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Localization of the active site of human tumour necrosis factor (hTNF) by mutational analysis

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In order to define the active site(s) of human tumour necrosis factor (hTNF), we mutagenized its gene at random and directly screened the resulting population for loss of cytotoxic activity on L929 cells. Four biologically inactive mutant proteins (Arg32 → Trp, Leu36 → Phe, Ser86 → Phe and Ala84 → Val) behaved similar to the wild-type in various physico-chemical assays. The residues were positioned on a 3D structural model and were found to cluster together at the base of the molecule at each side of the groove that separates two monomers in the trimeric structure. A very conservative mutation at one of these sites (Ala84 → Val) almost completely abolished cytotoxic activity. Amino acid alterations in three other residues in close proximity to this receptor binding site were introduced: replacements at positions 29 and 146 clearly reduced cytotoxicity only when non-conservative alterations were introduced (Leu29 → Ser and Glu146 → Lys), suggesting an indirect influence on the active site. However, a conservative mutation at position 91 (Val → Ala) caused a significant drop (500-fold) in bioactivity which suggests that Val91 may also play a direct role in receptor recognition. Our results favor a model in which each TNF molecule has three receptor-interaction sites (between the three subunits), thus allowing signal transmission by receptor clustering.

Key words: active site/cytotoxicity/random mutagenesis/structure-function analysis/tumour necrosis factor

Introduction

Tumour necrosis factor (TNF) is a polypeptide primarily produced by stimulated macrophages, which expresses a wide array of biological activities on various cells, explaining its key role in inflammation and immune responses (for an overview: see Fiers *et al.*, 1986, 1990; Beutler and Cerami, 1988). The primary polypeptide is 157 amino acids long (mol. wt 17 350), and the mature molecule exists in solution as a trimer (Wingfield *et al.*, 1987; Lewit-Bentley *et al.*, 1988). A striking homology in amino acid sequence (30%) was found between TNF and lymphotoxin (LT), a cytokine produced by a subset of lymphocytes (Gray *et al.*, 1984; Fiers *et al.*, 1986). The majority of these conserved residues turned out to cluster near the inner core at the base of the molecule (Tavernier *et al.*, 1989). The two cysteines (forming a disulfide bridge, Mark *et al.*, 1987; Narachi *et al.*, 1987) and the two tryptophan residues (which are very conserved both between the TNF species and between TNF and LT, Van Ostade *et al.*, 1988), were found not to be involved in the active centre of the molecule. The TNF protein was crystallized by several groups (Fiers *et al.*, 1986; Eck *et al.*, 1988; Hakoshima and Tomita, 1988; Lewit-Bentley *et al.*, 1988), permitting X-ray diffraction studies to 2.9 Å (Jones *et al.*, 1989) and 2.6 Å (Eck and Sprang, 1989) resolution. The three-dimensional structure revealed that the monomer was built up by two antiparallel β-pleated sheets, formed by the viral jelly-roll motif. Moreover, a striking structural homology with several viral coat proteins was found, especially with satellite tobacco necrosis virus.

Two receptors for TNF have been cloned, with mol. wts of ~55 kd (Loetscher *et al.*, 1990; Schall *et al.*, 1990) and 75 kd (Smith *et al.*, 1990), respectively. These results are in accordance with the findings of Hohmann *et al.* (1989) and Brockhaus *et al.* (1990), who showed that cells of epithelial or myeloid origin bear different major TNF receptors (55 and 75 kd respectively). In addition, soluble forms of the two species, capable of inhibiting TNF activity, have already been purified from human urine (Engelmann *et al.*, 1990) and from serum of cancer patients (Schall *et al.*, 1990).

A next step towards an understanding of the mechanism of receptor recogniton by TNF is a detailed analysis at the molecular level of its binding site. By making use of the high solubility of recombinant TNF in *Escherichia coli* lysates, we developed a method based on random mutagenesis of the TNF gene, followed by a phenotypic screening for mutants, inactive in a cytotoxicity assay. Several relevant amino acids were identified. On the three-dimensional trimeric structure, they were all situated at the base of the molecule, at both sides of each interface between two subunits.

Results

Selection of TNF mutants with reduced cytotoxity

The chemical mutagen sodium bisulfite converts a cytosine residue into uracil and hence leads to a C → T transition. Because this mutagen acts solely on single-stranded DNA (Shortle and Nathans, 1978; Pine and Huang, 1987), we used the bacterial expression phasmid pMaT4-hTNF-T as a gap-duplex with only the TNF coding sequence in an ss-DNA configuration (Figure 1a, see also Materials and methods). Also, as an additional control, gap-duplexes with a smaller ss-DNA region were tested. A series of different sodium bisulfite concentrations (4 M, 1 M, 500 mM, 250 mM, 100 mM, 20 mM and 0 mM) were investigated using a fixed incubation time (16 h, 37°C). After filling-in of the gap *in vitro*, the DNA was transformed into *E.coli* BW313 (a strain capable of incorporating uracil residues into DNA and thus deficient in a repair function), and the resultant clones were

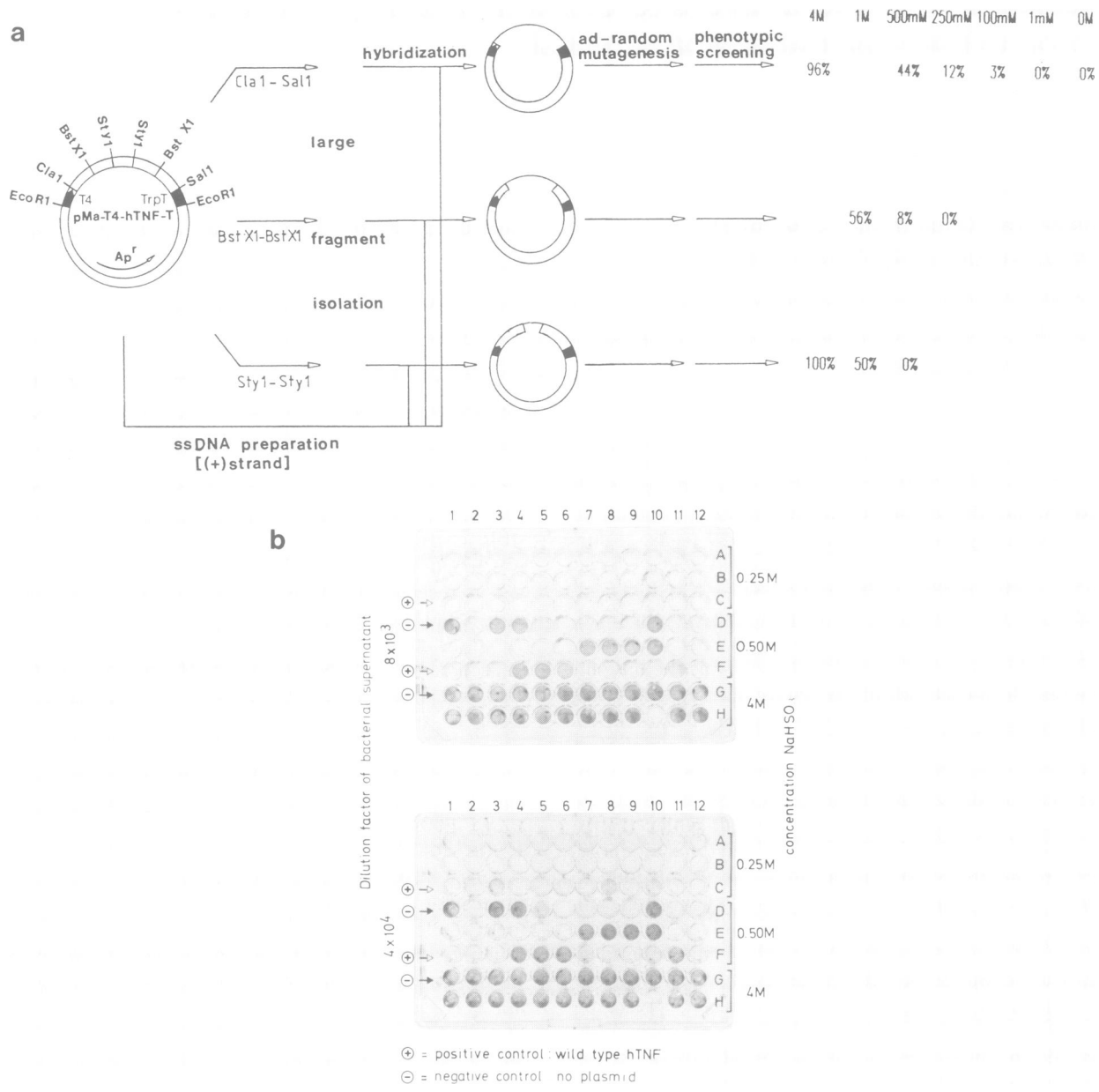

Fig. 1. Illustrations on the selection of inactive TNF mutants. (**a**) Brief schematic overview of the construction of the different gap-duplex structures and the resulting number of inactive mutants after random mutagenesis and phenotypic screening at a dilution factor of 4×10^4. Filled-in bars indicate bacteriophage T4 promoter and tryptophan terminator sequences. (**b**) Detection of mutant clones in a microtiter plate: two dilution factors (upper: 8×10^3 and below: 4×10^4) of the same supernatant of 96 colonies, resulting from treatment with 250 mM (lanes A,B,C), 500 mM (lanes D,E,F) or 4 M (lanes G,H) sodium bisulfite on the *ClaI–SalI* gap-duplex construction. Positive control wells (wild-type TNF): C1, F1; negative control wells (no plasmid); D1, G1. Additional mutants are detected at the higher dilution (lower plate, wells C3, C8, C10, D5, F7 and F11).

directly screened for their cytotoxic capacity. Each colony was inoculated, grown up, lysed and centrifuged in the well of a microtiter plate. Because the abundance of wild-type TNF in the *E.coli* lysate reached 30%, the screening test could easily be performed by diluting the supernatant into other microtiter plates and testing the cytotoxicity on mouse L929 cells. It was clearly shown that the higher the dilution, the more mutants with a partial activity appeared (Figure 1b). As expected, the number of colonies bearing an inactive protein was augmented with increasing concentrations of sodium bisulfite and with increasing gap width (Figure 1a). Treatment with 500 mM sodium bisulfite resulted in 44% mutants. This means that enough mutants could be produced from which presumably only a minority would contain multiple mutations. 310 colonies resulting from this type of mutagenesis were further screened for loss of TNF bioactivity. 132 colonies showed a clearly reduced cytotoxicity.

Characterization of the mutant proteins on denaturing polyacrylamide gels

Apart from an amino acid substitution, resulting in an alteration of the active site, at least three other events can result in a reduction of the bioactivity of the bacterial lysates

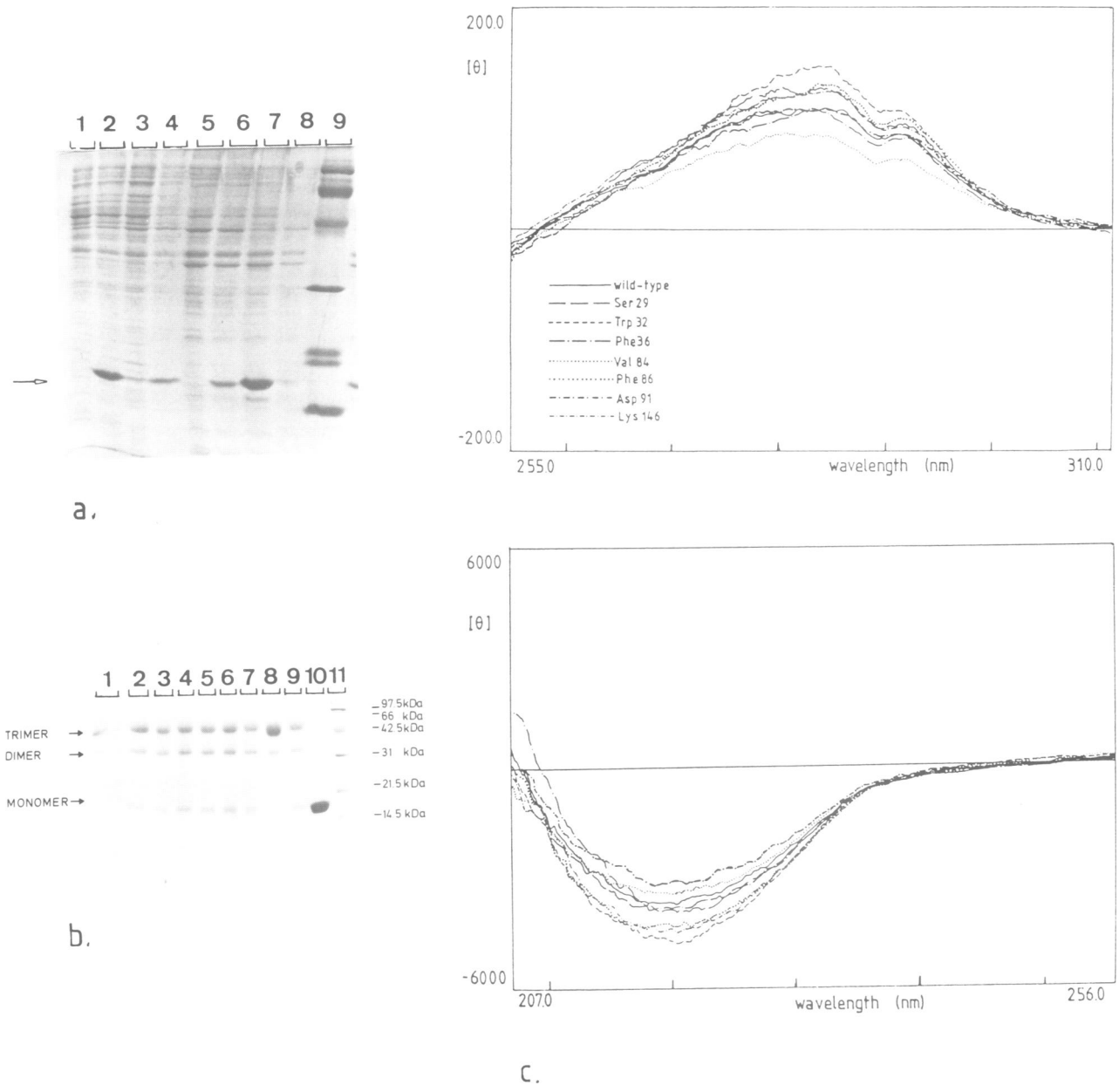

Fig. 2. Physico-chemical analysis of the mutants Arg32, Leu36, Ser86, Ala84 and Leu29, Val91, Glu146. (**a**) Different behaviour of TNF mutants on an SDS−PAA gel. Colonies were sonicated and the supernatant (lanes 1,2,3,4) and pellet fractions (lanes 5,6,7,8) were loaded onto the gel. Lanes 1 and 5: mutant with reduced expression level. Lanes 2 and 6: mutant with characteristics similar to those of the wild-type protein. Lanes 3 and 7: mutant with decreased solubility. Lanes 4 and 8: wild-type protein. Lane 9: mol. wt markers. The position of TNF is indicated by an arrow. (**b**) Cross-linking pattern of the purified proteins. The assay was performed as described in Materials and methods and mixtures were immediately loaded on an SDS−PAA gel. Lanes 1−9 all have BSOCOES added as the cross-linking agent. Lane 10 is without BSOCOES. Lane 1: wild-type; lane 2: Phe86; lane 3: Trp32; lane 4: Ser29; lane 5: Phe36; lane 6: Val84; lane 7: Asp91; lane 8: Lys146; lanes 9 and 10: wild-type; lane 11: mol. wt markers. (**c**) CD spectra of the different mutants in the near UV (I) or the far UV (II) region. The spectra are an average of 5−8 scans with the baseline subtracted. The protein concentration varied between 0.3 and 0.5 mg/ml in 0.01 M Tris, 0.09 M NaCl, 1 mM EDTA, pH 8.5. Recordings were performed at 20°C using cuvettes of 1 cm and 0.1 cm pathlength for the near and far UV regions, respectively.

after mutagenesis. The first and probably the most abundant phenomenon is insolubility. With the wild-type construct, ~75% of the TNF protein is present in the soluble fraction of the *E.coli* lysate. We have already shown that a single amino acid substitution, such as Trp28 → Phe (Van Ostade *et al.*, 1988) or Asp140 → Lys (Tavernier *et al.*, 1990) can convert the protein into an (almost) totally insoluble form. Because of purification problems, the value of these proteins for structure−function studies is limited. Secondly, mutants with a lowered expression level, possibly resulting from instability of the mutant mRNA or polypeptide can be generated, facing us with the same problems as mentioned above. A third possibility is the introduction of termination codons, resulting in shortened TNF molecules. Since deletion studies at the C-terminus have already been undertaken (Korobko *et al.*, 1989, see below), we were less interested in the latter mutant forms of TNF. Therefore, the supernatants of the 132 colonies were screened on SDS−polyacrylamide (SDS−PAA) gels, and only those mutant proteins showing similar mobility characteristics to

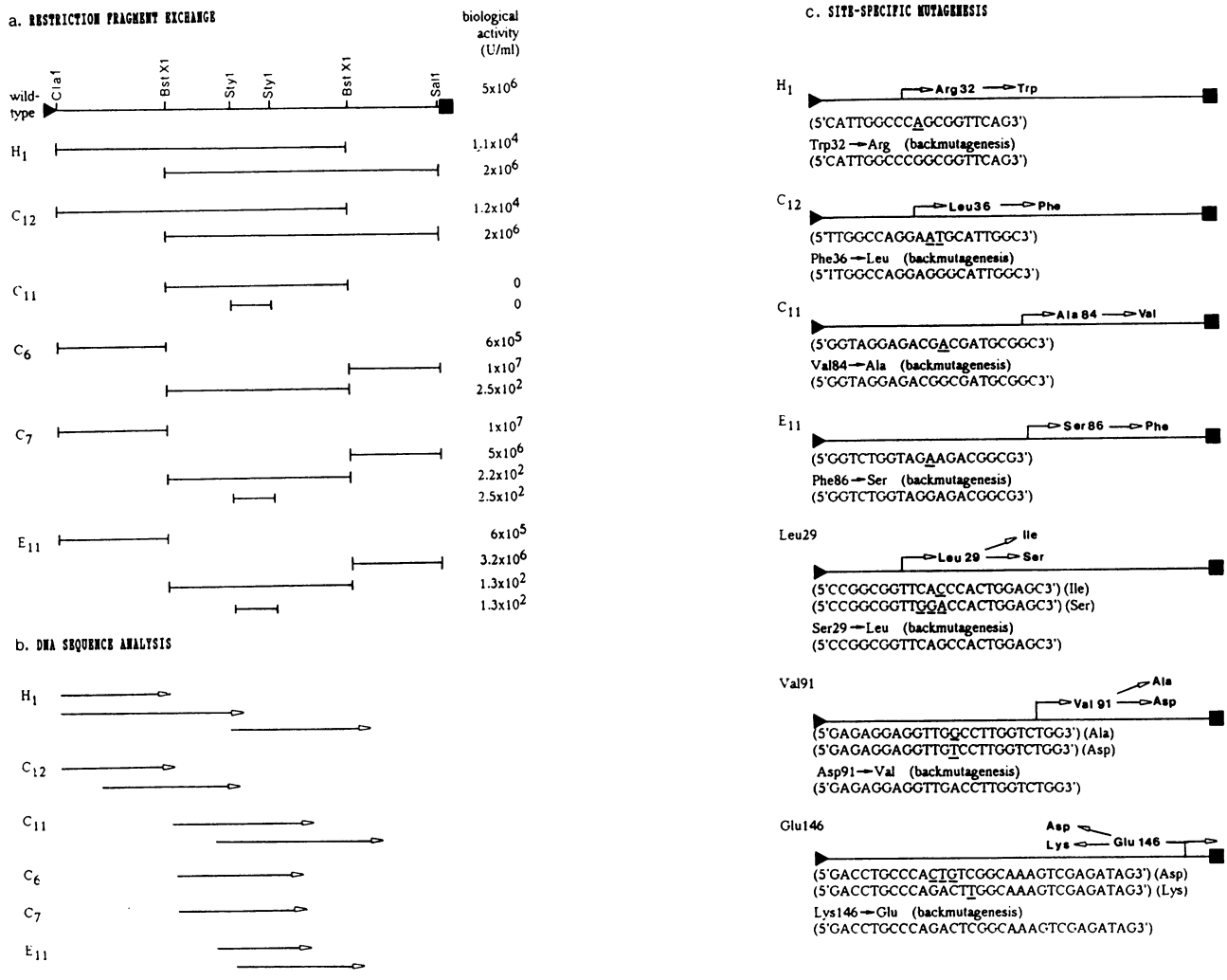

Fig. 3. Genetic analysis of TNF mutants. (**a**) Localization of the mutations by restriction fragment exchange. Restriction fragments, shown by bars, were derived from the mutants and were inserted into the wild-type gene or *vice versa*. The resulting constructs were tested for biological activity, expressed in U/ml (= the activity, present in the supernatant of a bacterial lysate after sonication and centrifugation; the concentration of TNF protein was checked by SDS–PAA gel electrophoresis and was comparable in all cases within a factor of 2). The arrowhead and the square indicate the T4 promoter and Trp terminator sequences, respectively. (**b**) DNA sequence analysis: arrows indicate sequenced regions. (**c**) Site-specific mutagenesis. Oligonucleotides used for the corresponding site-specific mutagenesis are shown. Base alterations that introduce the mutation and/or create or destroy a restriction site are underlined. All the back mutants and the two conservative mutants Leu29 → Ile and Glu146 → Asp showed wild-type activity ($2-8 \times 10^6$ U/ml bacterial lysate). Other mutants, clearly reduced in bioactivity, were purified and their specific activity was determined (Table I).

those of the wild-type were retained for further analysis (Figure 2a). We found 28 mutants of this type, out of which eight showed a slight reduction (2- to 5-fold) in expression level.

Localization of inactivating mutations in the TNF gene

The relevant amino acid change of six mutants with a high expression level and a clear reduction in cytotoxicity (10^5- to 10^6-fold for mutants C_6, C_7, C_{11} and E_{11} and 100-fold for C_{12} and H_1) was determined. Because several mutations in one TNF gene could have arisen during random mutagenesis, we first localized the relevant mutation by restriction fragment analysis. As can be seen in Figure 3a, different restriction fragments were transferred from the mutant to the wild-type gene or *vice versa*. Sequencing of the fragments (Figure 3b) clearly carrying the relevant mutation, revealed four base changes (all C → T transitions): one at position 84 (GCC → GTC), converting an alanine into a valine (Val84), which is a very conservative change. This mutation was only found in clone C_{11}. A second mutation was found at position 86 (TCC → TTC) and replaced a serine by a phenylalanine (Phe86), a non-

Table I. Specific activity of purified TNF mutants[a]

TNF molecule	Specific activity (U/mg)
Wild-type	2×10^7
Arg32 → Trp	6.4×10^4
Leu36 → Phe	8×10^4
Ala84 → Val	< 10
Ser86 → Phe	3.2×10^2
Leu29 → Ser	1×10^5
Val91 → Ala	3.2×10^4
Val91 → Asp	44
Glu146 → Lys	48

[a]The cytotoxic activity was measured on L929 cells and the protein concentration was determined by the Biorad protein assay (see Materials and methods).

Table II. Random amino acid insertion at positions 84 and 86

Original mutant	Codon introduced	Resulting amino acid	Biological activity (U/ml cell lysate)
Val84	–	–	4
	GCC	Ala	5×10^6
	(= back mutagenesis to wild-type)		
	CTC	Leu	32
	GAC	Asp	10^2
	ATC	Ile	75
Phe86	–	–	10^2
	AGC	Ser	4×10^6
	(= back mutagenesis to wild-type)		
	CTC	Leu	10^3
	GTG	Val	2×10^4
	ATC	Ile	4×10^3
Wild-type	–	–	5×10^6

conservative change. This alteration was clearly more abundant since it was detected in clones C_6, C_7 and E_{11}. Clone C_{12} showed a CTC → TTC transition at position 36, resulting in a fairly conservative change of leucine to phenylalanine (Phe36). Finally, the mutant H_1 contained a transition at position 32 (CGG → TGG), replacing arginine by tryptophan (Trp32), a non-conservative mutation. On the basis of this information, the four mutations were introduced into the human wild-type TNF gene by site-specific mutagenesis (Figure 3c). The activity of the resultant clones mimicked the activity of the primary mutants, resulting from random mutagenesis. These experiments confirmed that the observed mutations were indeed responsible for the drop in biological activity. A final confirmation was obtained by a site-specific mutagenesis of the mutant gene, converting it back to the wild-type sequence. In all four cases, wild-type activity was regained. We concluded that no other accidental mutations had been introduced by the mutagenesis.

Purification, specific activity determination and further physico-chemical characterization of the mutants Trp32, Phe36, Val84 and Phe86

The Trp32, Phe36, Val84 and Phe86 mutant proteins, derived from the site-specific mutagenesis, were purified by ion-exchange chromatography using a MONO-Q column (Pharmacia), resulting in protein preparations of >95% homogeneity. The residual biological activities are indicated in Table I and correlate well with previous biological activities, measured from the bacterial lysates (if one assumes a concentration of 250 μg/ml bacterial lysate, determined by staining on an SDS−PAA gel). This concentration varied only within a factor of 2 since one of the screening criteria was the maintenance of the wild-type TNF expression level. We were also interested in the degree of trimerization of the mutants, since only trimeric TNF has an active three-dimensional structure (Wingfield *et al.*, 1987) and is biologically active (Smith and Baglioni, 1987). Crosslinking of the mutants with the reagent bis[2-(succinimidooxy-carbonyloxy)ethyl]sulfone (BSOCOES), followed by separation of the products on an SDS−PAA gel, showed a similar pattern for all mutants as for wild-type TNF (Figure 2b). This means that all mutant proteins associated correctly to the trimeric form. Also, circular dichroism (CD) spectra could not discriminate between any mutant and the wild-type protein (Figure 2c). The minima at 219 nm in the far UV and the maxima around 285 nm with a minor peak at 292 nm in the near UV remain in general identical to the positions observed for the wild-type protein. Only slight differences can be seen, presumably due to minor local conformational distortions, removal or addition of aromatic amino acids or experimental errors. Together with the results we obtained from the SDS−PAA gel screening (see above), these experiments provide in different ways data on the physico-chemical characteristics of the mutant proteins, showing that no significant changes have taken place and hence that the overall 3D-conformation of the mutant proteins has largely been preserved.

Random mutagenesis at positions 84 and 86

The role of residues Ala84 and Ser86 was investigated in more detail. Site-specific mutagenesis was performed on the two mutant genes Val84 and Phe86. However, in this experiment, the probe contained a fully degenerate codon at position 84 or 86 respectively, resulting in random insertion of any of all possible amino acids at each position. Clones that did not bear the original mutation were selected on the basis of non-hybridization to the original oligonucleotide, and tested for biological activity as described above. By sequencing, it turned out that all mutants which showed wild-type bioactivity also had inserted the wild-type amino acid (Table II). This suggests that no other residue can functionally replace the wild-type residues. This is almost certainly true for position 84 where every other mutation had an extremely low bioactivity. Position 86 is somewhat more flexible, since the exchange of the aromatic Phe for Leu, Ile or Val increased the bioactivity. Furthermore, both positions seem to have a limited structural involvement, since only a few colonies (1/24 for position 84 and 1/10 for 86) contained an insoluble protein (data not shown).

Localization of positions 32, 36, 84 and 86 on a three-dimensional structural model—mutagenesis at positions 29, 91 and 146

The three-dimensional structure of TNF was modelled, making use of the coordinates provided by Eck and Sprang (1989). As can be seen in Figure 4a, the positions of the four amino acids Arg32, Leu36, Ala84 and Ser86 are all located in the lower half on the outside of the molecule. They fall into two groups, which are separated by the groove between two subunits (Figures 5a, b and 6). Ala84 and Ser86

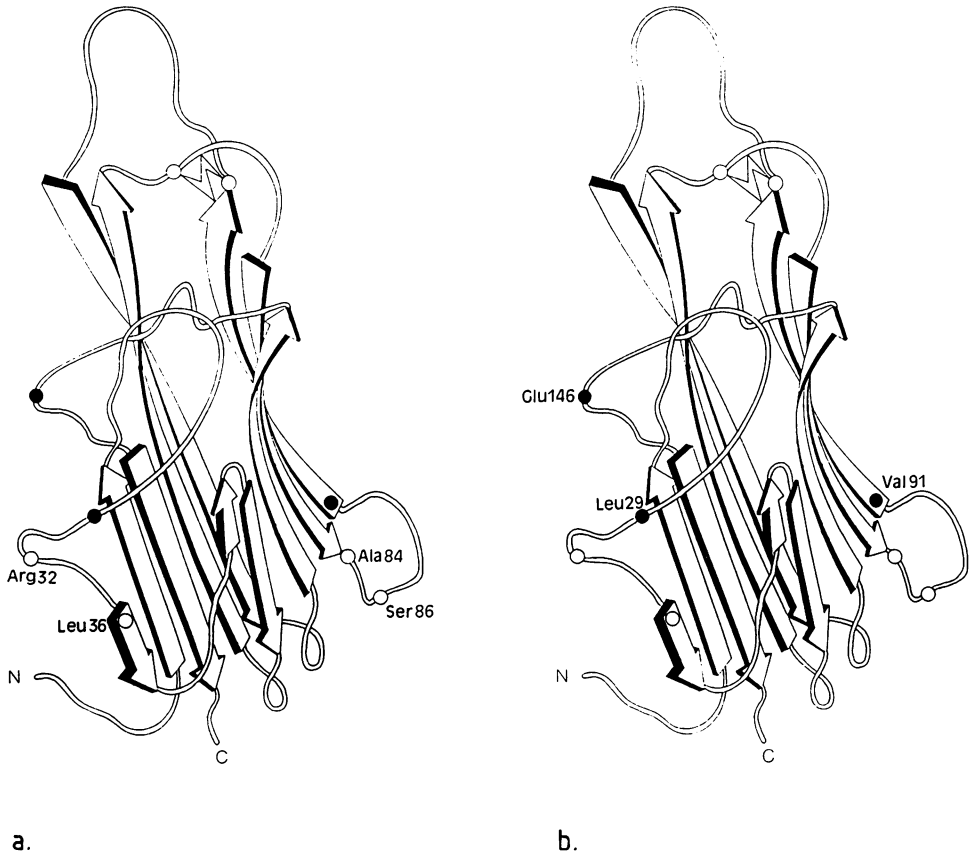

Fig. 4. Schematic drawing of a TNF monomer showing the positions of Arg32, Leu36, Ala84 and Ser86 as open circles (**a**) and Leu29, Val91 and Glu146 as black spots (**b**). The disulfide bridge at the top of the molecule is indicated by a lightning sign. Adapted from Jones *et al.* (1989).

are located on the β-turn that connects β-strands d and e [for nomenclature, see Eck and Sprang (1989)], one of which (e) flanks the groove at the left side. Arg32 lies in the β-turn, connecting β-strands a and a′. The latter contains Leu36 and flanks the groove at the right side. Based on these observations, we hypothesized that the groove could be an essential part of the active centre of TNF. On the basis of the computer graphics model, Glu146, which also flanks the groove, but is localized higher in the molecule, was selected for further investigation. In addition, Val91 (its side-chain pointing towards the solvent) and Leu29 were chosen to examine further the involvement of the two β-turns in the active site (Figures 4b and 5c and d). Conservative and non-conservative mutations were introduced at these positions (Figure 3c). The non-conservative mutations (Leu29 → Ser, Val91 → Asp, Glu146 → Lys) were further investigated regarding their physico-chemical properties (SDS−PAA gels, cross-linking, CD spectra) and they all showed the same characteristics as the wild-type TNF (Figure 2), indicating that the overall structure had been maintained. These alterations reduced the cytotoxicity drastically, especially for positions 91 and 146 (18 U/mg and 35 U/mg, respectively, Table I). It is possible that they have an indirect influence on the active site by disturbing the normal conformation of the groove (positions 91 and 146) or the local environment of the active site (all positions). Two of the three conservative replacements (Ala29 → Val, Glu146 → Asp) still result in a wild-type activity of the mutant, while the conservative mutation at position 91 caused a 500-fold drop in bioactivity. The fact that a very conservative alteration can reduce bioactivity only at position 91 and not at position 146, could be a further indication for the position of the active site near the bottom of the molecule. Hence, a direct involvement of Val91 in receptor recognition is quite possible.

Discussion

Due to the solubility of TNF in *E.coli* lysates, colonies resulting from random mutagenesis on the human TNF gene could be directly screened by a bioactivity assay and subsequently characterized on an SDS−PAA gel. This procedure enabled us to identify four mutants, with a strongly reduced bioactivity, while retaining wild-type characteristics as evidenced by various physico-chemical properties. The fact that all the mutations were clustered in one region on the 3D-structure (but not on the primary structure) is an intrinsic control on the random mutagenesis and the screening procedure, and strongly suggests that, as far as L929 cytotoxicity is concerned, no other distinct active sites are present on the TNF molecule. On the basis of these data, we propose that the receptor interaction site of TNF is localized in the lower part of the molecule, presumably at both sides of the cleft between two subunits (Figures 4, 5a, b and 6).

Particularly striking is the total loss of biological activity for the Ala84 → Val mutant (Table I). As the side-chain of Ala84 points towards the inside of the molecule, there is apparently no space available to accommodate the larger side-chain of valine. Hence, the loss of biological activity seems to be due to a local conformational change. But we cannot exclude, nevertheless, the possibility that Ala84 participates in a direct contact with the receptor, perhaps

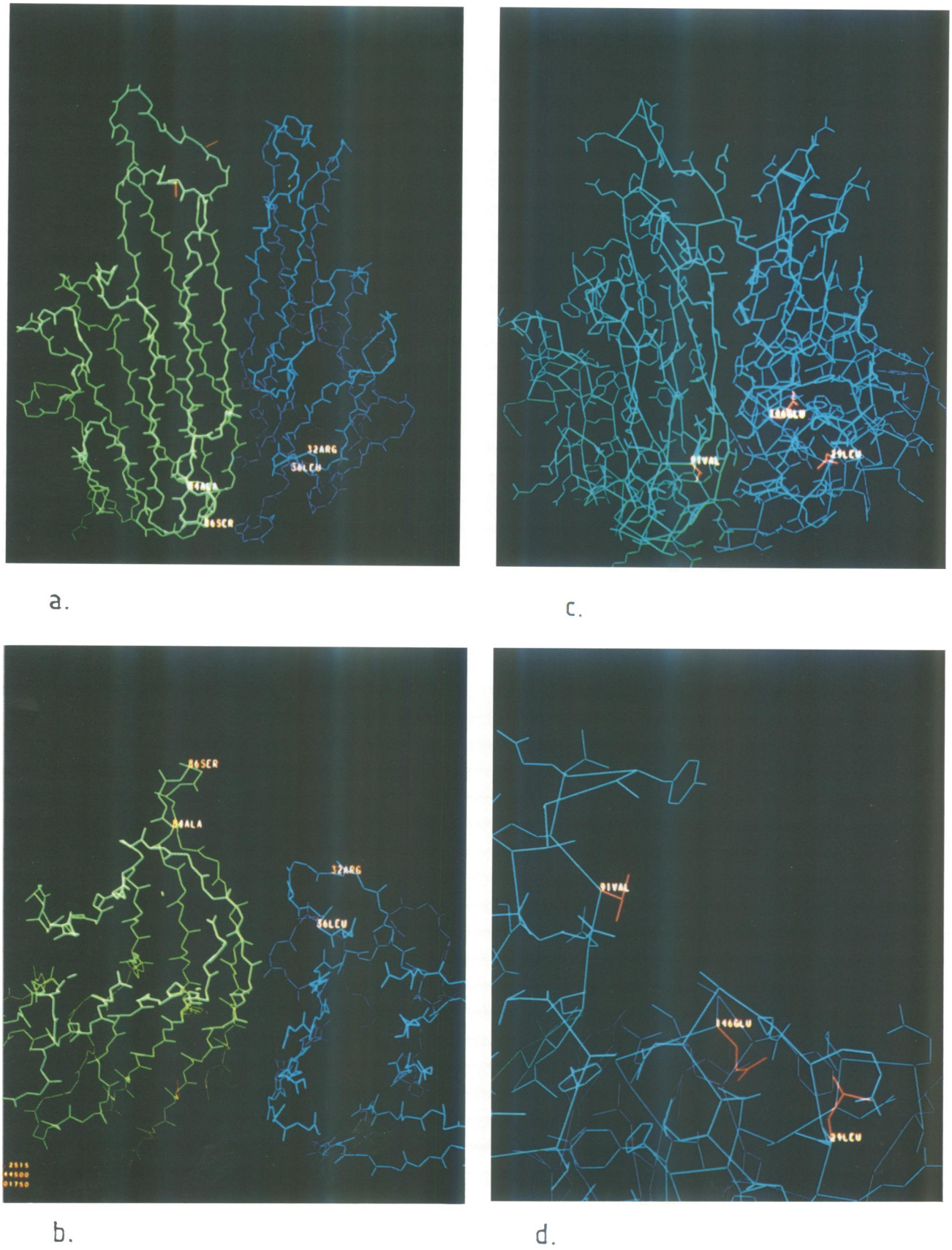

Fig. 5. Localization of positions 32, 36, 84, 86 (a,b) and 29, 91, 146 (c,d) on a three-dimensional computer simulation of the TNF structure. To avoid complexity, only two subunits of the TNF trimer are shown. (**a**) Side view with residues Arg32, Leu36, Ala84 and Ser86 indicated. (**b**) The same, but bottom view, showing the four residues at both sides of the interface. (**c**) Total image of the two subunits [as in (a)] but including all side-chains. The side-chains of residues Leu29, Val91 and Glu146 are coloured orange. (**d**) The same, but bottom view, showing the surfaces of each subunit facing the groove. Both Val91 and Glu146 are positioned at the edge of the interface and the side-chain of Val91 clearly points into the groove.

as a result of a binding-induced steric rearrangement. In order to elucidate this question, a detailed comparison of the Ala84 → Val mutant with the wild-type structure by means of X-ray diffraction analysis of crystals of the former protein is required (T.Prangé, in preparation). These crystals have already been obtained, showing in their morphology no difference from the wild-type crystals, a further indication for the preservation of the overall structure of the Val84 mutant. Further evidence for the involvement of the β-turn, connecting strands d and e and comprising Ala84 and Ser86, comes from two recent papers: Jones *et al.* (1990) pointed out that the coat protein VP1 from the picornavirus HRV14 is structurally related to TNF. The viral receptor binding regions were mapped on the TNF structure and it turned out that amino acids involved in the binding correspond to Ile83, Asp130 and Ser133 in the TNF sequence. One of these three residues is located in and the other two very near to the Ala84–Ser86 β-turn. Furthermore, we have previously shown that Arg131 is involved in the epitope of a neutralizing monoclonal antibody although it does not participate directly in the receptor interaction (Tavernier *et al.*, 1990).

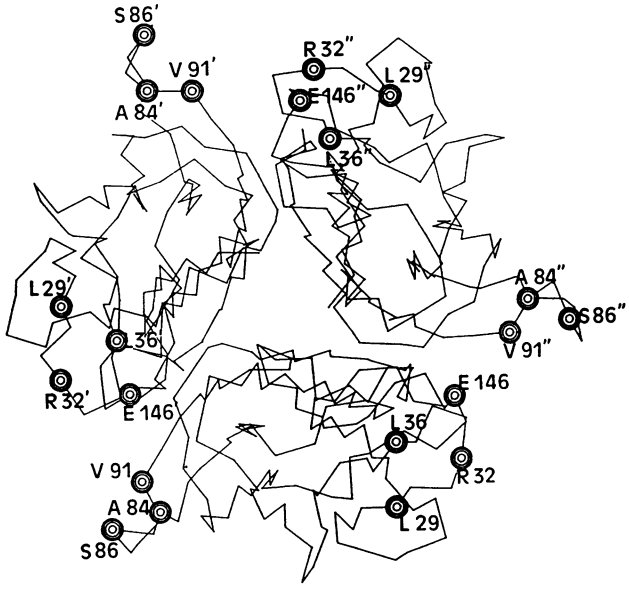

Fig. 6. Top view of the TNF molecule indicating the clustering of the seven mutant positions (29, 32, 36, 84, 86, 91 and 146) on the TNF trimer. To avoid complexity, only the lower section of the molecule is shown.

Hence, regions in the proximity of position 131 (like the Ala84–Ser86 loop) could also be topologically shielded by this antibody.

On the other side of the cleft, at least two amino acids are also likely to be involved in the receptor interaction. Leu36 is part of a β-sheet (a'), making it possible that replacement by phenylalanine could induce a local distortion in the conformation of the neighbouring receptor binding site, resulting in reduced activity. Arg32, positioned in the preceding β-turn, has previously been mutated to threonine by Tsujimoto *et al.* (1987). However, this TNF mutant contained also an Arg → Asn mutation at position 31. Nevertheless, the double mutant showed an activity on L929 cells of ~2%, a value close to the one we observed.

Concerning Leu29, it could be that a mutation to serine induces a small distortion which, in turn, could alter the constellation of the residues of the active site, positioned around the bottom of the groove.

Our results are in agreement with the data of Yamagishi *et al.* (1990), who showed, by means of random and site-directed mutagenesis, that the integrity of four regions [amino acids 32–34 (I), 84–91 (II), 117–119 (III) and 143–148 (IV)] is important for maintaining the biological activity. Three of these domains (I, II and IV) correlate well with our findings on the Ala84–Val91 β-turn, the Arg32–Leu36 β-turn and Glu146, respectively. Concerning the latter residue, it should be noted that a conservative mutation to Asp146 did not affect the biological activity (Figure 3c), suggesting that this residue has no direct contact with the receptor, but rather induces an indirect effect (via a positive charge interaction or a local conformational change). The conservative mutation (Val → Ala) at position 91 on the other hand, leads to loss of activity and concerns a residue on the surface and is exposed to the solvent (Table III). Therefore, this side-chain could very well be involved in a direct contact with the receptor. Furthermore, this residue is located in close proximity to residues 84 and 86, as discussed above.

In addition to this, several authors have also argued for an active site at the lower half of the molecule: (i) On the basis of the sequence conservation between TNF and lymphotoxin (LT), Tyr56 was hypothesized to be a potential participant in the ligand interaction (Eck and Sprang, 1989). The side-chain of this residue sticks out in a hollow at the base of the triangular pyramid; their detailed proposal for receptor interaction with this hollow does not, however, fit with our data. (ii) The histidine residue at position 15 has

Table III. Orientation of some residues implicated in biological activity

Residue	Direction of side-chain	Comments
Arg32	Surface	
Leu36	Groove interface + surface	Part of the hydrophobic cluster (Leu36/Leu55'/Val13)
Ala84	Groove interface + stacking in β-sheet	Implicated in a β-turn structure
Ser86	Surface	No contacts with other residues, but involved in a β-turn structure, as Ala84
Val91	Surface	Side-chain exposed to solvent
Glu146	Groove interface + surface	Oriented towards conserved residues Leu63 and Leu143

Residues of subunit 2 are labeled '.

been mutated to several other amino acids, most of them resulting in a marked reduction in biological activity (Yamamoto *et al.*, 1987, 1989). Since His15 is located very close to Leu36 (the NH36 → 015 distance is only 2.9 Å) but has a rather hidden position in the trimeric structure, replacement of this amino acid could result in a local conformational change, interfering with proper receptor binding events. Indeed, all the His15 mutants were insoluble, which not only suggests some conformational distortion, but also means that solubilization by denaturing agents followed by renaturation was necessary. Hence, if His15 is important for the correct overall 3D structure, inactivation of the protein could also be the result of faulty refolding of the polypeptide. (iii) Most of the conserved residues between TNF and LT are located inside the lower half of the molecule (Tavernier *et al.*, 1989), suggesting that this domain plays a crucial role in maintaining the correct conformation of the receptor binding site. (iv) Two neutralizing antibodies, one produced against the N-terminus of the molecule (Socher *et al.*, 1987) and another with Arg131 involved in its epitope (Tavernier *et al.*, 1990, see also above), bind near the bottom of the TNF molecule. (v) According to Eck and Sprang (1989), Lys11 and Ile157 (both positioned at the base of the molecule) form a salt bridge and hence are likely to fulfil a structural role. Therefore, deletions at the N-terminus, very close to Lys11 (Carlino *et al.*, 1987; Mark *et al.*, 1987; Soma *et al.*, 1987) or deletions and replacements at the C-terminus (Korobko *et al.*, 1989), result in a drop of biological activity.

Espevik *et al.* (1990) reported that antibodies generated against a TNF receptor could induce a TNF-like effect, indicating that cross-linking of receptors can account for the biological activity of TNF. As, according to our model, the native TNF molecule presumably contains three receptor binding sites (Figure 6), cross-linking of receptors could be the mechanism by which TNF exerts its various biological effects.

Further studies on amino acid residues facing the putative receptor interaction site are in progress. But ultimately the fine details may be solved by structural studies on ligand-soluble receptor complexes or crystals.

Materials and methods

Random mutagenesis

To carry out mutagenesis with the bisulfite mutagen which is specific for ss-DNA (Shortle and Nathans, 1978; Pine and Huang, 1987), 'gap-duplexes' were constructed in which only the human TNF gene was exposed as ss-DNA. Human TNF, under expression control of a constitutive T4 promoter and a Trp terminator, was inserted into the *Eco*RI site of the pMa and the pMc phasmids (Stanssens *et al.*, 1989) resulting in pMaT4-hTNF-T and pMcT4-hTNF-T, respectively (Figure 1a). The 'gap-duplexes' were constructed as described for site-specific mutagenesis (Stanssens *et al.*, 1989) with the exception that the (−) strand contained the same active antibiotic resistance gene (in this case the β-lactamase (bla) gene) as the (+) strand. This resulted in a homo-gap-duplex structure, while the construction, used for site-specific mutagenesis was a hetero-gap-duplex, in order to allow selection for the mutated strand. By making use of different restriction sites, variation of the width of the gap was possible: 470 nucleotides (*Cla*I−*Sal*I), 237 nucleotides (*Bst*XI−*Bst*XI) or 68 nucleotides (*Sty*I−*Sty*I) (see Figure 1a). Each of these constructions was treated with increasing concentrations (0−4 M) of bisulfite, followed by several dialysis steps, as described (Shortle and Nathans, 1978). About 10 ng of each sample was then transformed into the *E.coli* BW313 strain, allowing fixation of the mutations to uracil, generated on the (+) strand, by *in vivo* fill-in of the gap whereby an adenine base was inserted opposite the uracil residues.

Screening for inactive mutants and polyacrylamide gel electrophoresis of the mutant proteins

Each colony was inoculated in a microtiter plate in 200 μl LB containing 50 μg/ml carbenicillin and incubated at 37°C overnight. To lyse the cells 20 μl lysozyme (20 mg/ml in 10 mM Tris−HCl pH 8) was added and incubated for 15 min at 37°C, followed by five freeze−thaw cycles (−80°C, 15 min; 37°C, 20 min), with regular stirring in between. Nucleic acids were precipitated by polyethylenimine (4 μl of a 20% stock solution, 30 min, 4°C). Subsequently, the microtiter plates were centrifuged (1000 *g*) and from each well, the supernatant was diluted to other microtiter plates in L929 cell medium. Most frequently, dilution factors of 6×10^2, 8×10^3, 4×10^4, 4×10^5 and 2×10^6 were used. To 100 μl diluted supernatant, 100 μl L929 cell suspension (4×10^4 cells, containing 2 μg/ml actinomycin D) was added. Thus, the assay was carried out essentially as described by Ruff and Gifford (1981), with the only difference that one microtiter plate contains only one dilution of the supernatant of 96 different colony lysates (Figure 1b). Five microtiter plates, each containing an appropriate dilution of the bacterial lysate, gave in this way a crude estimation of the titer of 96 colonies. The number of mutants resulting from a given sodium bisulfite concentration on a given gap-duplex was determined from the results of the 4×10^4 dilution, as indicated in Figure 1a. This dilution factor was chosen because it provided us with a high number of mutants exhibiting a relatively low cytotoxic activity (< 2%). Colonies, which proved to contain an inactive, or a partially active protein, were grown in 5 ml LB medium containing 50 μg/ml carbenicillin at 37°C until saturation. 1 ml of this culture was centrifuged in an Eppendorf centrifuge for 10 min and the bacterial pellet was resuspended in phosphate buffer, pH 7. Sonication and centrifugation yielded a supernatant, a sample of which (30 μl) was directly analyzed by electrophoresis on 15% SDS−PAA gels (Laemmli *et al.*, 1970). The pellet was redissolved in 100 μl 'Laemmli buffer', from which 10 μl was used for electrophoresis on the same gel.

Sequencing

Sequencing was performed with the Pharmacia T7 Sequencing kit (Pharmacia) using T7 polymerase on supercoiled DNA. Each mutation was sequenced at least twice.

Site-specific mutagenesis

Constructs pMaT4-hTNF-T and pMcT4-hTNF-T also allowed a high expression of TNF in *E.coli* (±30% of the total protein in the soluble fraction when transformed into *E.coli* WK6). As a result, we were able to mutagenize and evaluate the cytotoxic activity of the mutants with the same plasmid.

Site-specific mutagenesis was performed according to the method of Stanssens *et al.* (1989). This procedure basically resembles the classical method of Kramer *et al.* (1984), but uses antibiotic resistance genes instead of M13 functional genes for selection to the mutation-containing strand. An advantage of this method lies in the capability to perform successive cycles of mutagenesis without the need to transfer the gene to a new mutagenesis vector: second round mutagenesis differs only in the selection to another antibiotic marker. We used the chloramphenicol acetyltransferase (*CAT*) gene to select for a mutant TNF gene. As a control on this experiment, however, the bla gene was used for site-specific back mutagenesis of the mutant to the wild-type TNF.

In most cases, an oligonucleotide, creating or destroying a restriction site in the TNF gene, was used in order to control the mutant not only by hybridization but also by the presence or absence of the restriction site (see Figure 3c).

A fairly accurate estimation of the activity of the mutant proteins could be achieved by measuring the cytotoxicity of the supernatant of a sonicated culture in an L929 assay and quantifying the amount of TNF on an SDS−PAA gel (see above).

Purification of the mutant proteins

Mutant proteins were purified according to the method described by Tavernier *et al.* (1990), using ammonium sulfate and polyethylenimine precipitation followed by a MONO-Q chromatography step. Only the wild-type TNF was purified by means of an additional MONO-S step. Elution was monitored by SDS−PAA gels. The purest fractions were immediately frozen at −80°C.

Cross-linking

6 μl of the crosslinking agent BSOCOES (Smith and Baglioni, 1987; 30 mg/ml freshly prepared in dimethylsulfoxide) was added to the mutant or wild-type TNF (25 μg/40 μl phosphate buffer, pH 7). Cross-linking was allowed to proceed for 30 min at room temperature and the reaction was terminated by the addition of 15 μl SDS−PAA gel sample buffer. The

mixture was heated to 95°C for 5 min and electrophoresed on a PAA gel according to the method of Laemmli *et al.* (1970).

CD spectra

Concentrations of mutant and wild-type proteins were determined on the basis of their absorbance at 280 nm, using an extinction coefficient of 1.62 (Narachi *et al.*, 1987). The concentration of mutant Trp32, where a tryptophan residue was introduced, was determined using the Biorad protein dye reagent. CD data were collected using a JASCO J-600 spectropolarimeter. These data were expressed as mean residue ellipticity, using a mean residue weight of 111 (Narachi *et al.*, 1987).

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