reducing conditions, but not under reducing conditions which would disrupt the three disulphide bonds in each repeat (data not shown). Table 1 shows the proportion of antibody-binding by each derivative relative to the ECD. All four derivatives were recognized by a variety of these antibodies, suggesting that they have retained native conformation. Variations in binding values reflect differences in antibody-binding epitopes. The mAb recognize a variety of epitopes. htr9 and H398 block TNF-binding to the receptor and are agonistic and antagonistic, repectively. 984 also blocks TNF-binding. 982, 986 and TBP1 bind the receptor in the presence of TNF. In addition, comparable antibody binding by appropriately concentrated supernatants suggests that low recognition of Δ1, Δ2 and Δ3 by the rabbit polyclonal serum was indeed due to low expression of the proteins, and not to reduced antibody recognition of the variants.

Characterization of soluble receptor derivatives

Binding of soluble receptor derivatives to a TNF-affinity column

The ability of ^{35}S-labelled COS-7 cell supernatants expressing soluble receptor derivatives to bind specifically to a TNF column was examined. The wild-type ECD exhibited a sharp elution peak at pH 5.2, followed by a second non spe-

Table 1. Binding of anti-(p55 TNF-R) mAb to soluble receptor derivatives. The mAb were raised against purified soluble p55 TNF-R as described [29–32], (Buurman, W., personal comunication). They bind to purified soluble p55 TNF-R under non-reducing, but not under reducing conditions, and are thus conformation dependent. Equivalent concentrations of the soluble receptor derivatives were captured by individual mAb in an ELISA. Relative mAb binding of the derivatives was expressed as a fraction of binding to the ECD.

TNF-R variant	Relative binding of mAb						
	982	984	986	TBP1	H398	HTR9	1H7
ECD	1.00	1.00	1.00	1.00	1.00	1.00	1.00
Δ4	0.53	0.78	0.06	0.02	1.18	1.29	0.08
Δ3	0.05	0.14	0.60	0.01	0.73	0.12	0.16
Δ2	0.13	0.17	0.71	0.85	0.16	0.17	0.82
Δ1	1.21	1.25	1.40	0.70	0.12	0.13	1.29
Mock	0.01	0.02	0.00	0.02	0.02	0.01	0.02

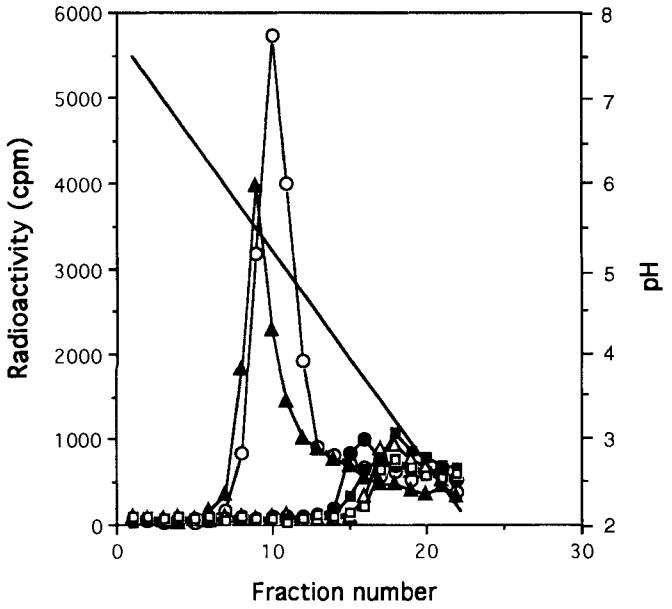

Fig. 3. Affinity binding of ^{35}S-labelled soluble receptor derivatives to a TNF-affinity column. ^{35}S-labelled COS-7 cell supernatants were applied to the column, followed by extensive washing with NaCl/P_i. Fractions were eluted using a pH gradient from pH 7.5 to pH 2.1. (○), ECD; (▲), Δ4; (●), Δ3; (△), Δ2; (■), Δ1; (□), vector alone.

cific peak at low pH (Fig. 3). Δ4 also eluted as a sharp peak at approximately the same pH. In contrast no radioactivity was recovered from the Δ3, Δ2, Δ1 or mock eluates until the pH gradient reached pH 4.0 and non-specific material was eluted. SDS/PAGE analysis of eluted peak fractions revealed TNF-R bands corresponding to those observed in Fig. 2A for ECD and Δ4, whereas no detectable bands were seen with Δ3, Δ2 or Δ1 (data not shown).

Biacore™ biosensor analysis

The binding of wild-type ECD and the four soluble derivatives to TNF was compared, by measuring changes in surface plasmon resonance caused by receptor binding to immobilized TNF [37]. Individual sensograms are overlaid for comparison (Fig. 4). The ECD and Δ4 bind TNF, as shown by the increase in resonance units (RU) over time. The sharp increase and decrease at the beginning and end of the sample pulse, respectively, are caused by the non-specific difference in refractive index between sample and running buffer. The binding of receptor is measured as the final difference in response signal before and after the sample pulse. The values for ECD and Δ4 were 630 RU and 625 RU, respectively. Under a range of conditions Δ3, Δ2 and Δ1 failed to produce an increase in RU, indicating that these derivatives do not bind TNF. An identical profile was observed with supernatant from mock-transfected cells (data not shown).

Inhibition of TNF cytotoxicity in the WEHI assay

The ability of the soluble receptor derivatives to bind and sequester TNF, thus inhibiting its cytotoxic effects, was tested using the TNF-sensitive murine cell line, WEHI 164 clone 13. In Fig. 5 the data is presented in terms of percentage inhibition of cytotoxicity compared with TNF (100 pg/ml) alone. Δ4 consistently inhibited TNF cytotoxicity to the same extent as the full-length ECD. The other three constructs inhibited cytotoxicity to a very small extent, which was however dose dependent and somewhat greater than the basal activity observed with supernatant from mock-transfected cells in four separate experiments.

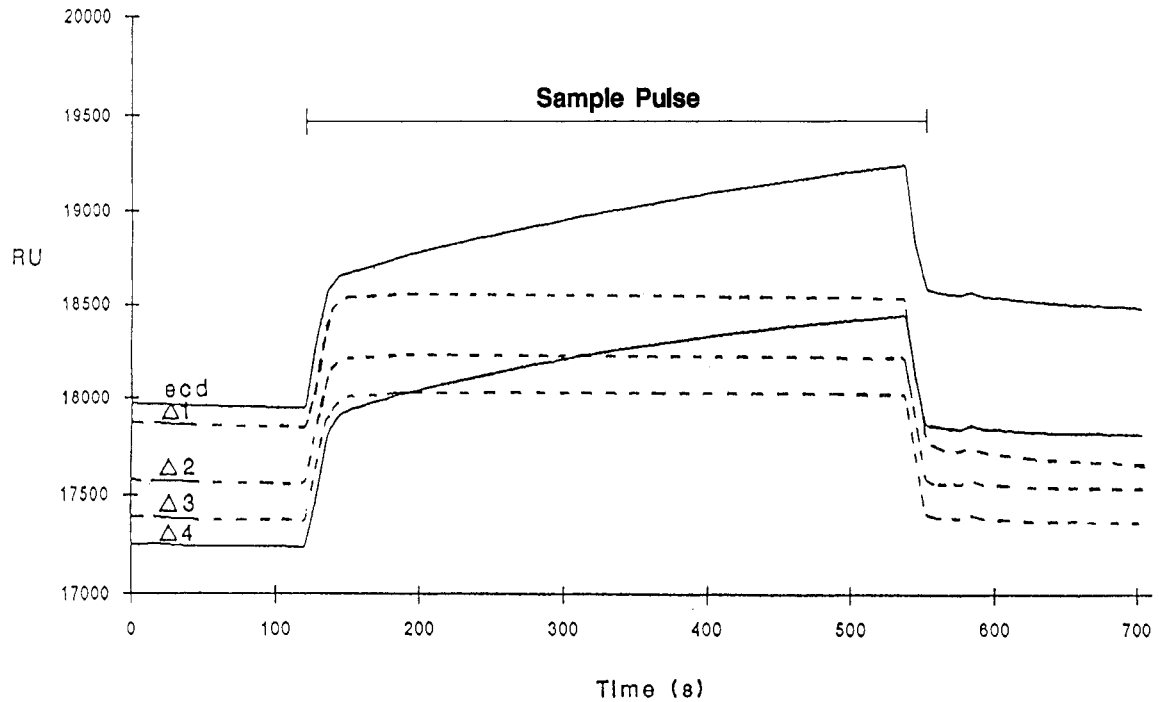

Fig. 4. Binding of soluble TNF-R derivatives to TNF immobilized on a BIAcore™ biosensor. The binding of soluble receptor derivatives to TNF was analysed by changes in surface plasmon resonance, measured in RU. 1 RU is approximately equivalent to 1 pg/mm^3 bound to the immobilized ligand. Individual sensograms are overlaid for comparison. ECD and Δ4 are represented by solid lines. Δ1, Δ2 and Δ3 are represented by dashed lines.

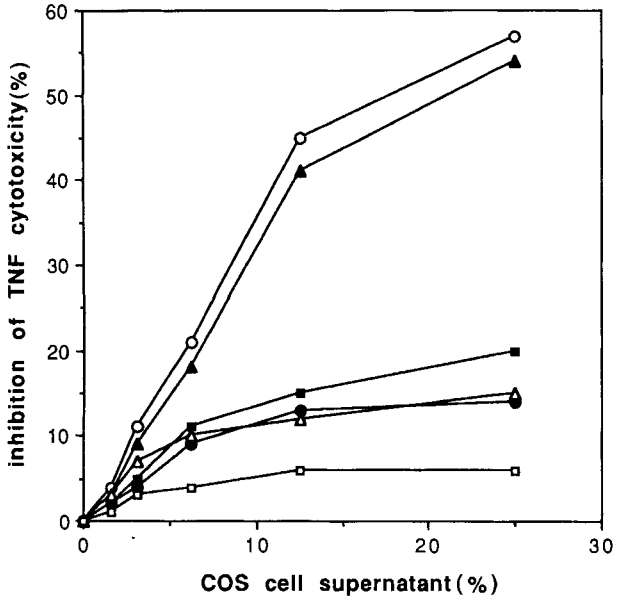

Fig. 5. Inhibition of TNF cytotoxicity in the WEHI 164 line. COS-7 cell supernatants containing p55 soluble receptor derivatives were incubated for 1 h with TNF (100 pg/ml) then incubated overnight in triplicate with WEHI cells. Cell survival was analysed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrozolium bromide dye uptake. Results are presented as percentage inhibition of cytotoxicity observed in the presence of the supernatants in comparison with TNF alone. Fig. 5 shows the results of a representative experiment which was repeated four times with similar results. (○), ECD; (▲), Δ4; (●), Δ3; (△), Δ2; (■), Δ1; (□), vector alone.

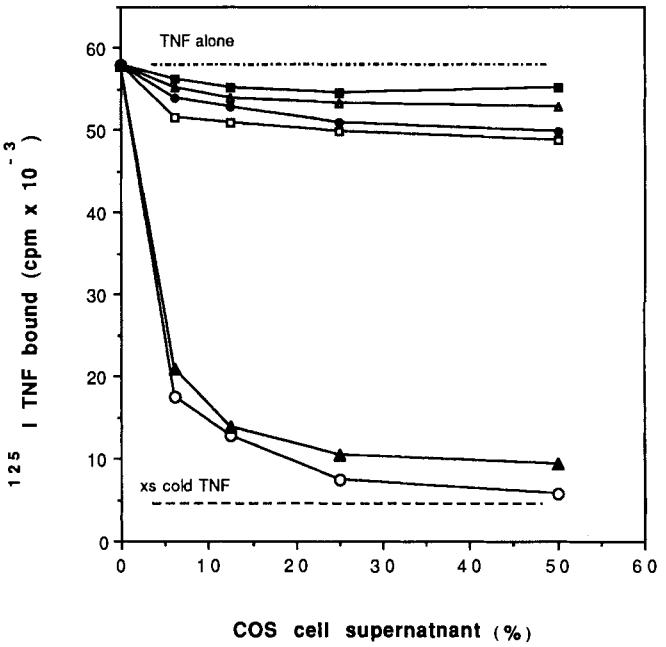

Fig. 6. Effect of soluble TNF-R derivatives on ^{125}I-TNF binding to U937 cells. U937 cells were incubated for 2 h at 4°C with 1 nM ^{125}I-TNF, either alone or following incubation with COS-7 cell supernatants containing soluble receptor derivatives. The cells were pelleted through a 20% sucrose gradient and analysed in a γ counter. Results are represented as reduction in bound radioactivity in the presence of receptor derivatives. (○), ECD; (▲), Δ4; (●), Δ3; (△), Δ2; (■), Δ1; (□), vector alone. Comparative samples contained equal amounts of immunoprecipitable protein.

Inhibition of binding of ^{125}I-labelled TNF to U937 cells

The U937 human monocytic cell line expresses both p55 and p75 TNF-R [32]. In Fig. 6, unconcentrated supernatants from COS cells expressing ECD or Δ4 exhibited very similar dose-dependent and almost complete inhibition of ^{125}I-TNF-binding to U937 cells. In contrast, concentrated supernatants expressing Δ3, Δ2 or Δ1 failed to inhibit ^{125}I TNF-binding to these cells.

Characterization of membrane-bound TNF-R derivatives

Plate binding of ^{125}*I-TNF and LT*

In this assay, relative expression of membrane-bound receptor protein was analysed by parallel immunoprecipitation (data not shown) and binding results were normalized to show binding to comparable numbers of expressed receptors. Fig. 7 shows that the truncated wild-type p55 receptor, ΔHindIIITM, binds both TNF and LT with high affinity. Δ4TM consistently bound both ligands with approximately 90% of the capacity of the wild-type receptor. Δ1TM, Δ2TM or Δ3TM failed to bind either ligand.

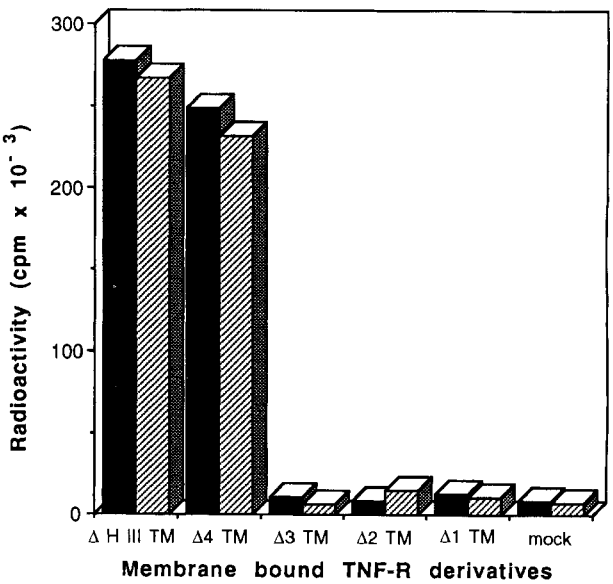

Fig. 7. Binding of ^{125}I-TNF and LT to COS-7 cells expressing membrane-bound TNF-R derivatives. COS-7 cells transfected with TNF-R constructs were incubated with 1 nM ^{125}I-TNF or LT, either alone or with excess unlabelled cytokine. Solubilized cells were analysed in a γ counter. Solid bars, TNF; hatched bars, LT.

Affinity of membrane TNF-R derivatives for TNF

The binding curves in Fig. 8 demonstrate specific saturable binding of TNF by both (A) wild-type ΔHindIIITM and (B) Δ4 TM derivative. Scatchard analysis (Fig. 8 A and B) revealed a considerable increase in binding sites (1.14×10^5 for ΔHindIII, 0.66×10^5 for Δ4) compared with endogenous TNF-R levels from mock-tranfected COS cells (2×10^3, data not shown). A single class of high-affinity binding sites was observed for both, with a K_d (dissociation constant) of 2.2 nM for ΔHindIIITM and a K_d of 2.0 nM for Δ4 TM. This indicates that removal of the fourth cysteine-rich repeat does not alter the affinity of the p55 TNF-R for TNF. The affinity value for the full-length receptor is in agreement with previous findings [6–8, 32, 36].

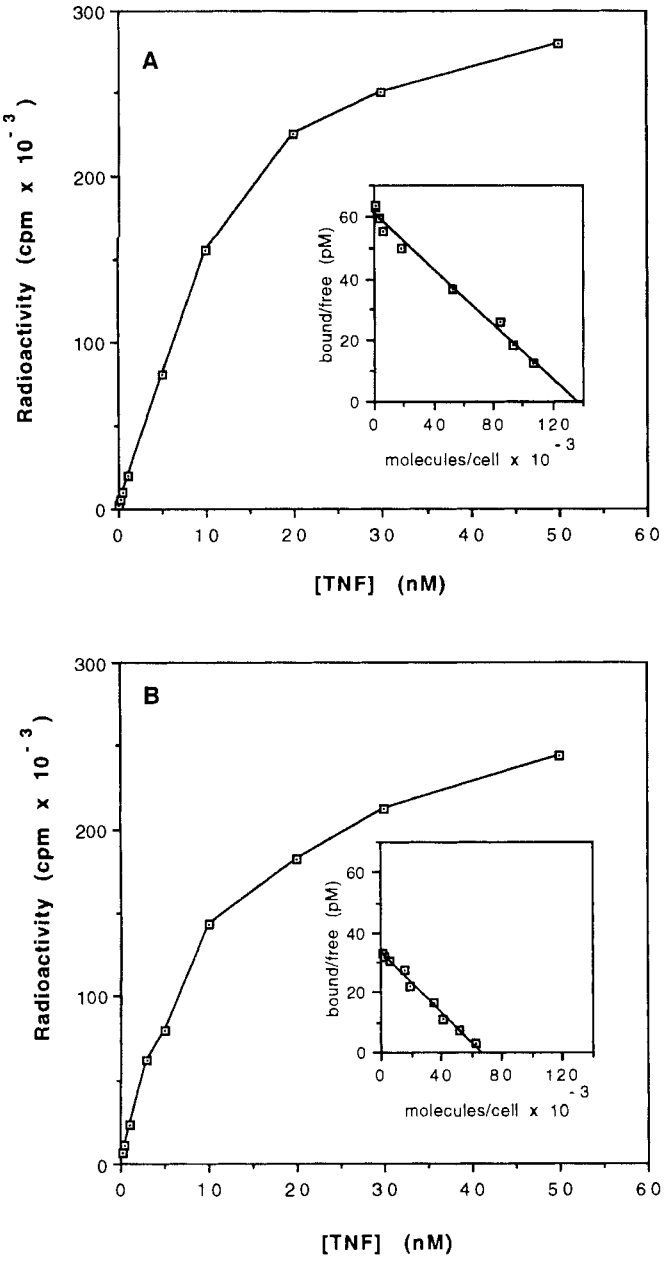

Fig. 8. Binding characteristics of membrane-bound p55 TNF-R derivatives. Saturation isotherms of the specific binding of ^{125}I-labelled TNF to COS-7 cells transiently transfected with either (A) ΔHindIIITM or (B) Δ4TM were performed. Insets show Scatchard analysis of the data. Binding assays were performed four times with similar results. A single representative experiment is shown.

DISCUSSION

The aim of this study was to identify which of the four extracellular cysteine-rich repeats of the p55 TNF-R are involved in TNF and LT binding by the membrane-bound and soluble receptor. We have deleted individual repeats by PCR mutagenesis and analysed the effect of these deletions on TNF and LT binding. In our soluble-receptor studies Δ4, the derivative lacking the fourth cysteine-rich repeat, bound TNF comparably with the wild-type ECD on a TNF-affinity column and a BIAcore™ biosensor and similarly competitively inhibited the binding and cytotoxicity of TNF in the U937 and WEHI assays, respectively. These results demonstrate that the fourth cysteine-rich repeat is not involved in TNF-binding by the soluble TNF-R. In contrast, the remaining three soluble receptor derivatives, lacking the first, second or third cysteine-rich repeat failed to bind to a TNF-affinity column, did not bind TNF immobilized on a BIAcore biosensor, and failed to competitively inhibit ^{125}I-TNF-binding to U937 cells. These derivatives exhibited a small, but reproducible inhibition of TNF cytotoxicity in the WEHI assay. However, it is possible that the longer time span of this assay may permit a small amount of low-affinity binding of TNF, which does not occur in the other three rapidly equilibrating systems. Alternatively, concentration of the supernatants may have concomitantly concentrated an unknown protective factor. The smallness of the effect precludes investigation of its mechanism.

In studies of transmembrane derivatives, Δ1 TM, Δ2 TM and Δ3 TM failed to bind TNF or LT, suggesting that each of the first three cysteine-rich repeats are also necessary for TNF and LT binding by the membrane-bound receptor. Thus it is clear that although the first three repeats exhibit significant structural similarity, they do not have interchangeable or redundant functions. In contrast, Δ4 TM bound TNF and LT efficiently in the plate-binding assay and exhibited an equivalent K_d for TNF to the wild-type ΔHindIIITM (2.0 nM and 2.2 nM, respectively). Thus we conclude that this repeat is not involved in TNF and LT binding by the cell surface receptor.

As the fourth repeat is adjacent to the transmembrane domain, its functional role may be chiefly as an anchor domain or spacer. Its lack of involvement in binding may confer conformational flexibility to the sterically complex interaction of a TNF or LT trimer with up to three TNF-R molecules at the cell surface. It may serve to orient the TNF-R/TNF complex for the intracellular clustering necessary for signalling. Additionally, the location of a putative Asn/Val proteolytic cleavage site three amino acids from the 3′ end of this repeat [38] may provide spatial access for a cleavage enzyme. This concept may be tested by comparing shedding of Δ4 TM and the full-length receptor.

An alternative explanation for loss of TNF-binding by removal of individual repeats, particularly the two second and third internal repeats, might be simply a disruption of receptor conformation. We believe that this is not the case for the following reasons. Each variant was secreted or expressed on the cell surface and specifically immunoprecipitated by a polyclonal anti-TBP1 serum. The soluble derivatives also bound a panel of conformation-dependent anti-(TNF-R) mAb, which recognize diverse epitopes on the soluble receptor (Table 1). In addition, recent crystallographic studies on a soluble human p55 TNF-R-LT complex [27] depict the four cysteine-rich repeats as discrete structural entities arranged end to end with little overlap or interaction between them. The six cysteines in each repeat form three intrachain disulphide bonds evenly spaced over the length of the repeat, indicating a high degree of rigidity. The second and third repeats are structurally superimposable and the amino acids at interdomain junctions are very similar. We thus postulate that removal of one repeat would not significantly alter the conformation of the rest of the receptor. However, the only definitive way to ascertain whether indirect conformational change had occurred would be, for example, to crystallize each derivative and this was not possible within the limits of this study. Thus we do not exclude the possibility that loss of ligand binding induced by removal of one of the first three repeats of the TNF-R may be due to non

specific conformational change, rather than removal of essential epitopes.

In the crystallographic model of LT/soluble TNF-R interaction [27], a number of residues in the second and third cysteine-rich repeats of each soluble receptor are predicted to be in direct contact with LT, which is in agreement with our functional observations of the loss of both LT and TNF-binding when either of these repeats was removed. The crystal structure does not demonstrate such a close involvement in LT binding by the first repeat, since this repeat partially protrudes beyond the base of the LT trimer. Our finding that TNF-binding is abrogated when this repeat is removed, suggests that it may have an important role in stabilizing the receptor-ligand complex. The crystallographic model is only complete as far as amino acid 150 and thus was unable to define the role of the fourth repeat in binding, but we postulated that it protruded beyond the LT trimer and was uninvolved in binding. Here we show functionally that this is indeed the correct prediction for the TNF-R/TNF interaction.

Comparison of TNF and LT binding by the membrane-bound derivatives indicates that despite their surface differences [21, 22], both cytokines interact with the first three cysteine-rich repeats and do not bind to the fourth repeat. We thus demonstrate experimentally that the crystallographic model of the interaction of the soluble receptor with LT [27] may also be extended to its interaction with TNF and may be transposed to the cell surface receptor interaction with both LT and TNF.

Previous analyses of TNF-binding by synthetic peptides spanning the extracellular domain of the p55 receptor suggested a putative binding site in the region of amino acids 159–178 [39], or amino acids 175–194 [40], which correspond to the end of the third repeat and part of the fourth repeat, and the fourth repeat, respectively. This is in contrast to our findings and to the crystallographic data [27]. In the former case, the data must be viewed with caution, as the cysteines were replaced with alanines to facilitate peptide synthesis, and the lack of intrachain disulphide bonds would be expected to change conformation significantly.

In a previous mutagenesis study [31], the first or the fourth cysteine-rich repeat were deleted from a p55 soluble-TNF-R-dimer–human-IgG-heavy-chain chimera, resulting in complete abrogation of biological function and a tenfold reduction in activity, respectively. In the present study, deletion of the first repeat also results in loss of TNF-binding. However, deletion of the fourth repeat does not appreciably alter biological activity. A possible explanation for this discrepancy may be that the IgG–TNF-R fusion protein, which lacks the Ig constant domain CH1, may be less flexible than the native transmembrane and soluble receptor. Thus without the fourth repeat, access of TNF to receptor may be sterically restricted. This hypothesis is supported by studies with $\Delta 4$ fused to an IgG backbone containing three CH domains, which shows comparable activity to soluble receptor (Corcoran, A. E., unpublished results).

A recent study [41] described chimeric receptors of the p55 TNF-R with the p75 NGF receptor, in which either the first two or all four of the TNF-R repeats replaced the corresponding NGF-R receptor sequences. Mutants lacking either pair of TNF-R repeats did not bind TNF whereas those containing all four repeats did. It was concluded that TNF-binding to each TNF-R required four cysteine-rich repeats. In our more detailed study, presented here, we have demonstrated that only three repeats i.e. the first three, are necessary for TNF and LT binding, and not all four as concluded by Hsu et al. [41].

In conclusion, the $\Delta 4$ derivative may be a useful inhibitor of TNF-mediated inflammatory processes, as it possesses the same TNF-binding capacity and has a lower molecular mass than the full-length soluble receptor. Although we cannot entirely exclude the possibility that repeat deletions may have caused indirect conformational change in the receptor, the first three repeats all appear to be integrally involved in TNF-binding by the soluble p55 TNF-R as they cannot substitute for one another despite their similar structure. We have also shown that TNF and LT both interact with the distal three repeats on the cell surface p55 TNF-R. These observations may further provide a model for the interaction of other members of the TNF-R/NGF-receptor family with corresponding members of the TNF ligand family.

We thank Dr Brian Foxwell for critical reading of the manuscript. We also thank D. Wallach for providing rabbit anti-TNF-R serum, and G. Adolf, M. Brockhaus, W. Buurman, D. Goeddel and K. Pfizenmaier for gifts of anti-(p55 TNF-R) mAb. This work was supported in part by the Arthritis and Rheumatism Council and by Centocor Inc., Malvern PA, USA.

REFERENCES

1. Beutler, B. & Cerami, A. (1988) Tumor necrosis, cachexia, shock, and inflammation: a common mediator, *Annu. Rev. Biochem. 57*, 505–518.
2. Grau, G. E., Fajardo, L. F., Piguet, P. F., Allet, B., Lambert, P. H. & Vassalli, P. (1987) Tumor necrosis factor (cachectin) as an essential mediator in murine cerebral malaria, *Science 237*, 1210–1214.
3. Brennan, F. M., Chantry, D., Jackson, A., Maini, R. & Feldmann, M. (1989) Inhibitory effects of TNFα antibodies on synovial cell interleukin-1 production in rheumatoid arthritis, *Lancet 2*, 244–247.
4. Feldmann, M., Brennan, F. M., Chantry, D., Haworth, C., Turner, M., Abney, E., Buchan, G., Barrett, K., Barkley, D., Chu, A., Field, M. & Maini, R. N. (1990) Cytokine production in the rheumatoid joint: implications for treatment, *Ann. Rheum. Dis. 49*, 480–486.
5. Stauber, G. B. & Aggarwal, B. B. (1989) Characterization and affinity cross-linking of receptors for human recombinant lymphotoxin (Tumour-necrosis factor-β) on a human histiocytic lymphoma cell line, U-937, *J. Biol. Chem. 264*, 3573–3576.
6. Gray, P. W., Barrett, K., Chantry, D., Turner, M. & Feldmann, M. (1990) Cloning of human tumor necrosis factor (TNF) receptor cDNA and expression of recombinant soluble TNF-binding protein, *Proc. Natl Acad. Sci. USA 87*, 7380–7384.
7. Loetscher, H., Pan, Y. C., Lahm, H. W., Gentz, R., Brockhaus, M., Tabuchi, H. & Lesslauer, W. (1990) Molecular cloning and expression of the human 55 kd tumor necrosis factor receptor, *Cell 61*, 351–359.
8. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., Kohr, W. J. & Goeddel, D. V. (1990) Molecular cloning and expression of a receptor for human tumor necrosis factor, *Cell 61*, 361–370.
9. Smith, C. A., Davis, T., Anderson, D., Solam, L., Beckmann, M. P., Jerzy, R., Dower, S. K., Cosman, D. & Goodwin, R. G. (1990) A receptor for tumor necrosis factor defines an unusual family of cellular and viral proteins, *Science 248*, 1019–1023.
10. Seckinger, P., Isaaz, S. & Dayer, J. M. (1989) Purification and biologic characterization of a specific tumor necrosis factor alpha inhibitor, *J. Biol. Chem. 264*, 11966–11973.
11. Engelmann, H., Novick, D. & Wallach, D. (1990) Two tumor necrosis factor-binding proteins purified from human urine.

Evidence for immunological cross-reactivity with cell surface tumor necrosis factor receptors, *J. Biol. Chem. 265*, 1531–1536.
12. Porteu, F., Brockhaus, M., Wallach, D., Engelmann, H. & Nathan, C. F. (1991) Human neutrophil elastase releases a ligand-binding fragment from the 75-kDa tumor necrosis factor (TNF) receptor. Comparison with the proteolytic activity responsible for shedding of TNF-R from stimulated neutrophils, *J. Biol. Chem. 266*, 18846–18853.
13. Cope, A., Aderka, D., Doherty, M., Engelmann, H., Gibbons, D., Jones, A. C., Brennan, F. M., Maini, R. N., Wallach, D. & Feldmann, M. (1992) Increased levels of soluble tumour necrosis factor receptors in the sera and synovial fluids of patients with rheumatic diseases, *Arthritis Rheum. 35*, 1160–1169.
14. Aderka, D., Englemann, H., Hornik, V., Skornick, Y., Levo, Y., Wallach, D. & Kushtai, G. (1991) Increased serum levels of soluble receptors for tumor necrosis factor in cancer patients, *Cancer Res. 51*, 5602–5607.
15. Johnson, D., Lanahan, A., Buck, C. R., Sehgal, A., Morgan, C., Mercer, E., Bothwell, M. & Chao, M. (1986) Expression and structure of the human NGF receptor, *Cell 47*, 545–554.
16. Stamenkovic, I., Clark, E. A. & Seed, B. (1989) A B-lymphocyte activation molecule related to the nerve-growth-factor receptor and induced by cytokines in carcinomas, *EMBO J. 8*, 1403–1410.
17. Smith, C. A., Davis, T., Wignall, J. M., Din, W. S., Farrah, T., Upton, C., McFadden, G. & Goodwin, R. G. (1991) T2 open reading frame from the Shope fibroma virus encodes a soluble form of the TNF-R, *Biochem. Biophys. Res. Commun. 176*, 335–342.
18. Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. & Nagata, S. (1991) The polypeptide encoded by the cDNA for human cell surface antigen Fas can mediate apoptosis, *Cell 66*, 233–243.
19. Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., Hession, C., O'Brine-Greco, B., Foley, S. F. & Ware, C. F. (1993) Lymphotoxin β, a novel member of the TNF family that forms a heteromeric complex with lymphotoxin on the cell surface, *Cell 72*, 847–856.
20. Armitage, R. J., Fanslow, W. C., Strockbine, L., Sato, T. A., Clifford, K. N., Macduff, B. M., Anderson, D. M., Gimpel, S. D., Davis-Smith, T., Maliszewski, C. R., Clark, E. A., Smith, C. A., Grabstein, K. H., Cosman, D. & Spriggs, M. K. (1992) Molecular and biological characterization of a murine ligand for CD40, *Nature 357*, 80–82.
21. Jones, E. Y., Stuart, D. I. & Walker, N. P. (1989) Structure of tumour-necrosis factor, *Nature 338*, 225–228.
22. Eck, M. J., Ultsch, M., Rinderknecht, E., de Vos, A. M. & Sprang, S. R. (1992) The structure of human lymphotoxin (tumor necrosis factor-β) at 1.9-Å resolution, *J. Biol. Chem. 267*, 2119–2122.
23. Smith, R. A. & Baglioni, C. (1987) The active form of tumor necrosis factor is a trimer, *J. Biol. Chem. 262*, 6951–6954.
24. Engelmann, H., Holtmann, H., Brakebusch, C., Avni, Y. S., Sarov, I., Nophar, Y., Hadas, E., Leitner, O. & Wallach, D. (1990) Antibodies to a soluble form of a tumor necrosis factor (TNF) receptor have TNF-like activity, *J. Biol. Chem. 265*, 14497–14504.
25. Zhang, X.-M., Weber, I. & Chen, M.-J. (1992) Site-directed mutational analysis of human tumour-necrosis factor-α receptor binding site and structure-function relationship, *J. Biol. Chem. 267*, 24069–24075.
26. Van Ostade, X., Vandenabeele, P., Everaerdt, B., Loetscher, H., Gentz, R., Brockhaus, M., Lesslauer, W., Tavernier, J., Brouckaert, P. & Fiers, W. (1993) Human TNF mutants with selective activity on the p55 receptor, *Nature 361*, 266–269.
27. Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H.-J., Broger, C., Loetscher, H. & Lesslauer, W. (1993) Crystal structure of the soluble human 55 kd TNF-R-human TNFβ complex: implications for TNF-R activation, *Cell 73*, 431–445.
28. Yan, H. & Chao, M. V. (1991) Disruption of cysteine-rich repeats of the p75 nerve-growth-factor receptor leads to loss of ligand binding, *J. Biol. Chem. 266*, 12099–12104.
29. Adolf, G. R. & Fruhbeis, B. (1992) Monoclonal antibodies to soluble human TNF-R (TNF-binding protein) enhance its ability to block TNF toxicity, *Cytokine 4*, 180–184.
30. Thoma, B., Grell, M., Pfizenmaier, K. & Scheurich, P. (1990) Identification of a 60-kD tumour-necrosis factor (TNF) receptor as the major signal transducing component in TNF responses, *J. Exp. Med. 172*, 1019–1023.
31. Marsters, S. A., Frutkin, A. D., Simpson, N. J., Fendly, B. M. & Ashkenazi, A. (1992) Identification of cysteine-rich domains of the type 1 tumor necrosis factor receptor involved in ligand binding, *J. Biol. Chem. 267*, 5747–5750.
32. Brockhaus, M., Schoenfeld, H. J., Schlaeger, E. J., Hunziker, W., Lesslauer, W. & Loetscher, H. (1990) Identification of two types of tumor necrosis factor receptors on human cell lines by monoclonal antibodies, *Proc. Natl Acad. Sci. USA 87*, 3127–3131.
33. Klein-Hitpass, L., Ryffel, G. U., Heitlinger, E. & Cato, A. C. (1988) A 13 bp palindrome is a functional estrogen responsive element and interacts specifically with estrogen receptor, *Nucleic Acids Res. 16*, 647–663.
34. Espevic, T. & Nissen-Meyer, J. (1986) A highly sensitive cell line, WEHI 164, clone 13, for measuring cytotoxic factor/tumour-necrosis factor from human monocytes, *J. Immunol. Methods 95*, 99–105.
35. Lantz, M., Gullberg, U., Nilsson, E. & Olsson, I. (1990) Characterization in vitro of a human tumor necrosis factor-binding protein. A soluble form of a tumor necrosis factor receptor, *J. Clin. Invest. 86*, 1396–1402.
36. Pennica, D., Kohr, W. J., Fendly, B. M., Shire, S. J., Raab, H. E., Borchardt, P. E., Lewis, M. & Goeddel, D. V. (1992) Characterization of a recombinant extracellular domain of the type 1 tumor necrosis factor receptor: evidence for tumor necrosis factor-alpha induced receptor aggregation, *Biochemistry 31*, 1134–1141.
37. Jonsson, U., Fagerstam, L., Varsson, B., Johnsson, B., Karlsson, R., Lundh, K., Lofas, S., Persson, B., Roos, H., Ronnberg, I., Sjolander, S., Sterberg, E., Stahlberg, R., Urbaniczki, C., Ostlin, H. & Malmquist, M. (1991) Real-time biospecific interaction analysis using surface plasmon resonance and a sensor chip technology, *Biotechniques 11*, 620–627.
38. Gullberg, U., Lantz, M., Lindvall, L., Olsson, I. & Himmler, A. (1992) Involvement of an Asn/Val cleavage site in the production of a soluble form of a human tumor necrosis factor (TNF) receptor. Site-directed mutagenesis of a putative cleavage site in the p55 TNF-R chain, *Eur. J. Cell Biol. 58*, 307–312.
39. Lie, B.-L., Tunemoto, D., Hemmi, H., Mizukami, Y., Fukuda, H., Kikuchi, H., Kato, S. & Numao, N. (1992) Identification of the binding site of 55 kDa tumour-necrosis factor receptor by synthetic peptides, *Biochem. Biophys. Res. Commun. 188*, 503–509.
40. Hwang, C. D., Gatanaga, M., Innins, E. K., Yamamoto, R. S., Granger, G. A. & Gatanaga, T. (1991) A 20 amino acid synthetic peptide of a region from the 55 kDa human TNF-R inhibits cytolytic and binding activities of recombinant human tumour-necrosis factor in vitro, *Proc. R. Soc. Lond. Ser. B. Sci. Biol. 245*, 115–119.
41. Hsu, K. C. & Chao, M. V. (1993) Differential expression and ligand binding properties of tumor necrosis factor receptor chimeric mutants, *J. Biol. Chem. 268*, 16430–16436.