TNFα and the TNF Receptor Superfamily: Structure-Function Relationship(s)

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ABSTRACT Tumour Necrosis Factor α (TNFα), is an inflammatory cytokine produced by macrophages/monocytes during acute inflammation and is responsible for a diverse range of signalling events within cells, leading to necrosis or apoptosis. The protein is also important for resistance to infection and cancers. TNFα exerts many of its effects by binding, as a trimer, to either a 55 kDa cell membrane receptor termed TNFR-1 or a 75 kDa cell membrane receptor termed TNFR-2. Both these receptors belong to the so-called TNF receptor superfamily. The superfamily includes FAS, CD40, CD27, and RANK. The defining trait of these receptors is an extra cellular domain comprised of two to six repeats of cysteine rich motifs. Additionally, a number of structurally related "decoy receptors" exist that act to sequester TNF molecules, thereby rescuing cells from apoptosis. The crystal structures of TNFα, TNFβ, the extracellular domain of TNFR-1 (denoted sTNFR-1), and the TNFβ sTNFR-1 complex have been defined by crystallography. This article will review the structure/function relationships of the TNFα and the TNF receptor superfamily. It will also discuss insights as to how structural features play a role in the pleiotropic effects of TNFα. *Microsc. Res. Tech. 50:184–195, 2000.*

BACKGROUND

Hints of the existence of a biological factor mediating tumour necrotic activity date back to the 18th century following observations that cancer patients, exposed to severe bacterial infection, underwent occasional shrinkage of their tumours (Coley, 1893). Lymphotoxin was discovered in 1968 as a soluble protein produced by T-lymphocytes (Granger and Williams, 1968). In 1975, experiments demonstrated that a protein, termed "Tumour Necrosis Factor" (TNFα), was released into the circulation of animals subsequent to stimulation of their reticuloendothelial system and lipopolysaccharide challenge (Carswell et al., 1975). This protein has been demonstrated to cause rapid necrotic regression of certain forms of tumours (hence its name). The necrotic ability of TNFα did not prove true for all tumour types and the therapeutic potential of TNFα was hampered by its systemic toxicity at effective concentrations. This discriminatory TNFα mediated cytotoxicity may be related to the tumour cell's ability to undergo nitrotyrosinylated-tubulin mediated microtubule dysfunction (Idriss, 2000). However, the specific necrotic effects of TNFα on tumour cells were increased by the presence of Interferon (Fiers, 1991). Both TNFα and lymphotoxin (termed TNFβ and also as LTα) define two structurally and functionally related proteins (Aggarwal et al., 1991; Armitage, 1994; Bazzoni and Beutler, 1996; Beyaert and Fiers, 1994; Fiers, 1991; Vilcek and Lee, 1991). We use the abbreviation TNF to refer to both proteins. Both proteins were isolated in 1984 from activated macrophages and T cells, respectively (Gray et al., 1984; Pennica et al., 1984). Their cDNA sequence revealed 30% homology in the encoded amino acid residues. The structure of TNFα and TNFβ have been determined and both proteins were found as a "pear"/"cone" shaped trimers (Fig. 1; Eck and Sprang, 1989; Eck et al., 1992; Jones et al., 1989). TNF receptors, TNFR-1 and TNFR-2, were cloned and expressed as 55 and 75 kDa proteins, respectively (Dembic et al., 1990; Loetscher et al., 1990; Schall et al., 1990; Smith et al., 1990). The X-ray structure of TNFβ in complex with TNFR-1 provided a break through in the molecular understanding of how TNF functions (Fig. 2; Banner et al., 1993). The TNF trimer binds three receptor molecules, one at each of three TNF monomer-monomer interfaces (Fig. 3). The extracellular domain of the receptor is an elongated molecule composed of 3 disulphide-containing 40 residue motifs (Banner et al., 1993). Only three of four extracellular modules of TNFR-1 were visualised. The first structure of the receptor on its own also contained disorder in the C-terminus but did hint at a possible mechanism for signal transduction (Naismith et al., 1995). This was followed up by a high-resolution structure of the entire extracellular domain of receptor (Naismith et al., 1996). This work determined the structure of the fourth motif, which is different in structure to the other three. This led to the novel method of aligning and predicting the structure of related receptors (Naismith and Sprang, 1998). Such an approach has been recently used in modelling the CD40/CD40L interaction (Singh et al., 1998).

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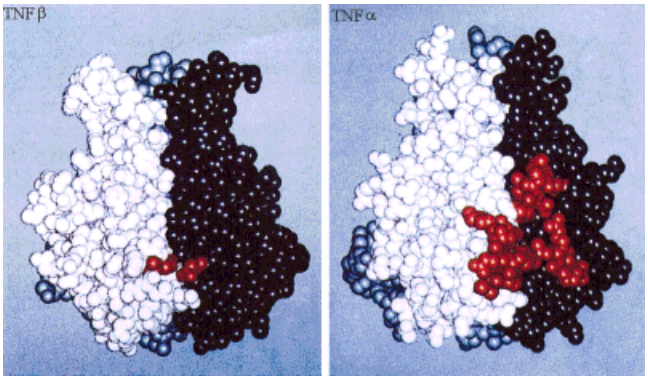


Fig. 1. Van der Waals surface of the lymphotoxin-TNFβ (***left***) and the TNFα (***right***) trimers. Subunits are coloured white, grey, and charcoal. Mutagenic "hot spots" associated with changes in cytotoxicity or receptor binding, are shown at the front in grey. On TNFβ, these are Tyr-108 (on white subunit) and Asp-50 (on black subunit). In TNFα, mutagenesis of residues 84–91 (on white subunit), 30–40 (lower group on charcoal subunit), and 143–149 (upper group on charcoal subunit) can alter cytotoxicity. Reproduced from Eck et al. (1992) with permission of the publisher. (Note: color version of artwork is available online.)

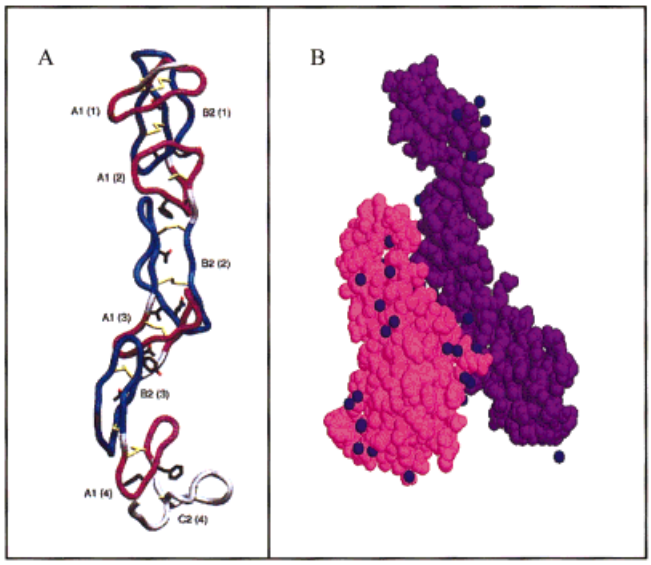


Fig. 2. **A:** Complete structure of the extracellular domain of TNFR-1. Reproduced from Naismith et al. (1996) with permission of the publisher. **B:** Van der Waals surface of the TNFβ trimer (magenta) complexed with its receptor (purple); water molecules are shown in blue. From Banner et al. (1993), prepared using Rasmol. (Note: color version of artwork is available online.)

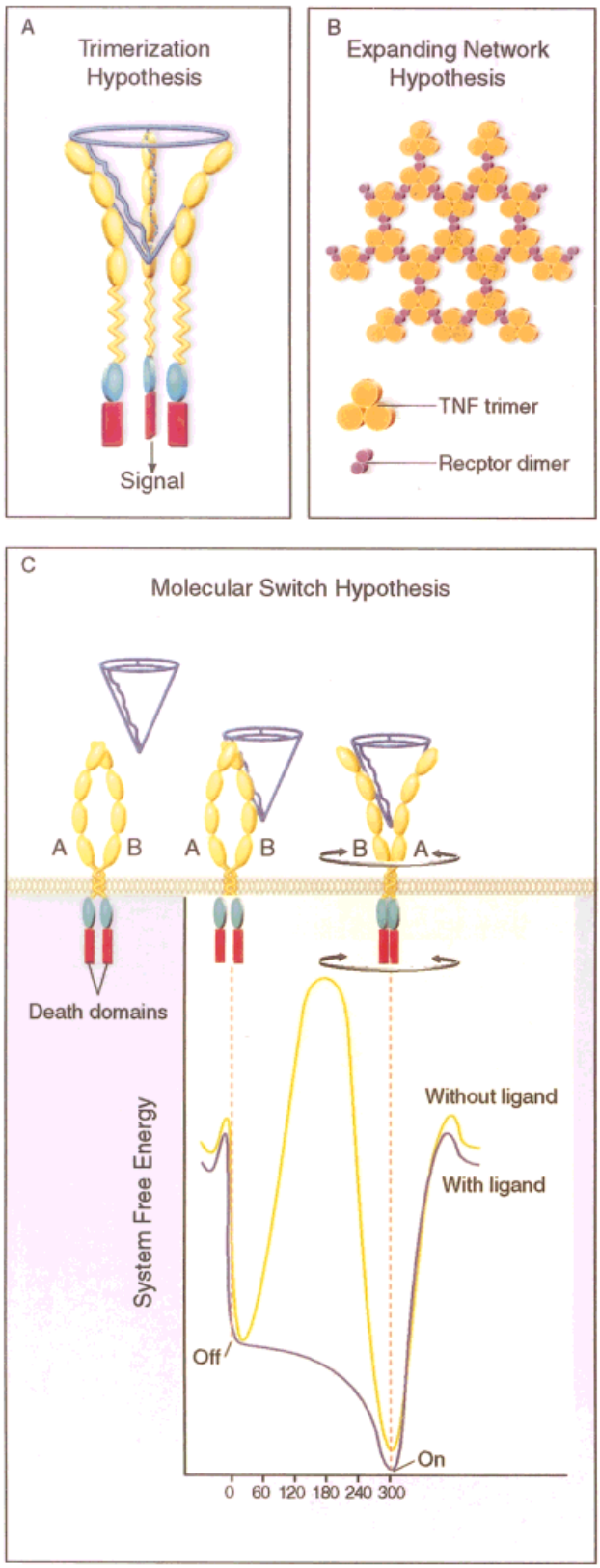
BIOLOGICAL ROLE(S) OF TNFα

The biological functions of TNFα are varied and the mechanism of action is somewhat complex. This protein, conferring resistance to certain type of infections on the one hand and causing pathological complications on the other, carries out contradictory roles (Fiers, 1991). This may be related to the varied signalling pathways that are activated. Interestingly, mutant phenotype of knock-out mice lacking the gene for TNFα showed no resistance to leishmania and failed to form germinal centres in their spleens (Goldfeld and Tsai, 1996). Moreover, TNFR knockout mice displayed an increased susceptibility to microbial infection and a suppressed inflammatory response upon challenge with bacterial endotoxins (Acton et al., 1996; Steinshamn et al., 1996). This clearly points to an important role for TNFα in conferring immunity and a proper inflammatory response.

TNFα plays several therapeutic roles within the body, which include immunostimulation, resistance to infection agents, resistance to tumours (Aggarwal et al., 1991; Vilcek and Lee, 1991), sleep regulation (Krueger et al., 1998), and embryonic development (Wride and Sanders, 1995). On the other hand, parasitic, bacterial and viral infections become more pathogenic or fatal due to TNF circulation (Fiers, 1991). However, the major role of TNF seems to be as an important mediator in resistance against such infections. Indeed, in experimental leishmaniasis, insufficient TNFα is associated with progressive disease and death (Goldfeld and Tsai, 1996). It seems that only abhorrent situations, such as overreaction of the host or dysfunction of natural autoregulatory networks, lead to the aforementioned deleterious effects (Fiers, 1991). Many cell types infected with viruses become sensitised to TNF effects implicating this cytokine in viral disease (Czarniecki, 1993). TNF may contribute towards resistance of infection through activation of neutrophils and platelets, enhancement of macrophage/NK cell killing abilities, and stimulation of the immune system (Fiers, 1991). TNF also plays a possible pathological role in a number of autoimmune diseases such as graft vs. host rejection, and rheumatoid arthritis (Beutler and Bazzoni, 1998; Beutler, 1999). Additionally, TNF shows anti-malignant cell cytotoxicity especially in combination with Interferon (Fiers, 1991; Gruss and Dower, 1995). Such a combination can cure aggressive non-immunogenic tumours in animal models. However, as high concentrations of TNFα are toxic to the host (maximum tolerated dose in humans 200 $\mu g/m^2$), it is necessary to increase the therapeutic index either by decreasing toxicity (e.g., pre-treatment with IL-1) or by increasing effectiveness. This may be possible through mutations that reduce systematic cytotoxicity and increase TNF's effectiveness in selectively eliminating tumour cells (Cha et al., 1998). Efficacy may be heightened with some chemotherapuetic drugs (Balkwill, 1992). So far clinical trials using human TNFα or TNFα/Interferon γ, reported no success (Hieber and Heim, 1994).

TNFα is also involved in physiologic sleep regulation. Administration of exogenous TNFα was shown to induce increased sleep, whilst inhibition of TNFα reduced spontaneous sleep. TNFα mRNA is produced in diurnal rhythm in brain with the highest levels occurring during peak sleep periods. Absence of the TNFR-1 receptors resulted in reduced sleep in animal models. Similar effects were observed with the cytokine IL-1 (Shoham et al., 1987). TNF mediated apoptosis also seems a part of normal embryonic development (Wride and Sanders, 1995).

TNF can induce necrotic or apoptotic cell death (Beyaert and Fiers, 1994). Necrosis is characteristic with cell swelling, organelle destruction, and cell lysis.



Apoptosis is perceived as a conserved and biochemically driven process of cell death with distinct morphological features (Steller, 1995). Apoptosis is characterised by cell shrinkage, formation of apoptotic bodies, and, typically, formation of inter nucleosomal DNA fragmentation. Necrosis is observed with the necrotic haemorrhage of transplantable methylcholanthrene-induced sarcomas in animal models (Beyaert and Fiers, 1994). Apoptosis is seen as a general TNF mediated cytotoxic phenomenon occurring through the TNFR-1 signalling pathway. However, it has been proposed that TNFR-2 indirectly mediates cytotoxicity through endogenous production of TNF and autotropic or paratropic activation of TNFR-1 (Grell et al., 1999). Many adaptor proteins are involved in the complex signalling pathways, which initiate apoptosis (Baker and Reddy, 1996, 1998; Yuan, 1997). However, the full signalling network leading to, and controlling, apoptosis remains to be elucidated. Activation of proteases is postulated as a general mechanism of apoptosis (Thornberry and Lazebnik, 1998). TNFα can also lead to cell survival through activation of NF-κB . Many TNF related ligands have been identified thus far (Armitage, 1994; Bazzoni and Beutler, 1996; Orlinick and Chao, 1998). TNF related proteins such as RANKL are required for (osteoclast) cell differentiation, which is necessary for bone resorption (Takahashi et al., 1999). Certain TNF-like receptors (e.g., HVEM) serve as cell entry points into cells during infection (Marsters et al., 1997; Montgomery et al., 1996).

MECHANISM(S) OF ACTION

TNFα exerts its effect(s) by binding to, as a trimer, and clustering high-affinity receptors present in great numbers on most cell membranes (Loetscher et al., 1991a; Schoenfeld et al., 1991). The ligand/receptor complex is rapidly internalised via clathrin-coated pits and ends up in secondary lysosymes where it is degraded. Binding of TNFα to the 75 kDa TNFR-2 is not sufficient to attain cytotoxicity (Thoma et al., 1990), but rather binding to the 55 kDa TNFR-1 is sufficient to attain TNFα mediated cell killing (Van Ostade et al., 1993). TNFα exerts its effects by activating a number of secondary proteins that provoke a variety of re-

Fig. 3. Three models describing TNF-induced receptor signalling. **A:** Trimerisation hypothesis: the juxtaposition of the three receptors results from binding of a single TNF trimer, leading to cell signalling. **B:** Expanding network hypothesis: binding of the TNF trimer to the receptor dimer generates an expanding hexagonal array of ligand/receptor complex. Each TNF trimer engages three receptor dimers. "Capping" of the receptors triggers the biological response. **C:** Molecular-switch hypothesis: each receptor dimer is an activatable unit. Receptor activation occurs in response to: (1) TNF ligand binding to subunit B of the receptor, (2) Subunit A disengaging from subunit B, resulting in the receptor binding to another part of the TNF trimer. These events lead to conformational changes (e.g., homodimerisation of the receptor's cytoplasmic "death domains"), resulting in signal transduction. The graph shows a hypothetical profile depicting the free energy associated with conformational changes between "On" and "Off" states of the receptor. A large energy barrier preventing transition from the "Off" to the "On" states, is lowered upon ligand binding. The free energy of the "On" state (defining the stable ligand/receptor complex), may be lower than the energy of the "Off" state and this may be irreversible. Reproduced from Bazzoni and Beutler (1996) with permission of the publisher. (Note: color version of artwork is available online.)

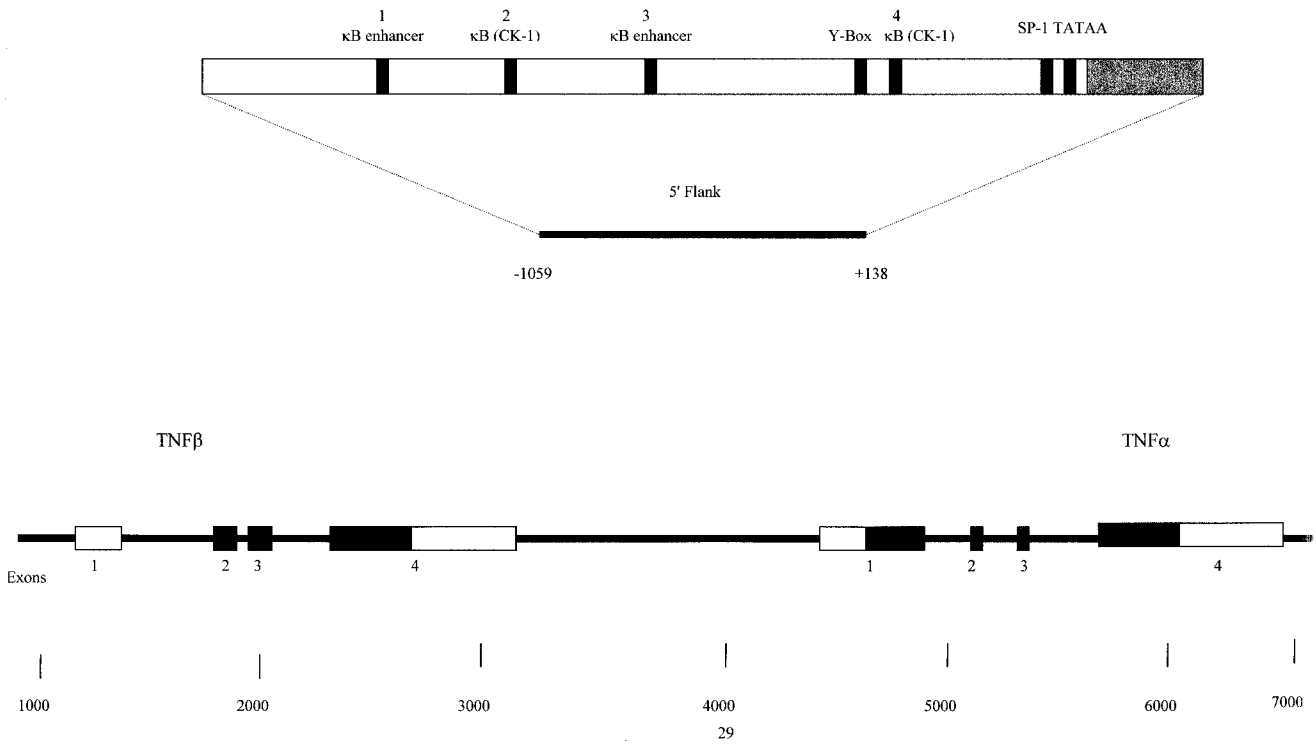


Fig. 4. Structure of the murine TNF locus. Coding portion of the exons are shaded and inset shows location of TNFα 5′ flanking region elements potentially involved in transcriptional regulation of TNFα gene. Reproduced from Shakhov et al. (1990), with permission of the publisher.

sponses within the cell such as activation of gene transcription and/or production of reactive oxygen or nitrogen radicals (e.g., NO). Activated proteins include G-Protein, transcription factors (e.g., NF-κB, AP-1), protein kinases (e.g., CK II, erk-1, erk-2, and MAP2), phospholipases (e.g.PLA_2, PLC, PLD and sphingomyelinase) mitochondrial proteins (e.g., manganese superoxide dismutase), and serine and cysteine proteases, known as caspases (Beyaert and Fiers, 1994; Darnay and Aggarwal, 2000; Thornberry and Lazebnik, 1998; Vilcek and Lee, 1991). Several members of the TNF receptor superfamily possess intracellular "death domains," which are protein interaction domains each consisting of 65–80 amino acids that participate in TNFα mediated apoptosis. These "death domains" function to activate caspases (cysteine proteases that cleave certain proteins after specific aspartic acid residues). Caspases are recruited to the activated receptor (together with other signalling molecules) by adaptor proteins such as TRADD and FADD (for review see Ashkenazi and Dixit, 1998, 1999; Yuan, 1997). Interestingly, the TNFα trimer undergoes, at a relatively low pH and in the absence of receptor binding, conformational change that allows it to form a functional ion channel (Baldwin et al., 1996; Kagan et al., 1992). This shows that the molecule performs many subtle and extraordinarily diverse functions. Additionally, TNF binding mediates alterations in cell volume associated with necrosis/apoptosis by inducing regulation of the activities of a number of anion/cation channels (Lang et al., 1998).

CHARACTERISATION OF THE TNFα GENE AND MOLECULE:

The TNFα gene is a single copy gene, preceded by the TNFβ on the short arm of human chromosome 6 and murine chromosome 17 (Spriggs et al., 1992). It is closely linked within the major histocompatibility complex genes. TNFα is always 3′ to the TNFβ gene and is around 3 Kb long. It consists of four exons, arranged over approximately 3 kb of DNA, interrupted by three introns. More than 80% of the mature TNF sequence is encoded in the fourth exon, while exons I and II contain almost entirely leader peptide sequence. The TNFα mRNA seems to be transcribed in a wide variety of cells. The 5′ flanking region of the TNFα gene contains several κB homologous sequence enhancer elements and a MHC class II like "Y box" (Fig. 4; Shakhov et al., 1990). These sequences seem to be involved in transcriptional activation. The gene also seems to be regulated at the translational level through the UA-rich sequence in the 3′ untranslated region of the human TNFα mRNA. The mRNA translates into a 157 amino acid protein in human and 156 in murine cells (Spriggs et al., 1992; Vilcek and Lee, 1991).

TNFα exists in two forms, a membrane bound and a soluble form; each form possibly carries out a distinct physiological role (Beyaert and Fiers, 1994; Watts et al., 1997). Human TNFα is expressed as a precursor molecule of a 157 amino acid, preceded by a 76 amino acid presequence (79 in murine). This presequence is highly conserved and seems to serve to anchor the

TABLE 1. Consensus phosphorylation sites† for TNFα and TNFR-1

hTNFα 233 A.A (precursor molecule):

```
  1 MSTESMIRDV ELAEEALPKKT*GGPQGS*RRC LFLSLFSFLI VAGATTLFCL LHFGVIGPQR
 61 EEFPRDLS*LI SPLAQAVRSS*  S*RT*PS*DKPVA HVVANPQAEG QLQWLNRRAN ALLANGVELR
121 DNQLVVPSEG LYLIYSQVLF KGQGCPSTHV LLTHTISRIA VSYQTKVNLL SAIKSPCQRE
181 TPEGAEAKPW YEPIYLGGVF QLEKGDRLSA EINRPDY*LDF AESGQVYFGI IAL
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hTNFR-1 (55):476 A.A (precursor molecule):

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  1 MGLST*VPDLL LPLVLLELLV GIYPSGVIGL VPHLGDREKR DS*VCPQGKYI HPQNNSICCT
 61 KCHKGTYLYN DCPGPGQDTD CRECESGSFT ASENHLRHCL SCSKCRKEMG QVEISSCTVD
121 RDT*VCGCRKN QYRHYWSENL FQCFNCSLCL NGTVHLS*CQE KQNTVCTCHA GFFLRENECV
181 SCSNCKKSLE CTKLCLPQIE NVKGTEDSGT TVLLPLVIFF GLCLLSLLFI GLMYRYQRWK
241 S*KLYSIVCGK ST*PEKEGELE GTT*TKPLAPN PSFSPTPGFT PTLGFSPVPS STFTSSSTYT
301 PGDCPNFAAP RREVAPPYQG ADPILATALA SDPIPNPLQK WEDSAHKPQS* LDTDDPATLY*
361 AVVENVPPLR WKEFVRRLGL SDHEIDRLEL QNGRCLREAQ YSMLATWRRR TPRREATLEL
421 LGRVLRDMDL LGCLEDIEEA LCGPAALPPA PSLLR
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†Phosphorylation consensus sequences are based on those described by Pearson and Kemp (1991):

CK II sites	(***S/T*** XX E/DX)
PKA sites	(***K/R*** XX S/T)
PKC sites	(***S/T*** X R/K, R/K X ***S/T***, R/K XX ***S/T***)
Tyr kinase sites	(X E/D ***Y*** X).

*Phosphorylatable residue.

precursor polypeptide in the membrane (Vilcek and Lee, 1991). Hence unlike TNFβ, which is secreted, TNFα is first produced as a type II membrane protein. Amino acids -44 to -26 of the TNFα presequence comprise the hydrophobic transmembrane region and residues -76 to -50 comprise the intracytoplasmic region. The unprocessed protein has a molecular mass of 26 kDa and is proteolytically cleaved (possibly with a serine protease between amino acids -14 and -1) to give a 17 kDa active form. The mature form of TNFα lacks any methionine residues. Processed (cleaved) TNFα exists in solution as a homotrimer of total molecular mass of 52 kDa (other estimates between 45–65 kDa) and this is the form that binds and cross-links receptors (Smith and Baglioni, 1987). Each of the three subunits contains a disulphide bridge, which is not essential for biological activity. Human TNFα binds strongly to TNF receptors with a disassociation constant K_d of around 0.5 and 0.1 nM for TNFR-1 and TNFR-2, respectively. Binding to the TNFR-1 receptor selectively mediates the toxic effects of TNFα (Beyaert and Fiers, 1994; Fiers, 1991; Vilcek and Lee, 1991). A TNFβ related ligand termed LTβ has been identified, which is capable of forming heterotrimer with TNFβ ($LT\alpha_2\beta$ or $LT\beta_2\alpha$) and binding to TNF receptors (Browning et al., 1993).

Not many post-translational modifications have been reported to occur on TNFα. Human TNFα lacks N-glycosylation, which is observed in TNFβ (Smith and Baglioni, 1992; Vilcek and Lee, 1991). The primary sequence of TNFα shows the presence of a number of phosphorylation consensus sites (Table 1),offering a potential mechanism for regulating trimer formation and/or mode of receptor binding. Indeed, phosphorylation of the 26-kDa membrane bound human TNFα has been previously reported in monocytes (Pocsik et al., 1995). Further, a recent study demonstrated that membrane bound TNFα is phosphorylated by CK I and dephosphorylated in the presence of soluble dimeric TNFR-1, thereby offering a mechanism for reverse signalling. Dephosphorylation occurs through phosphatase activation as this was sensitive to phospahtase inhibitors (Watts et al., 1999).

TNFα exists in a more diverse range of cells than TNFβ. TNFα is produced in a wide variety of hematopoietic and non-hematopoietic cells, both normal and malignant. These include macrophages, $CD4^+$ and $CD8^+$ T-lymphocytes, B-lymphocytes, LAK cells, NK cells, neutrophils, astrocytes, endothelial cells, smooth muscle cells, and a number of non-hematopoietic tumour cell lines (Vilcek and Lee, 1991). Indeed, the classical cell line for testing TNF cytotoxicity is the murine fibrosarcoma cell line L929, although others (such as the WEHI164 clone) show heightened sensitivity. Some cells can be induced to produce the two forms of TNF. Many of TNFα's activities are species-independent, but some are species specific (Fiers, 1991).

TNF RECEPTORS AND THE TNF RECEPTOR SUPERFAMILY

TNFα binds to two cognate receptors of molecular weight 55 and 75 kDa (Loetscher et al., 1991b). Human TNFR-1 consists of 434 amino acids whilst TNFR-2 comprises 439 residues. These receptors share very limited homology in the extracellular region. The TNF receptor superfamily is defined by the presence of repeating units of cysteine clusters. No homology is seen in the intracellular regions of the proteins (reviewed in Beutler and Bazzoni, 1998). Lack of homology in the intracellular regions suggests the two receptors activate distinct signalling pathways. TNF receptors are

TABLE 2. Summary of TNF Receptor Family Members

Receptor	Other names	A.A.	M.wt. kDa	Ligand	Death domain	Reference
CAR 1	N/A	368	39	Unknown	Yes	Brojatsch et al. (1996)
CD 27	N/A	240	45–55	CD27L	No	Camerini et al. (1991); Armitage (1994)
CD 30	N/A	577	120	CD30L	No	Durkop et al. (1992); Armitage (1994)
CD 40	N/A	256	40	CD40L	No	Armitage (1994)
CD 95	FAS, APO-1	319	45	CD95L	Yes	Itoh et al. (1991); Armitage (1994)
CD134	OX 40	272[1]	50	OX 40L	No	Mallett et al. (1990)
CD137	4-1BB	256	35	4-1BBL	No	Vinay and Kwon (1998)
DR 3	WSL1,TRAMP, Apo3, LARD	417	54	Apo3L	Yes	Screaton et al. (1997)
DR 4	TRAILR-1	468[2]	32	Apo2L/TRAIL	Yes	Pan et al. (1997b)
DR 5	TRICK2, TRAILR-2, Apo2, KILLER	411	45	Apo2L/TRAIL	Yes	MacFarlane et al. (1997)
DR 6	N/A	655	72	Unknown	Yes	Pan et al. (1998a)
GITR	AITR	228	25	GITRL	No	Nocentini et al. (1997)
HveA	HVE1, HveM	283	30.5	TRAF	No	Marsters et al. (1997)
LTβR	N/A	435	60	LTβ	No	Crowe et al. (1994)
NGFR	N/A	425	75	NGF	Yes	Radeke et al. (1990)
RANK	TRANCE-R	616	80[3]	RANKL	No	Anderson et al. (1997)
TNFR-1	CD120a, R55,R60	460	55	TNFα, TNFβ	Yes	Loetscher et al. (1990); Armitage (1994)
TNFR-2	CD120b, R75,R80	476	75	TNFα, TNFβ	No	Smith et al. (1990); Armitage (1994)
DcR1	TRID,TRAIL-R3, LIT	299	50	TRAIL	No	Pan et al. (1997a); MacFarlane et al. (1997)
DcR2	TRUNDD, TRAIL-R4	386	42	Apo2L	No	Pan et al. (1998b)
DcR3	N/A	300	33	FasL	No	Pitti et al. (1998)
OCIF	OPG, TR1	401	60	OPGL/RANKL	No	Tsuda et al. (1997); Simonet et al. (1997)
CrmB	N/A	355	48	TNFα, TNFβ	No	Hu et al. (1994)
CrmC	N/A	186	25	TNFα	No	Smith et al. (1996)

[1]Mouse.
[2]Including 24 A.A. signal peptide.
[3]60 kDa predicted (Murine).

present on almost all known cell types with few exceptions, such as erythrocytes and unstimulated T lymphocytes. Receptor density ranges from 200–10,000 per cell. There seems to be no correlation between the number of receptors present on a cell and the magnitude or direction of the TNF-induced response (Beyaert and Fiers, 1994). Soluble forms of TNFR-1 and TNFR-2 have been identified in human urine and in the serum of cancer patients. These probably regulate TNF's activity by decoying the ligand, thereby down-regulating the generated signals (Engelmann et al., 1990).

Many membrane bound receptors (including TNFR-1 and 2) have been identified thus far, forming what is known as the TNF receptor superfamily (Bazan, 1993). Their molecular weight is in the range of 50–120 kDa (Table 2). Each molecule contains several disulphide bonds, essential for activity. Members of the TNF receptor family belong to the type I transmembrane glycoproteins. They share a common cysteine-rich motif (about 40 residues) repeated two (e.g., CAR 1) to six (e.g., CD 30) times in the extracellular N-terminal domain (Fig. 2; Beutler and Bazzoni, 1998; Naismith and Sprang, 1998). The average homology in these repeats is around 25% between the various members. The receptors differ in the possession of a cytoplasmic "death domain," which is absent in a number of the many members of the TNFR family (Table 2).

All of these receptors have been cloned from various species. The mode of action of these receptors relies on being cross-linked upon binding of ligand oligomers (e.g., TNF trimers, NGF dimers). They can be activated through cross-linking with specific monoclonal antibodies (Shalaby et al., 1990). Viruses can utilise many of these receptors as entry points to the cell. A number of related receptors termed "decoy receptors" have also been identified, which function to sequester secreted ligands (Ashkenazi and Dixit, 1999; Beutler, 1999). Decoy receptors (DcR1, DcR2, and DcR3) sequester Trail (DcR1 and DCR2) and Fas (DcR3) ligands, thereby preventing apoptosis (Ashkenazi and Dixit, 1999). In addition, a soluble non-membrane bound decoy receptor termed osteoprotegrin (OPG) has also been identified (Simonet et al., 1997).

Not many posttranslational modifications have been reported for the TNFR-1 family. TNFR-1 and TNFR-2 are both N-glycosylated, but only TNFR-2 is O-glycosylated (Fiers, 1991). All other membrane receptors are also glycosylated. Neither has intrinsic kinase activity or is known to be phosphotylated by a membrane bound or cytoplasmic kinases, but recently murine TNFR-1 has been shown to be preferentially phosphorylated by mitogen activated protein kinase (MAPK) on Thr-236 and Ser-270 (Van Linden et al., 2000) and humanTNFR-1 to be phosphorylated on Tyr-331 (Darnay and Aggarwal, 1997). The primary sequence of TNFR-1 suggests the presence of a number of potential phosphorylation consensus sites for PKC or CK II (Table 1).

TNFR-1 has ubiquitous distribution present on cells such as fibroblasts and epithelial cells, whilst TNFR-2 presence is more restricted, being confined mainly to cell of hematopoietic origins (Armitage, 1994; Byffel

and Mihatsch, 1993). Certain cell types (e.g., Hep2) express only TNFR-1 (Hohmann et al., 1989). CD40 is expressed as a 50-kDa glycoprotein on a variety of cells including B cells, thymic epithelium, dendritic cells, monocytes, and at a low level on T cells (Armitage, 1994). Fas receptor is expressed on activated T cells, B cells, monocytes, and neutrophils. Fas mRNA is expressed in various organs in mice (Armitage, 1994). CD30 receptor was originally identified in Hodgkin's cells. It is also expressed on a number of non-Hodgkin's lymphoma cells as well as a several virally transformed B- and T-cell lines (Armitage, 1994). CD27 is expressed on the majority of medullary thymocytes and peripheral blood T cells. It is also present on some B and NK cells (Armitage, 1994). The receptor 4-1BB is expressed as a 35 kDa protein on activated $CD4^+$ and $CD8^+$ T cells and intestinal intraepithelial T lymphocytes and human lung epithelial cells (Armitage, 1994; Vinay and Kwon, 1999). Ox40 on the other hand was originally described as a 50-kDa protein on activated rodent $CD4^+$ T cells and thymocytes and hence shows a relatively restricted profile of expression (Armitage, 1994). CAR1 was identified as a chicken cell-surface receptor for cytopathic avian leukosis sarcoma viruses (Brojatsch et al., 1996). LTβR is expressed in a wide range of cell types, except lymphocytes (VanArsdale et al., 1997). However, expression of its ligand is restricted to activated lymphocytes. Human GITRL mRNA is found in several peripheral tissues, and hGITRL protein is detected on cultured vascular endothelial cells. The levels of hGITR mRNA in tissues are generally low; in peripheral blood T cells, however, antigen-receptor stimulation leads to substantial induction of hGITR transcripts (Nocentini et al., 1997). HVEM mRNA is expressed mainly in lymphocyte-rich tissues, e.g., spleen and also in several non-lymphoid tissues (Marsters et al., 1997). DR3 is abundantly expressed on thymocytes and lymphocytes, expression is restricted predominantly to lymphoid tissues, spleen, thymus, and PBL (Screaton et al., 1997). DR4 and DR5 are present in many tissues, including spleen, thymus, peripheral blood lymphocytes (PBLs), prostate, testis, ovary, uterus, and multiple tissues along the gastrointestinal tract (Pan et al., 1997a,b; MacFarlane et al., 1997; Walczak et al., 1997). TRAIL-R3 (Dc1) is a decoy receptor (MacFarlane et al., 1997); its mRNA has been found in heart, placenta, lung, liver, kidney, spleen, peripheral blood leukocytes, and bone marrow, but at substantially lower amounts in most transformed cell lines (Frank et al., 1999; Golstein, 1997). TRAIL-R4 (DcR2), shows widespread tissue distribution, being expressed both in normal human tissue and tumour cell lines (Degli-Esposti et al., 1997). RANK is expressed on the cell surface of precursor bone cells (Anderson et al., 1997; Nakagawa et al., 1998).

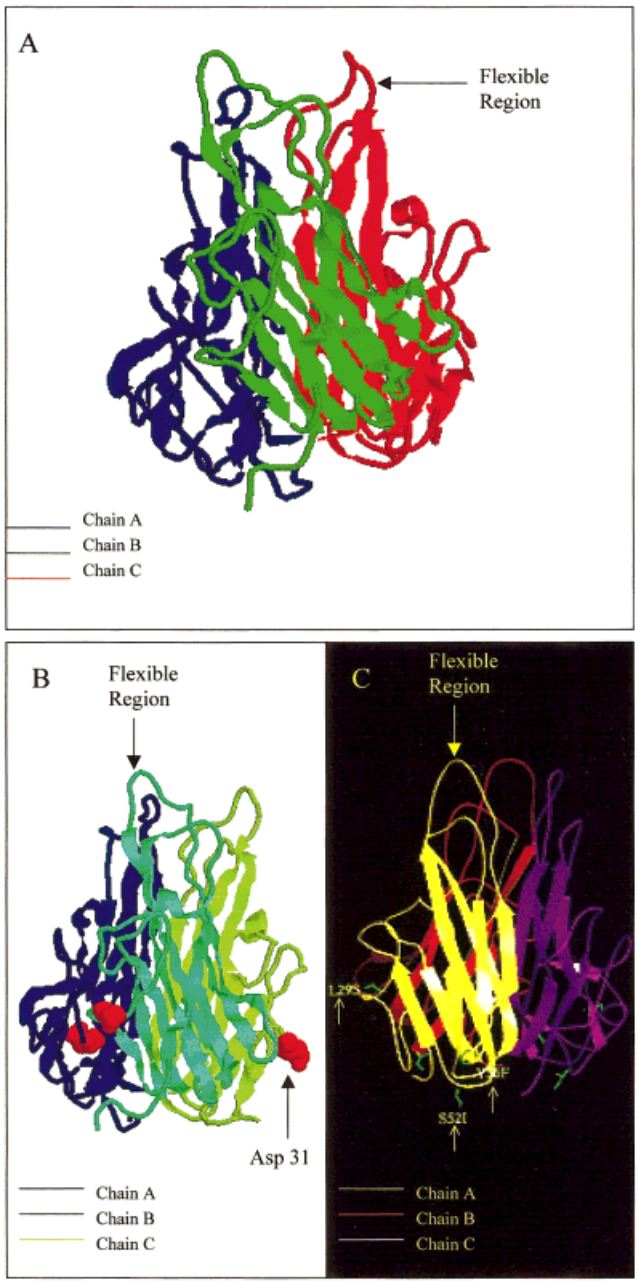

Fig. 5. Ribbon diagram showing the "cone-shaped" TNFα trimer (**A**, Eck et al., 1989) and two TNFα mutants (**B**, Reed et al., 1997; **C**, Cha et al., 1998). Mutated residues are marked with arrows. Panel (**C**) was reproduced from Cha et al. (1998) with permission of the publisher. (Note: color version of artwork is available online.)

STRUCTURAL ASPECTS OF THE TNFα PROTEIN

The crystal structure for TNFα was solved almost simultaneously by two research groups (Eck and Sprang, 1989; Jones et al., 1989). Recently, the structure has been described to a very high resolution (Baeyens et al., 1999). TNFα exists as a timer arranged like a triangular cone, such that each molecule contacts the other two. The interfaces between the monomers in this analogy would be the vertical edge of the cone. Each monomer consists of two packed β-pleated sheets each of eight, anti-parallel β-strands arranged in a β-jellyroll topology with an N-terminal insertion that contains three additional β-strands (Fig. 5A). The monomer is approximately 60Å long and 30Å wide. The outer sheet is rich in hydrophilic residues, whilst the inner sheet is hydrophobic and contains the C-terminal segment, which is located close to the trimer's central

axis. The jellyroll has a wedged shape with a broad base and a tapering peak and is reminiscent of viral capsid proteins. The "inner" sheet, hidden in the trimer complex, is formed by strands B"-B-I-D-G (based on the historical nomenclature of strands) in correct spatial order. The exposed "outer" sheet is formed by strands C'-C-H-E-F. Murine TNF has now been determined to 1.4Å (Baeyens et al., 1999); although it is materially the same as the human TNF structure, it does provide a more accurate and detailed description of side chain positions. Site-directed mutagenesis identified many residues within the TNF sequence that are necessary for the TNF's cytotoxicity and receptor binding (reviewed by Jones et al., 1991; Sprang and Eck, 1991).

The related cytokines TNFβ (Banner et al., 1993; Eck et al., 1992) and CD40L (Karpusas et al., 1995) have both been structurally characterised. As was expected, both share the same fold as TNFα. However, there are considerable differences in the surface properties of the three molecules. The differences were particularly marked for CD40L, which binds to CD40 and does not bind to TNFR-1 or TNFR-2.

The crystal structures of TNF mutants that show greater affinity for TNFR-1 binding compared to TNFR-2 or that have diminished binding to both receptors, have been elucidated (e.g., Cha et al., 1998; Reed et al., 1997). One TNFα mutant, where Arg31 was substituted with Asp, shows preferentially decreased binding to TNFR-2 (Fig. 5B). Model complexes of TNFα with receptors TNFR-1 and TNFR-2 suggest that Arg31 of TNFα forms an ionic interaction with a Glu residue in both receptors. Asp31 of the TNFα mutant was postulated to form hydrogen bond interactions with Ser59 or Cys70 of TNFR-1, but not in TNFR-2. The loss of the strong ionic interactions of Arg31 and the electrostatic repulsion of Asp31 explains the reduced binding of the Arg31Asp mutant to TNFR-2. The replacement of the ionic interactions by a weaker hydrogen bond interactions between Asp31 of the TNFα mutant and TNFR-1, compared with no interactions with TNFR-2, explains the observed preferential binding of the Arg31Asp mutant to TNFR-1 over TNFR-2 (Reed et al., 1997).

Another TNFα mutant, termed M3S, contained four changes: a hydrophilic substitution of Leu29Ser, two hydrophobic substitutions of Ser52Ile and Tyr56Phe, and a deletion of the N-terminal seven amino acids that is disordered in wild-type TNFα (Fig. 5C). The mutant exhibited 11- and 71-fold lower binding affinities for the human TNFR-1 and TNFR-2, respectively. It also showed 20 and 10 times lower in vitro cytotoxic effect and in vivo systemic toxicity, respectively, resistance to trypsin proteolysis and increased thermal stability (Cha et al., 1998). The decreased affinity and altered thermal stability were due to the Leu29Ser mutation causing substantial restructuring of the loop containing residues 29–36 into a rigid segment, resulting from the formation of intra- and intersubunit interactions. This rigidity also explained resistance to proteolysis. The Ser52Ile and Tyr56Phe mutations did not induce a noticeable conformational change. This and similarly mutated TNF molecules may be beneficial for cancer therapy, due to their reduced systematic cytotoxicity.

Recently, the structure of the TNF related ligand TRAIL (Apo2L) was independently solved by several groups to a resolution of 2.8–1.3 Å, both in a complex with DR5 receptor (Hymowitz et al., 1999; Mongkolsapaya et al., 1999) and as a free uncomplexed trimer (Cha et al., 1999; Hymowitz et al., 2000). The structure shows the expected B-sandwich jellyroll topology and has high degree of structural homolgy to the other TNF family members (TNFα , TNFβ, and CD40L). Conflicting reports emerged concerning the presence of a unique zinc-binding site within the trimer. Hymowitz et al. (2000) reported the presence of such site, which they suggested was important for both the structure and function of the protein. However, the zinc binding site was not observed by the other investigators (Cha et al., 1999; Mongkolsapaya et al., 1999). Mutagenesis studies identified at least five different residues (Gln 205, Val 207, Tyr 216, Glu 236, and Tyr 237) within TRAIL that are essential for high-affinity receptor binding and/or apoptotic function (Hymowitz et al., 2000).

STRUCTURE ASPECTS OF THE RECEPTOR-LIGAND COMPLEX AND THE RECEPTOR

The complex between TNFβ and the extracellular domain of TNFR-1 was the first view of the receptor (Fig. 2B). The complex confirmed cross-linking studies that TNF functioned by aggregating receptors upon binding them. The receptors are highly elongated, almost rod shaped (Fig. 2A). The receptors align their long axis parallel to each other and parallel to and in contact with the three TNFβ subunit interfaces (Banner et al., 1993). To continue with our earlier cone analogy, three rods lie along the vertical edges of the cone. The extracellular domain of the receptor can be split by sequence analysis into four similar sub-domains. Each sub-domain consists of between 30 to 40 amino acids (Banner et al., 1993; D'Arcy et al., 1993). The structure, which only visualised the N-terminal 3 and 1/2 domains, confirmed this analysis. Each subdomain contained 3 disulphide bonds, which held together three strands connected by loops. The interface between the receptor and TNFβ involved residues from two TNF monomers and from the second and third subdomain of the receptor. The interface splits into two almost separate contact surfaces. The lower region (Fig. 2B) consists of receptor residues 56 to 73, which form an extended loop like structure, which spans the interface between two TNFβ molecules. Residues 35–53 from one TNFβ subunit (denoted A) and residues 106–112 from the other (denoted C subunit) are involved in the contacts (Banner et al., 1993). Two regions of the receptor (residues 77–81 and 107–114) form the upper region and these sit in a cleft formed at the TNFβ monomer interface. Residues 82–84, 126–130, and 155–161 from A subunit and residues 97, 118–120 and150–152 of the C subunit of TNFβform this interface. The interface is a mixture of both directional hydrophilic contacts and hydrophobic contacts. This mix probably confers both the exquisite specificity and high affinity that characterises the receptor complex. The extracellular domains of the receptors made no contact with each other in the crystal structure. However, it seemed likely that by bringing the receptors close together this would promote aggregation below the membrane.

Shortly after the determination of the complex, the structure of the free receptor was reported at neutral pH (Naismith et al., 1995). The receptor showed extensive hinging movements between the complex and the free form (Naismith et al., 1995) but was unchanged in the overall description of the receptor structure. In this structure again only 3 and 1/2 domains were visualised. The receptor was found in the crystals as two different dimers, both with extensive contacts. This study suggested for the first time that receptors may dimerise in the absence of TNF. It was postulated that this may regulate the signalling by enforcing an "off" state and/or pre-assembling the receptors for signalling. Some biological evidence for this came from separate studies (Boldin et al., 1995). This suggested that in the absence of the extracellular domain the receptor constitutively signals. Further indirect evidence came from Remy et al. (1999), who showed that the erythropoietin receptor (unrelated in structure to the TNF receptor superfamily) dimerises in the absence of ligand and plays a role in signal regulation. Another dimer seen in the crystal structure would be capable of binding TNFα and may allow a 2-D array of receptor complexes to form on the cell surface (Fig. 3B). This is the so-called expanding network hypothesis (Naismith et al., 1995).

The high-resolution structure of the complete extracellular domain of the receptor was determined by Naismith et al. (1996). This structure was determined at low pH but again showed the protein to be a dimer with extensive contacts, but different to the one observed at neutral pH. The fourth module of the extracellular domain was revealed as having a very different structure than the other three modules. The structure emphasised that the elongated receptor deforms, in response to binding another molecule (either TNFβ or itself), by a series of hinging movements. These movements are very pronounced and can lead to a difference of nearly 20Å in atomic positions (Naismith et al., 1996). This led to a new method being advanced for modelling and aligning the extracellular domains of the TNF receptor superfamily. This new approach radically alters the location of certain residues and allows modelling of CD40 binding of its ligand (Singh et al., 1998). This study is particularly relevant to TNF as both the receptor and ligand share similar structures. Singh et al. (1998) highlighted the importance of the polar residues in controlling the interaction.

A consistent picture of the receptor superfamily emerges. The disulphide bonds form a structural scaffold that can be decorated with short regions of variable length and amino acid sequence. These variable regions confer specificity for the appropriate ligand. The combination of structural conservation and sequence variability allows each member of this large receptor superfamily to recognise its cognate ligand with exquisite specificity, whilst utilising a very small array of topologically distinct units. This recognition is enhanced by the inherent flexiblity of the receptor molecule, which can deform to optimise its interaction with ligand through a series of hinging movements.

Most recently the structure of extracellular fragment of DR5 (residues 58–184 or 1–130) complexed with its TRAIL/Ap02L ligand (residues 91–281 or 114–281) emerged from two different groups (Hymowitz et al., 1999; Mongkolsapaya et al., 1999) . The complex crystallised as a hexameric unit containing three receptors and a ligand trimer. Like TNFR1, DR5 forms an extended rod-like structure consisting of three cysteine-rich domains (CRDs), rather than the four observed for TNFR1. DR5 contains seven disulfide bridges; six were present in structural regions corresponding to CRD2 and CRD3 (three each) of TNFR1 and only one in the region corresponding to the C-terminal half (last disulfide bridge) of CRD1. Binding of Trail to DR5 buries 2,750 Å^2 of solvent-accessible surface area (1,400–1,540 Å^2 from the receptor and 1,350–1,420 Å^2 from the ligand). The residues of contact between the receptor and ligand (i.e., interface), were located in two main patches, covering a total area of 1,790 Å^2 and 890 Å^2, respectively. The structure suggests common structural features amongst all of the TNF receptors.

The structure of certain accessory proteins that associate with TNF receptors, such as TRAFs (Pullen et al., 1999), have also been determined. TRAF2 (TNFR associated factor 2) binds to the cytoplasmic domain of

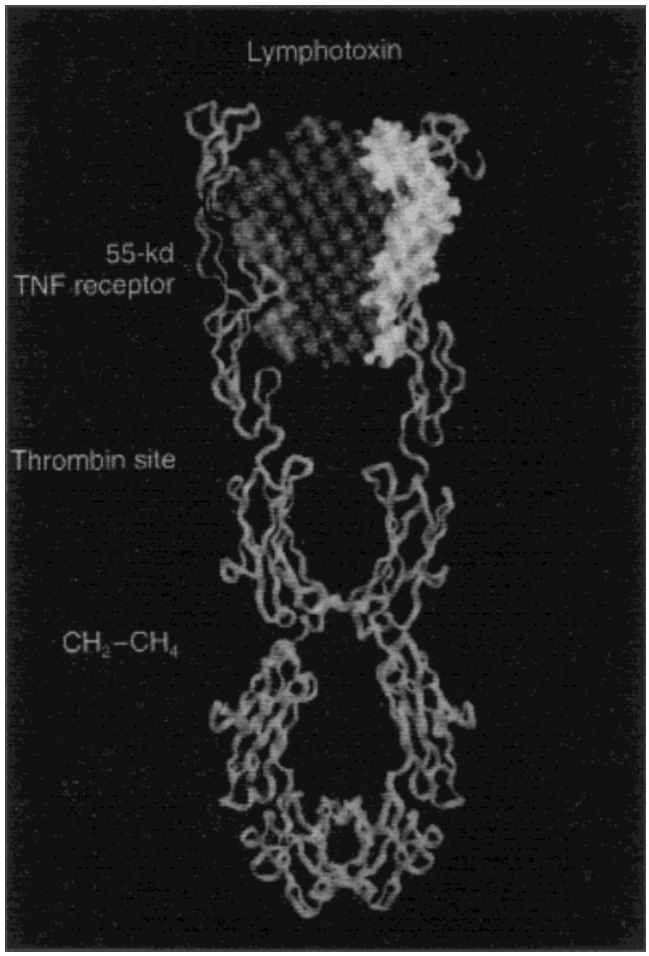

Fig. 6. Predicted tertiary and quaternary structures of a chimeric TNF inhibitor molecule generated by linking the extracellular domain of TNFR-1 to domains CH_2, CH_3, and CH_4 of an IgG Heavy Chain. Reproduced from Bazzoni and Beutler (1996), with permission of the publisher.

TNF receptors such as TNFR-1 and CD40 and mediates signal transduction. The structure of TRAF2 receptor binding domain, complexed with CD40 peptid, showed that TRAF2 forms a mushroom-shaped trimer consisting of a coiled coil and a unique beta-sandwich domain (McWhirter et al., 1999). The CD40 peptide binds in an extended conformation with every side chain in contact with a complementary groove on the rim of each TRAF monomer. The trimer may pre-organise the receptors to ensure recognition of three distinct sites on the TRAF trimer (McWhirter et al., 1999).

FUTURE PROSPECTS

The structure of many other TNF receptors should soon emerge. Elucidation of the crystal structure of the TNF related receptor, receptor activated NFκB (RANK), is currently underway. RANK is the sole signalling receptor for the ligand RANKL (also known as TRANCE/ODF) and its activation is important for differentiation and activation of osteoclasts, which are necessary for bone resorption and remodelling (Nakagawa et al., 1998; Takahashi et al., 1999). Signalling through RANK leads to activation of the transcription factor NFkB and the protein kinase JNK. Activation of these factors seems to be important for osteoclastogenesis (formation of mature osteoclasts from their precursor stem cells). Understanding the structure/function relationship for the RANK molecule and residues involved in binding RANKL or adaptor protein during signalling will be valuable for designing future drugs that may relieve diseases associated with perturbations of bone density, such as osteopetrosis or osteoporosis.

Generating TNF mutants with decreased systematic cytotoxicity and increased tumour-specific cytotoxicity is also a useful approach in terms of increasing the overall TNF potency against aggressive tumours. Similarly, TNF inhibitor molecules aimed at sequestering "surplus" TNF molecules in the circulation is a useful approach to reduce any harmful side effects of this cytokine. Of particular interest is a chimeric inhibitor molecule engineered such that the ligand binding domain of the TNFR is linked to parts of an antibody molecule, serving as a form of "artificial" decoy receptor (Peppel et al., 1991). Such chimeric inhibitors have now been produced for clinical use in diseases such as rheumatoid arthritis (Bazzoni and Beutler, 1996; Beutler, 1999)and can be delivered using adenovirus vector (Kolls et al., 1994). Chimeric TNFR-IgG molecules (Fig. 6) may prove useful in a range of other diseases. Continued emergence of the crystal structure of TNF ligands and relevant receptors will be invaluable for efficient design of such chimeras.

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