Two conserved tryptophan residues of tumor necrosis factor and lymphotoxin are not involved in the biological activity

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Each of the two highly conserved tryptophan residues in hTNF (positions 28 and 114) was converted into phenylalanine by site-directed mutagenesis and the mutant proteins were partially purified. A cytotoxicity assay on mouse L929 cells showed only a slight reduction in biological activity, strongly suggesting that neither of the two amino acids is involved in the active site.

Tumor necrosis factor; Site-specific mutagenesis; Cytotoxicity

1. INTRODUCTION

Human and murine tumor necrosis fractors (hTNF and mTNF), products derived from macrophages, exist as trimers consisting of three equal subunits of 17 kDa [1,2]. The monomers have a length of 157 and 156 amino acids and show a homology of 79%. Both contain an unusually long prepeptide of 76 and 79 amino acids respectively, with an even higher degree of amino acid conservation of 86% [3]. The amino acid sequence of the rabbit TNF (rTNF) revealed a monomer of 154 amino acids with 80% homology, as compared with hTNF. Lymphotoxin (LT), also referred to as hTNF-β, a related lymphokine produced by lymphocytes, is preceded by a classical signal peptide [4]; the mature human and mouse contain 172 and 169 amino acids, giving a homology of 28 and 31%, respectively, with the corresponding TNF [5,6].

Comparing these 5 related sequences, four strongly conserved regions can be deduced (positions 11–15, 48–64, 119–133, 152–157; hTNF numbering) (fig.1). TNF contains a central region which has no similarity to the lymphotoxins and is almost completely enclosed between the two conserved cysteine residues (positions 69, 101). Cys 69 can still be found in mLT. These cysteine residues in TNF form a disulfide bridge, that is probably not necessary for maintaining the overall conformation as shown by CD spectra [7,8]. Fluorescence spectra and quenching, however, indicated that, although the biological activity was not reduced significantly, the local conformation around the two tryptophan residues was affected after removing this disulfide bond [9].

Our attention was attracted by these two tryptophan residues which are highly conserved among all the molecules sequenced so far. Here, we evaluate whether this conservation is related to their functional importance, or whether these residues play a merely structural role. We converted each tryptophan residue by site-specific mutagenesis into phenylalanine (Phe) and subsequently examined the effect on the cytotoxic activity of hTNF for malignant cells. It was found that the two mutant proteins, 28F and 114F, only exhibit a somewhat reduced specific activity: 45 and 30% of wild type, respectively. This indicates that these residues are not involved in the active site.

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                                                                    1                                          10
MOUSE LT   LEU SER GLY VAL  -   -  ARG PHE SER ALA ALA ARG THR ALA HIS PRO LEU PRO GLN LYS HIS LEU THR HIS GLY ILE LEU
HUMAN LT   LEU PRO GLY VAL GLY LEU THR PRO SER ALA ALA GLN THR ALA ARG GLN HIS PRO LYS MET HIS LEU ALA HIS SER THR LEU
HUMAN TNF                                                                  VAL ARG SER SER SER ARG THR PRO SER ASP
MOUSE TNF                                                                  LEU ARG SER SER SER GLN ASN SER SER ASP
RABBIT TNF                                                                          SER ALA SER ARG ALA LEU SER ASP

                                     20                                      30                                     40
LYS PRO ALA ALA HIS LEU VAL GLY TYR PRO SER LYS GLN ASN SER LEU LEU TRP ARG ALA SER THR ASP ARG ALA PHE LEU ARG HIS GLY
LYS PRO ALA ALA HIS LEU ILE GLY ASP PRO SER LYS GLN ASN SER LEU LEU TRP ARG ALA ASN THR ASP ARG ALA PHE LEU GLN ASP GLY
LYS PRO VAL ALA HIS VAL VAL ALA ASN PRO GLN ALA GLU GLY GLN LEU GLN TRP LEU ASN ARG ARG ALA ASN ALA LEU LEU ALA ASN GLY
LYS PRO VAL ALA HIS VAL VAL ALA ASN HIS GLN VAL GLU GLU GLN LEU GLU TRP LEU SER GLN ARG ALA ASN ALA LEU LEU ALA ASN GLY
LYS PRO LEU ALA HIS VAL VAL ALA ASN PRO GLN VAL GLU GLY GLN LEU GLN TRP LEU SER GLN ARG ALA ASN ALA LEU LEU ALA ASN GLY

                                     50                                      60                                     70
PHE SER LEU SER ASN ASN SER LEU LEU ILE PRO THR SER GLY LEU TYR PHE VAL TYR SER GLN VAL VAL PHE SER GLY GLU SER CYS SER
PHE SER LEU SER ASN ASN SER LEU LEU VAL PRO THR SER GLY ILE TYR PHE VAL TYR SER GLN VAL VAL PHE SER GLY LYS ALA TYR SER
VAL GLU LEU ARG ASP ASN GLN LEU VAL VAL PRO SER GLU GLY LEU TYR LEU ILE TYR SER GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO
MET ASP LEU LYS ASP ASN GLN LEU VAL VAL PRO ALA ASP GLY LEU TYR LEU VAL TYR SER GLN VAL LEU PHE LYS GLY GLN GLY CYS PRO
MET LYS LEU THR ASP ASN GLN LEU VAL VAL PRO ALA ASP GLY LEU TYR LEU ILE TYR SER GLN VAL LEU PHE SER GLY GLN GLY CYS ARG

                                     80                                      90                                    100
PRO ARG ALA ILE PRO THR PRO ILE TYR LEU ALA HIS GLU VAL GLN LEU PHE SER SER GLN TYR PRO PHE HIS VAL PRO LEU LEU SER ALA
PRO LYS ALA THR SER SER PRO LEU TYR LEU ALA HIS GLU VAL GLN LEU PHE SER SER GLN TYR PRO PHE HIS VAL PRO LEU LEU SER SER
SER THR HIS VAL LEU LEU THR HIS THR ILE SER ARG ILE ALA VAL SER TYR GLN THR LYS VAL ASN LEU LEU SER ALA ILE LYS SER PRO
ASP TYR  -  VAL LEU LEU THR HIS THR VAL SER ARG PHE ALA ILE SER TYR GLN GLU LYS VAL ASN LEU LEU SER ALA VAL LYS SER PRO
SER TYR  -  VAL LEU LEU THR HIS THR VAL SER ARG PHE ALA VAL SER TYR PRO ASN LYS VAL ASN LEU LEU SER ALA ILE LYS SER PRO

                                     110                                     120                                    130
GLN LYS SER VAL TYR PRO GLY LEU GLN GLY  -   -  PRO TRP VAL ARG SER MET TYR GLN GLY ALA VAL PHE LEU LEU SER LYS GLY ASP
GLN LYS MET VAL TYR PRO GLY LEU GLN GLU  -   -  PRO TRP LEU HIS SER MET TYR HIS GLY ALA ALA PHE GLN LEU THR GLN GLY ASP
CYS GLN ARG GLU THR PRO GLU GLY ALA GLU ALA LYS PRO TRP TYR GLU PRO ILE TYR LEU GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP
CYS PRO LYS ASP THR PRO GLU GLY ALA GLU LEU LYS PRO TRP TYR GLU PRO ILE TYR LEU GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP
CYS HIS ARG GLU THR PRO GLU GLY ALA GLU PRO MET ALA TRP TYR GLU PRO ILE TYR LEU GLY GLY VAL PHE GLN LEU GLU LYS GLY ASP

                                     140                                     150              157
GLN LEU SER THR HIS THR ASP GLY ILE SER HIS LEU HIS PHE SER PRO SER SER  -  VAL PHE PHE GLY ALA PHE ALA LEU
GLN LEU SER THR HIS THR ASP GLY ILE PRO HIS LEU VAL LEU SER PRO SER THR  -  VAL PHE PHE GLY ALA PHE ALA LEU
ARG LEU SER ALA GLU ILE ASN ARG PRO ASP TYR LEU ASP PHE ALA GLU SER GLY GLN VAL TYR PHE GLY ILE ILE ALA LEU
GLN LEU SER ALA GLU VAL ASN LEU PRO LYS TYR LEU ASP PHE ALA GLU SER GLY GLN VAL TYR PHE GLY ILE VAL ILE ALA LEU
ARG LEU SER THR GLU VAL ASN GLN PRO GLU TYR LEU ASP LEU ALA GLU SER GLY GLN VAL TYR PHE GLY ILE ILE ILE ALA LEU
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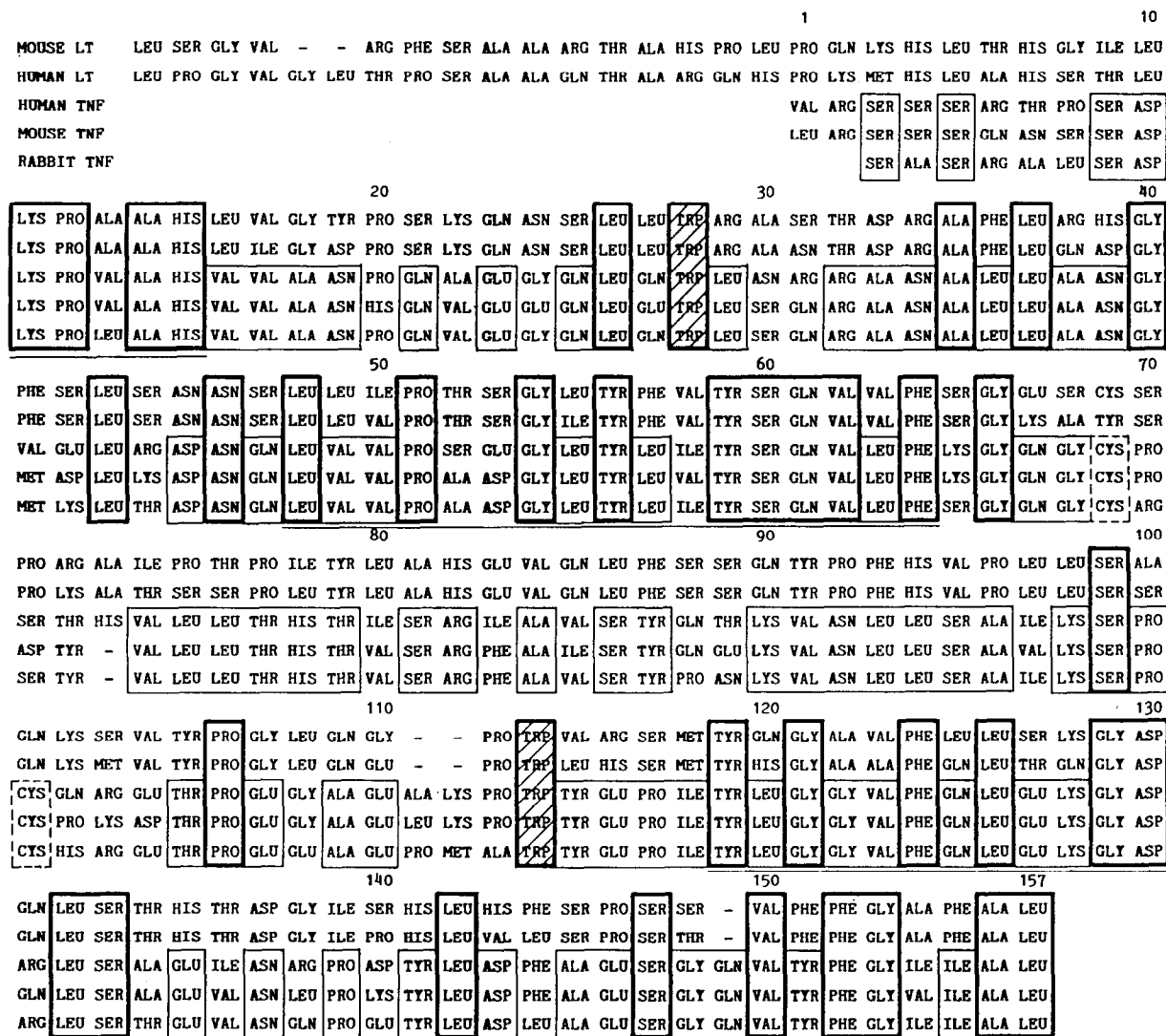
Fig.1. Amino acid sequence comparison of TNFs and LTs. Numbering refers to the mature hTNF sequence. Homology between TNFs, or between TNFs and LTs is represented by fine and bold boxes, respectively, Cys residues by dashed boxes and the two strongly conserved Trp residues by hatched boxes. The four strongly conserved regions are underlined. Adapted from Tavernier et al. [5].

2. MATERIALS AND METHODS

2.1. Insertion of the hTNF gene into M13 mp11 DNA

The entire hTNF gene, coupled to the tryptophan A terminator sequence (TA), was inserted into the M13 mp11 vector which was opened by EcoRI and HindIII. This was done using an EcoRI-BstE2 fragment (from the pP$_L$c-mu236-hTNF1 plasmid [3]) and the BstE2-HindIII fragment from the pP$_L$-T$_4$-hTNF-T vector (fig.2).

2.2. Site-specific mutagenesis of the recombinant hTNF gene

Mutagenesis of the two tryptophan residues was performed using a selection method based on uracil-containing single-stranded templates of M13 DNA [10]. Hybridization was performed with oligonucleotides containing the proposed mutations and removing or introducing a restriction site (fig.3, see also section 3).

2.3. Subcloning of the mutant genes into an expression vector and control of expression

The genes containing either the 28F or 114F mutations were transferred as AvaI-HindIII fragments from the M13 vector to the AvaI-HindIII-opened pMa-T$_4$-hTNF-TA vector (fig.2), resulting in the phasmids pMa-T$_4$-hTNF28FTA or pMa-
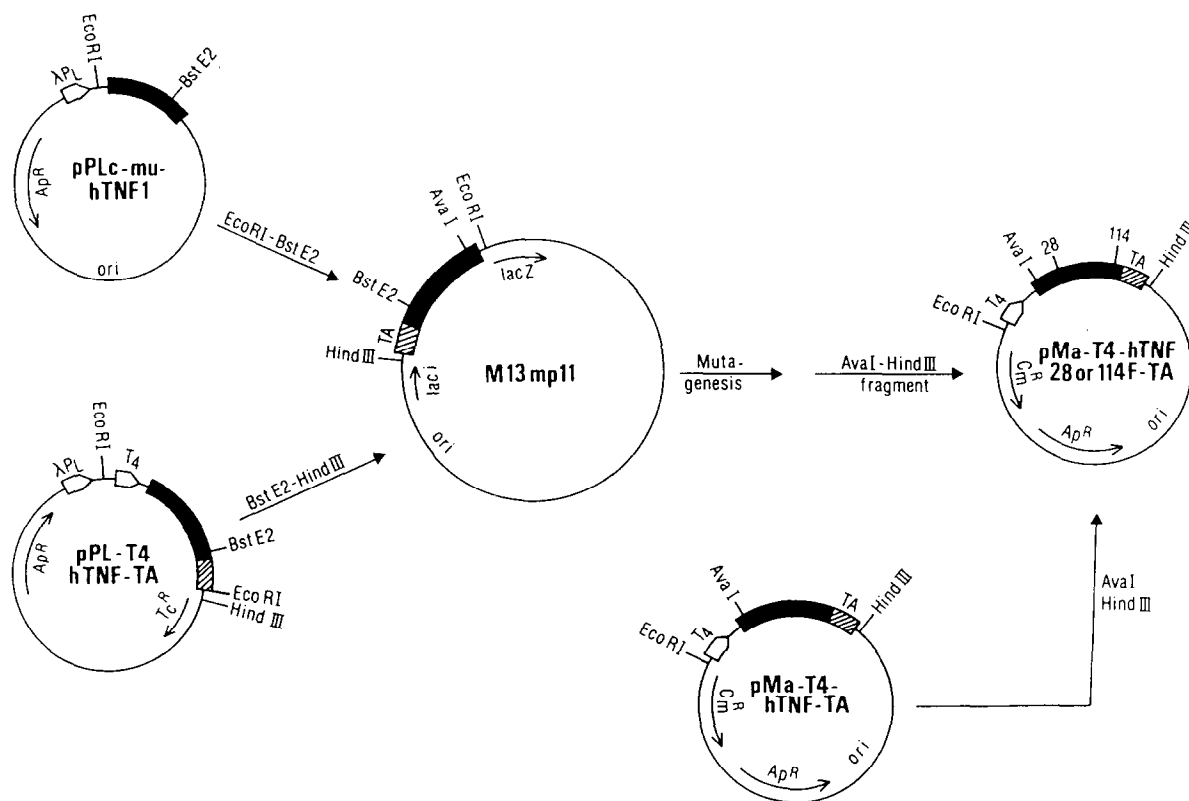
Fig.2. Insertion of the wild-type hTNF gene into the M13 mp11 vector from the plasmids pP$_L$c-mu236-hTNF1 and pP$_L$-T$_4$-hTNF-TA followed by insertion of the mutant genes into the pMa-T$_4$-hTNF-TA expression vector. Only relevant restriction sites are shown. The hTNF cDNA information is indicated as a filled-in bar. Open arrows represent promoters; the hatched box is the Trp A terminator sequence. Ap^R, ampicillin resistance; Tc^R, tetracyclin resistance; Cm^R, chloramphenicol resistance.
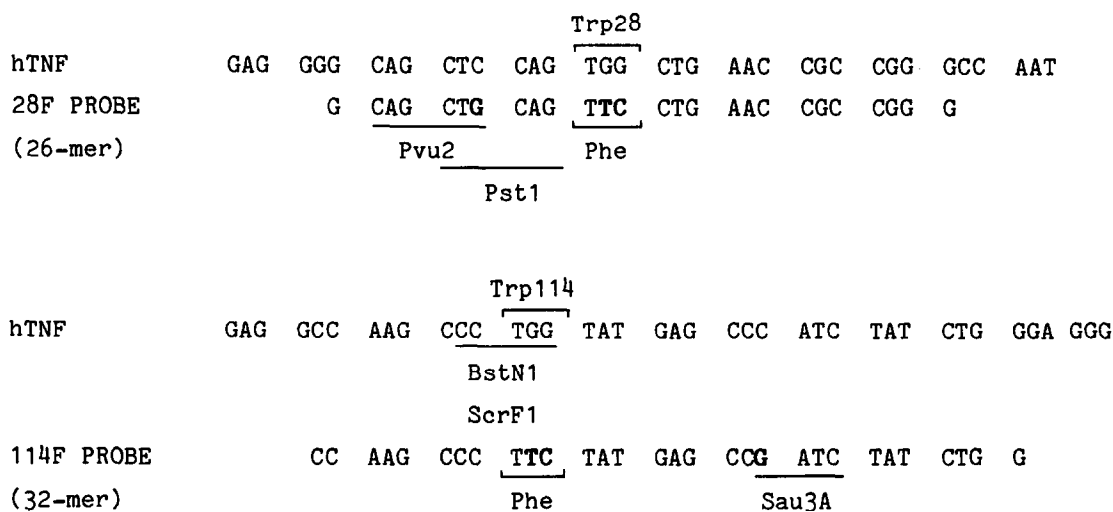
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                                    Trp28
                                   ┌────┐
hTNF      GAG  GGG  CAG  CTC  CAG   TGG   CTG  AAC  CGC  CGG  GCC  AAT

28F PROBE       G    CAG  CTG  CAG   TTC   CTG  AAC  CGC  CGG  G

(26-mer)             └─ Pvu2 ─┘     Phe
                 └──── Pst1 ────┘
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                              Trp114
                             ┌────┐
hTNF      GAG  GCC  AAG  CCC   TGG   TAT  GAG  CCC  ATC  TAT  CTG  GGA  GGG

                             BstN1
                             ScrF1

114F PROBE     CC   AAG  CCC  TTC   TAT  GAG  CCG  ATC  TAT  CTG  G

(32-mer)                     Phe                   Sau3A
```

Fig.3. Oligonucleotide sequences used for mutagenesis and screening of mutants. Differing bases appear in bold on the probe sequence. Disappearing and appearing restriction sites are underlined on the wild-type hTNF sequence and the oligonucleotide sequence, respectively. The Trp and Phe codons are indicated by horizontal square brackets.

T4-hTNF114F-TA, and transformed to E. coli MC1061. The pMa phasmid was originally constructed for generating ssDNA of an inserted gene; it can however, also be used as an expression system [11]. To test the solubility and expression level, 1 ml culture was sonicated and centrifuged. The pellet, containing the insoluble fraction, was dissolved in 100 μl Laemmli buffer [12] whereas the supernatant was precipitated with 5 vols ethanol and spun down. The resulting pellet was also redissolved in 100 μl Laemmli buffer. 10 μl of each sample was run on an SDS-polyacrylamide gel (15%) as described [12].

2.4. Purification of the mutated hTNF proteins

The procedure described by Tavernier et al. [13] was followed, with the exception that the mutant proteins were only purified over a Mono-Q column (Pharmacia, Sweden), while the wild-type protein used as a control was further purified by means of an additional Mono-S chromatography step. Elution of the 28F and 114F mutants was controlled by a cytotoxicity assay [14] and/or by SDS-polyacrylamide gel electrophoresis.

2.5. Determination of the specific activity

An aliquot (2 μl) of the partially purified product was diluted in 800 μl H$_2$O and 200 μl of the Biorad protein dye reagent was added in order to measure the total protein concentration in a Beckmann DU-7 spectrophotometer. To calculate the concentrations of mutant proteins, their purity percentage was measured by running the mixture on a 15% SDS-polyacrylamide gel followed by scanning on a chromatogram densitometer CD-50 (Desaga, Heidelberg).

The cytotoxic activity of the samples was tested on L929 mouse fibroblast cells [14].

3. RESULTS

3.1. Conversion of the position 28 and 114 tryptophan residues into phenylalanine by site-specific mutagenesis

Using the selection method based on uracil-containing single-stranded templates of M13 DNA [10] for site-specific mutagenesis as described above, we generated 8% transitions to the Phe residue at position 28 and 50% at position 114, as tested by hybridization with the same [32]P-labeled oligonucleotide sequence as used for mutagenesis. These probes also generated a PstI and a PvuI restriction site in addition to the 28F mutation (28F probe, 26-mer); they also generated a Sau3A site and deleted a ScrFI as well as a BstNI site adjacent to the 114F mutation (114F probe, 32-mer) (fig.3). As a result of this, we could simply monitor the presence of the mutations by restriction analysis.

The mutated genes were then transferred to the expression vector pMa and analysis of the resulting constructions pMa-T4-hTNF28F-TA and pMa-T4-hTNF114F-TA in E. coli MC1061 revealed

mainly insoluble 28F and mainly soluble 114F mutant proteins, respectively, both constituting about 30% of the total protein content (fig.4).

3.2. Purification of the mutant proteins

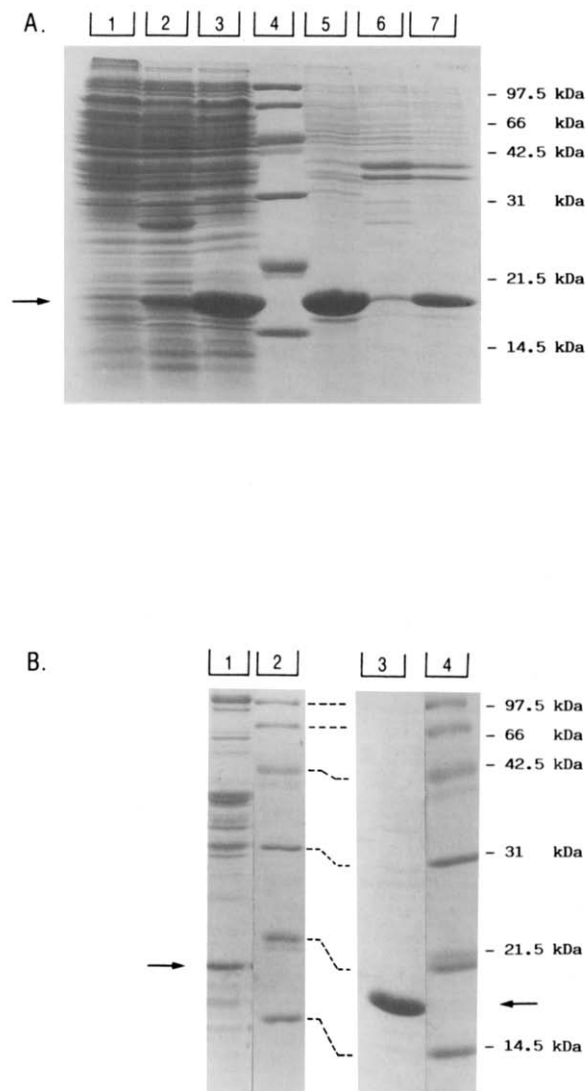Although the 28F mutation resulted in a mainly

Fig.4. Expression of the 28F and 114F mutant proteins. Samples were prepared as described in section 2 and analyzed on a 15% SDS-polyacrylamide gel. (A: lysed cultures) Lanes: 1,5, hTNF 28F; 2,6, hTNF (wild-type); 3,7, hTNF 114F; 1–3, soluble fractions; lanes 5–7, insoluble fractions of the lysates; 4, Biorad molecular mass markers. (B: purified proteins) Lanes: 1, hTNF 28F; 2,4, Biorad molecular mass markers; 3, hTNF 114F.

insoluble TNF protein, the soluble fraction still contained sufficient biological activity to warrant purification. hTNF-28F eluted at 160 mM NaCl from the Mono Q column with a purity of about 12% relative to the total protein concentration of the fraction (fig.4). The eluted samples were tested for the presence of hTNF-28F in a cytotoxicity assay and checked for purity on an SDS-polyacrylamide gel.

The more soluble 114F mutant protein eluted at 280 mM NaCl from the Mono-Q column, with a purity of about 90% relative to the total protein concentration of the fraction, as judged by SDS-polyacrylamide gel electrophoresis (fig.4).

3.3. Cytotoxic activity of the 28F and 114F mutant proteins

The same samples as used for SDS-polyacrylamide gel electrophoresis were tested in an L929 cytotoxicity assay. This allowed us to determine the specific activity to be 8.5×10^6 and 5×10^6 U/μg for the 28F and 114F mutant TNF proteins, respectively. As the specific activity of the wild-type protein is about 2×10^7 U/mg [5], those of the 28F and the 114F mutant proteins can be estimated at 45 and 30%, respectively, of the wild-type activity.

4. DISCUSSION

We have shown that the replacement of either of the two highly conserved tryptophan residues in hTNF by phenylalanine does not markedly lower the cytotoxic activity of the molecule. Because tryptophan and phenylalanine have similar chemical characteristics, we hoped to cause minimal structural distortion in order to maintain the hTNF molecule in the native conformation. As observed for the 28F mutation, even a single conservative mutation is, however, sufficient to convert a soluble protein into a mainly insoluble form. Also, the 114F mutation seems to convert the wild-type protein, normally eluting from Mono Q at 140 mM NaCl, into a clearly different structure, eluting at 280 mM NaCl.

If the Trp residues were part of the active site responsible for cytotoxicity, one would expect that conversion into another amino acid, even with similar characteristics, would result in a drastic drop in cytolytic activity. Thus, it is likely that these two Trp residues play only a structural role, and that they perhaps are located sufficiently far from the active site that a local structural change does not interfere with cytotoxic activity. However, because the mutant proteins eluted on a gel filtration column (TSK, G 3000 SW, BRL) at the same position as the wild-type protein, it is unlikely that the Trp residues affect the oligomerization and that the slightly reduced activity should be due to a monomeric form. Also, these tryptophan residues might play an important role during the biosynthesis of TNF in eucaryotic cells or could affect post-translational events, such as secretion.

Similar results were obtained by Valenzuela et al. [15], who examined three amino acids in the human interferon-α protein, two of which were highly conserved in the different human interferon-α species and even the human interferon-β protein (intraspecies), and one was strictly conserved between man, mouse and livestock (interspecies).

Proteins mutated into these three amino acids did, however, maintain their antiviral activity. Pielak reported that the phylogenetically conserved phenylalanine 87 in yeast iso-1-cytochrome c is not directly involved in electron transfer, but performs a structural function by controlling the polarity of the heme environment [16]. Also, α-globin, mutated at the strongly conserved positions 127 and 141, seems to be functionally active, and persons carrying these mutations are asymptomatic [17,18].

So far, no satisfactory explanation for the conservation of the tryptophan residues in TNF and LT has been advanced. More insight into this phenomenon may be gained, however, when the two amino acids have been located on a three-dimensional model of the molecule. Crystals of hTNF have already been obtained and X-ray diffraction studies are in progress [19].

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