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Soluble forms of tumor necrosis factor receptors (TNF-Rs). The cDNA for the type I TNF-R, cloned using amino acid sequence data of its soluble form, encodes both the cell surface and a soluble form of the receptor

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Two proteins which specifically bind tumor necrosis factor (TNF) have recently been isolated from human urine in our laboratory. The two proteins cross-react immunologically with two species of cell surface TNF receptors (TNF-R). Antibodies against one of the two TNF binding proteins (TBPI) were found to have effects characteristic of TNF, including stimulating phosphorylation of specific cellular proteins. Oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBPI were used to clone the cDNA for the structurally related cell surface type 1 TNF-R. It is notable that although this receptor can signal the phosphorylation of cellular proteins, it appears from its amino acid sequence to be devoid of intrinsic protein kinase activity. The extracellular domain of the receptor is composed of four internal cysteine-rich repeats, homologous to structures repeated four times in the extracellular domains of the nerve growth factor receptor and the B lymphocyte surface antigen CDw40. The amino acid composition and size of the extracellular domain of the type I TNF-R closely resemble those of TBPI. The COOH-terminal amino acid sequence of the four cysteine rich repeats within the extracellular domain of the type I TNF-R matches the COOH-terminal sequence of TBPI. Amino acid sequences in the extracellular domain also fully match other sequences found in TBPI. On the other hand, amino acid sequences in the soluble form of the type II TNF-R (TBPII), while indicating a marked homology of structure, did not suggest any identity between this protein and the extracellular domain of the type I TNF-R. CHO cells transfected with type I TNF-R cDNA produced both cell surface and soluble forms of the receptor. The receptor produced by CHO cells was recognized by several monoclonal antibodies against TBPI, reacting with several distinct epitopes in this molecule. These data suggest that the soluble forms of the TNF-Rs are structurally identical to the extracellular cytokine binding domains of these receptors and are consistent with the notion that the soluble forms are, at least partly, derived from the same transcripts that encode the cell surface receptors.

Key words: CDw40 antigen/cytokines/nerve growth factor/receptors/tumor necrosis factor

Introduction

Tumor necrosis factors TNF-α and TNF-β (lymphotoxin) are structurally related polypeptide cytokines, produced primarily by mononuclear leukocytes, whose effects on cell function constitute a major factor in the elicitation of the inflammatory response. The TNFs affect cells in different ways, some of which resemble the functional modes of other inflammatory mediators, like interleukin 1 (IL-1) and interleukin 6 (IL-6). What appears most distinctive regarding the activity of the TNFs is that many of their effects can result in cell and tissue destruction. Increasing evidence that over-induction of these destructive activities contributes to the pathogenesis of a number of diseases makes it of particular interest to elucidate their mechanisms and the ways they are regulated (Beutler and Cerami, 1988; Old, 1988). High affinity receptors, to which both TNF-α and TNF-β bind (Baglioni *et al.*, 1985; Beutler *et al.*, 1985; Kull *et al.*, 1985; Tsujimoto *et al.*, 1985; Aggarwal *et al.*, 1986; Israel *et al.*, 1986) play a key role in the initiation and control of the cellular response to these cytokines. These receptors are expressed on the surfaces of a variety of different cells. Studies showing that antibodies reacting with their extracellular portions affect cells in a manner very similar to the TNFs demonstrate that the receptors and cellular components associated with them are sufficient to provide the intracellular signalling for the effects of the TNFs (Engelmann *et al.*, 1990a; Espevik *et al.*, 1990). Other studies have shown that molecules related to the TNF receptors (TNF-Rs) also exist in soluble forms. Two immunologically distinct species of such soluble TNF-Rs, TBPI and TBPII, were recently isolated from human urine (Engelmann *et al.*, 1989, 1990b; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a). Immunological evidence indicated that the two proteins are structurally related to two molecular species of the cell surface TNF-R (the type I and type II receptors, respectively). Antibodies to each of the two soluble proteins were shown to block specifically the binding of TNF to one of the two receptors and could be used to immunoprecipitate the receptors. Antibodies against one of the two soluble proteins (TBPI) were also found to induce effects characteristic of TNF in cells which express the immunologically cross-reactive cell receptors (Engelmann *et al.*, 1990a, 1990b). Like the cell surface receptors for TNF, the soluble forms of these receptors specifically bind TNF and can thus interfere with its binding to cells. It was suggested that they function as physiological inhibitors of TNF activity (Engelmann *et al.*, 1989; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a).

In the present study we explored the structural relationship of the soluble and cell surface forms of the TNF-Rs further by determining amino acid sequences of the soluble forms and by using amino acid sequence data for one of the soluble receptors to clone the cDNA which encodes this protein. Initial information on the mechanism of formation

of the soluble receptors was gained by examining the expression of this cDNA in transfected CHO cells.

Results

Cloning of the cDNA for the type I TNF-R

To clone the cDNAs which code for the TNF-binding protein, TBPI, and its related TNF receptor, we screened several cDNA libraries, using three overlapping oligonucleotide probes designed on the basis of the NH_2-terminal amino acid sequence of TBPI (Figure 1A). In a λGT11 library derived from the mRNA of human colon (randomly primed, Clontech, Palo Alto, CA), we detected four recombinant phages which hybridized with the three probes. The inserts in these four phages were similar in size, and were found to overlap by restriction mapping and sequence analysis. Complete analysis of the sequence of the longest of the four (C2 in Figure 1B) revealed an open reading frame extending over its entire length. A polypeptide chain encoded in this reading frame fully matches the NH_2-terminal amino acid sequence of TBPI. Neither an initiation nor a stop codon was found in the C2 insert. Rescreening the colon cDNA library using another probe corresponding to a sequence found in C2 (see Materials and methods) yielded several other recombinant phages containing inserts that overlap with the C2 insert. However, none of them provided further sequence information on the cDNA in the 5′ or the 3′ directions. In a λZAP cDNA library derived from the mRNA of CEM lymphocytes (Foley *et al.*, 1965) [oligo (dT) and randomly primed, Clontech] five phages hybridizing with this probe were detected, which contained significantly longer inserts than C2. The longest insert (E13, Figure 1B) was sequenced in its entirety (Figure 1D) and was found to contain the C2 sequence (nucleotides 346–1277 in Figure 1D) within one long open reading frame of 1365 bp, flanked by untranslated regions of 255 and 556 nucleotides at its 5′ and 3′ ends respectively. The potential ATG initiation site, occurring at positions 256–258 in the nucleotide sequence, (denoted by an asterisk in Figure 1d) is preceded by an upstream in-frame termination codon at bases 244–246. The start location is in conformity with one of the possible alternatives for the translation initiation consensus sequence (GGCATGG, nucleotides 253–259; Kozak, 1987).

There is no characteristic poly(A) addition signal near the 3′ end of the cDNA. The sequence ACTAAA, at nucleotides 2045–2050, may serve as an alternative to this signal, but with low efficiency (Sheets and Wickens, 1989). At nucleotides 1965–2000, there are six consecutive repeats of the sequence G(T)n (n varying between 4 and 8). Similar sequences have also been observed in the 3′ noncoding regions of the cDNAs of some members of the *jun* family, which are also devoid of the characteristic poly(A) signal (Ryder *et al.*, 1988, 1989). The 3′ end has a 15 base poly(A) tail.

The size of the protein encoded by the cDNA (~50 kd) is significantly larger than that of TBPI (Engelmann *et al.*, 1989; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a). A hydropathy index computation (Kyte and Doolittle, 1982) of the deduced amino acid sequence of the protein (Figure 1C) revealed two major hydrophobic regions (see round-ended boxes in Figure 1D). One, at its NH_2-terminus, is apparently the signal peptide whose most likely cleavage site

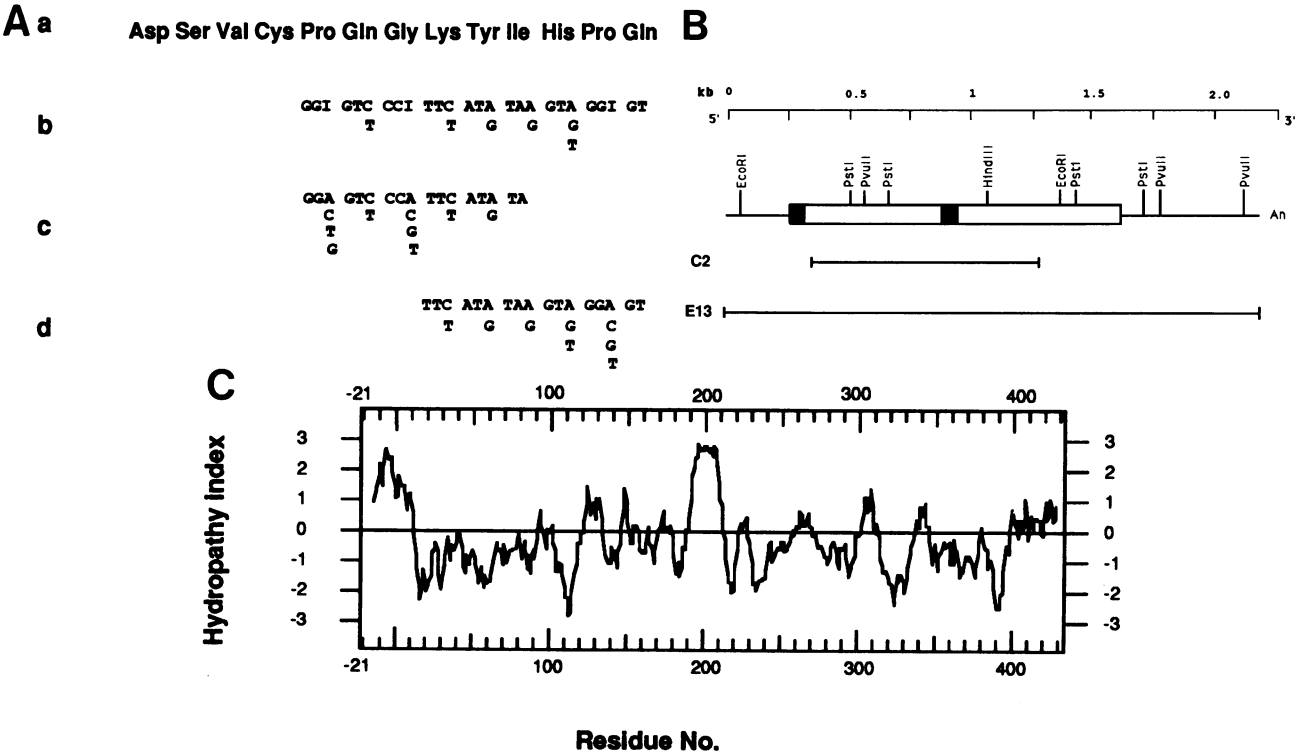

Fig. 1. Nucleotide sequence of the type I TNF receptor cDNA and the predicted amino acid sequence of the encoded protein. (**A**) The probes used for screening for the cDNA: (**a**) The NH_2-terminal amino acid sequence of TBPI. (**b**) Synthetic oligonucleotide probes, designed on the basis of the NH_2-terminal amino acid sequence, used for initial screening. (**c**) and (**d**) Probes overlapping with the probes presented in b, used to confirm the validity of clones isolated in the initial screening. (**B**) Schematic presentation of the cDNA clones isolated from a human colon (C2) and from CEM-lymphocyte (E13) libraries and a diagram of the complete cDNA structure. Untranslated sequences are represented by a line. Coding regions are boxed. The shaded portions represent the sequences which encode the signal peptide and the transmembrane domains. (**C**) Hydropathy profile of the predicted amino acid sequence of the TNF receptor. Hydrophobicity (above the line) and hydrophilicity (below the line) values were determined using the sequence analysis software package of the University of Wisconsin genetic computer group (UWGCG) according to Kyte and Doolittle

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                                                                                      1  CGGCCCAGTGATCTTGA      17
  18 ACCCCAAAGGCCAGAACTGGAGCCTCAGTCCAGAGAATTCTGAGAAAATTAAAGCAGAGAGGAGGGGAGAGATCACTGGGACCAGGCCGTGATCTCTATGCCCGAGTCTCAACCCTCAA   136
 137 CTGTCACCCCAAGGCACTTGGGACGTCCTGGACAGACCGAGTCCCGGGAAGCCCCAGCACTGCCGCTGCCACACTGCCCTGAGCCCAAATGGGGGAGTGAGAGGCCATAGCTGTCTGGC   255

     -21                                     -10                                     -1  +1
     Met Gly Leu Ser Thr Val Pro Asp Leu Leu Leu Pro Leu Val Leu Leu Glu Leu Leu Val Gly Ile Tyr Pro Ser Gly Val Ile Gly Leu
 256 ATG GGC CTC TCC ACC GTG CCT GAC CTG CTG CTG CCG CTG GTG CTC CTG GAG CTG TTG GTG GGA ATA TAC CCC TCA GGG GTT ATT GGA CTG   345
      *
     10                                      20                                      30
     Val Pro His Leu Gly Asp Arg Glu Lys Arg Asp Ser Val Cys Pro Gln Gly Lys Tyr Ile His Pro Gln Asn Asn Ser Ile Cys Cys Thr
 346 GTC CCT CAC CTA GGG GAC AGG GAG AAG AGA GAT AGT GTG TGT CCC CAA GGA AAA TAT ATC CAC CCT CAA AAT AAT TCG ATT TGC TGT ACC   435

     40                                      50                                      60
     Lys Cys His Lys Gly Thr Tyr Leu Tyr Asn Asp Cys Pro Gly Pro Gly Gln Asp Thr Asp Cys Arg Glu Cys Glu Ser Gly Ser Phe Thr
 436 AAG TGC CAC AAA GGA ACC TAC TTG TAC AAT GAC TGT CCA GGC CCG GGG CAG GAT ACG GAC TGC AGG GAG TGT GAG AGC GGC TCC TTC ACC   525

     70                                      80                                      90
     Ala Ser Glu Asn His Leu Arg His Cys Leu Ser Cys Ser Lys Cys Arg Lys Glu Met Gly Gln Val Glu Ile Ser Ser Cys Thr Val Asp
 526 GCT TCA GAA AAC CAC CTC AGA CAC TGC CTC AGC TGC TCC AAA TGC CGA AAG GAA ATG GGT CAG GTG GAG ATC TCT TCT TGC ACA GTG GAC   615

     100                                     110                                     120
     Arg Asp Thr Val Cys Gly Cys Arg Lys Asn Gln Tyr Arg His Tyr Trp Ser Glu Asn Leu Phe Gln Cys Phe Asn Cys Ser Leu Cys Leu
 616 CGG GAC ACC GTG TGT GGC TGC AGG AAG AAC CAG TAC CGG CAT TAT TGG AGT GAA AAC CTT TTC CAG TGC TTC AAT TGC AGC CTC TGC CTC   705

     130                                     140                                     150
     Asn Gly Thr Val His Leu Ser Cys Gln Glu Lys Gln Asn Thr Val Cys Thr Cys His Ala Gly Phe Phe Leu Arg Glu Asn Glu Cys Val
 706 AAT GGG ACC GTG CAC CTC TCC TGC CAG GAG AAA CAG AAC ACC GTG TGC ACC TGC CAT GCA GGT TTC TTT CTA AGA GAA AAC GAG TGT GTC   795

     160                                     170                                     180
     Ser Cys Ser Asn Cys Lys Lys Ser Leu Glu Cys Thr Lys Leu Cys Leu Pro Gln Ile Glu Asn Val Lys Gly Thr Glu Asp Ser Gly Thr
 796 TCC TGT AGT AAC TGT AAG AAA AGC CTG GAG TGC ACG AAG TTG TGC CTA CCC CAG ATT GAG AAT GTT AAG GGC ACT GAG GAC TCA GGC ACC   885

     190                                     200                                     210
     Thr Val Leu Leu Pro Leu Val Ile Phe Phe Gly Leu Cys Leu Leu Ser Leu Leu Phe Ile Gly Leu Met Tyr Arg Tyr Gln Arg Trp Lys
 886 ACA GTG CTG TTG CCC CTG GTC ATT TTC TTT GGT CTT TGC CTT TTA TCC CTC CTC TTC ATT GGT TTA ATG TAT CGC TAC CAA CGG TGG AAG   975

     220                                     230                                     240
     Ser Lys Leu Tyr Ser Ile Val Cys Gly Lys Ser Thr Pro Glu Lys Glu Gly Glu Leu Glu Gly Thr Thr Thr Lys Pro Leu Ala Pro Asn
 976 TCC AAG CTC TAC TCC ATT GTT TGT GGG AAA TCG ACA CCT GAA AAA GAG GGG GAG CTT GAA GGA ACT ACT ACT AAG CCC CTG GCC CCA AAC  1065

     250                                     260                                     270
     Pro Ser Phe Ser Pro Thr Pro Gly Phe Thr Pro Thr Leu Gly Phe Ser Pro Val Pro Ser Ser Thr Phe Thr Ser Ser Ser Thr Tyr Thr
1066 CCA AGC TTC AGT CCC ACT CCA GGC TTC ACC CCC ACC CTG GGC TTC AGT CCC GTG CCC AGT TCC ACC TTC ACC TCC AGC TCC ACC TAT ACC  1155

     280                                     290                                     300
     Pro Gly Asp Cys Pro Asn Phe Ala Ala Pro Arg Arg Glu Val Ala Pro Pro Tyr Gln Gly Ala Asp Pro Ile Leu Ala Thr Ala Leu Ala
1156 CCC GGT GAC TGT CCC AAC TTT GCG GCT CCC CGC AGA GAG GTG GCA CCA CCC TAT CAG GGG GCT GAC CCC ATC CTT GCG ACA GCC CTC GCC  1245

     310                                     320                                     330
     Ser Asp Pro Ile Pro Asn Pro Leu Gln Lys Trp Glu Asp Ser Ala His Lys Pro Gln Ser Leu Asp Thr Asp Asp Pro Ala Thr Leu Tyr
1246 TCC GAC CCC ATC CCC AAC CCC CTT CAG AAG TGG GAG GAC AGC GCC CAC AAG CCA CAG AGC CTA GAC ACT GAT GAC CCC GCG ACG CTG TAC  1335

     340                                     350                                     360
     Ala Val Val Glu Asn Val Pro Pro Leu Arg Trp Lys Glu Phe Val Arg Arg Leu Gly Leu Ser Asp His Glu Ile Asp Arg Leu Glu Leu
1336 GCC GTG GTG GAG AAC GTG CCC CCG TTG CGC TGG AAG GAA TTC GTG CGG CGC CTA GGG CTG AGC GAC CAC GAG ATC GAT CGG CTG GAG CTG  1425

     370                                     380                                     390
     Gln Asn Gly Arg Cys Leu Arg Glu Ala Gln Tyr Ser Met Leu Ala Thr Trp Arg Arg Arg Thr Pro Arg Arg Glu Ala Thr Leu Glu Leu
1426 CAG AAC GGG CGC TGC CTG CGC GAG GCG CAA TAC AGC ATG CTG GCG ACC TGG AGG CGG CGC ACG CCG CGG CGC GAG GCC ACG CTG GAG CTG  1515

     400                                     410                                     420
     Leu Gly Arg Val Leu Arg Asp Met Asp Leu Leu Gly Cys Leu Glu Asp Ile Glu Glu Ala Leu Cys Gly Pro Ala Ala Leu Pro Pro Ala
1516 CTG GGA CGC GTG CTC CGC GAC ATG GAC CTG CTG GGC TGC CTG GAG GAC ATC GAG GAG GCG CTT TGC GGC CCC GCC GCC CTC CCG CCC GCG  1605

     430
     Pro Ser Leu Leu Arg End
1606 CCC AGT CTT CTC AGA TGA GGCTGCGCCCCTGCGGGCAGCTCTAAGGACCGTCCTGCGAGATCGCCTTCCAACCCCACTTTTTTCTGGAAAGGAGGGGTCCTGCAGGGGCAAGC  1718
                         *
1719 AGGAGCTAGCAGCCGCCTACTTGGTGCTAACCCCTCGATGTACATAGCTTTTCTCAGCTGCCTGCGCGCCGCCGACAGTCAGCGCTGTGCGCGCGGAGAGAGGTGCGCCGTGGGCTCAA  1837
1838 GAGCCTGAGTGGGTGGTTTGCGAGGATGAGGGACGCTATGCCTCATGCCCGTTTTGGGTGTCCTCACCAGCAAGGCTGCTCGGGGGCCCCTGGTTCGTCCCTGAGCCTTTTTCACAGTG  1956
1957 CATAAGCAGTTTTTTTTGTTTTTGTTTTGTTTTGTTTTGTTTTTAAATCAATCATGTTACACTAATAGAAACTTGGCACTCCTGTGCCCTCTGCCTGGACAAGCACATAGCAAGCTGAA  2075
2076 CTGTCCTAAGGCAGGGGCGAGCACGGAACAATGGGGCCTTCAGCTGGAGCTGTGGACTTTTGTACATACACTAAAATTCTGAAGTTAAAAAAAAAAAAAAA  2176
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(1982). The curve is the average of the hydrophobicity index for each residue over a window of nine residues. (**D**) Nucleotide and predicted amino acid sequences of the TNF receptor. The presumptive start and stop signals are denoted by asterisks; the three sequences derived from TBPI by broken overlining; the transmembrane and leader domains by round-ended boxes; and the four repetitive sequences in the extracellular domain by thick underlining. Cysteine residues are boxed. Glycosylation sites are overlined and the presumptive polyadenylation signal is underlined.

Table I. Amino acid sequences of TBPI and TBPII

TBP I:

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
CNBr-1 (=N-terminus)	NH_2	Asp	Ser	Val	Cys	Pro	Gln	Gly	Lys	Tyr	ILe	His	Pro	Gln	---		
CNBr-2	NH_2	Gly	Gln	Val	Glu	ILe	Ser	Ser	Cys	Thr	Val	Asp	Arg	Asp	Thr	Val	---
C-terminus	---	ILe	Glu	Asn	COOH												

TBP II:

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	
N-terminus	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg	Leu	Arg	Glu	Tyr	Tyr	---
TRP 35	NH_2	Leu	Cys	Ala	Pro	Leu	Arg	Lys																
TRP 39/1	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 39/2	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---											
TRP 44/1	NH_2	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	Ser	---										
TRP 44/2	NH_2	Ser	Cys	Gly	Pro	Ser	Tyr	Pro	Asp	---														
TRP 46/1	NH_2	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg										
TRP 46/2	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 50	NH_2	Leu	Arg	Glu	Tyr	Tyr	Asp	Gln	Thr	Ala	Gln	Met	Cys	Cys	---									
TRP 53/1	NH_2	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg								
TRP 53/2	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg														
TRP 54/1	NH_2	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	Lys														
TRP 54/2	NH_2	Ala	Gln	Val	Ala	Phe	Thr	Pro	Tyr	Ala	Pro	Glu	Pro	Gly	Ser	Thr	Cys	Arg						
TRP 60	NH_2	Ile	Cys	Thr	Cys	Arg	Pro	Gly	Trp	Tyr	Cys	Ala	Leu	Ser	---									
TRP 62	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	---			
TRP 65	NH_2	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys	Pro	Cys	Ala	Pro	Gly	Thr	Phe	Ser	---			
TRP 67	NH_2	Cys	Arg	Pro	Gly	Phe	Gly	Val	Ala	Arg	Pro	Gly	Thr	Glu	Thr	Ser	Asp	Val	Val	Cys	Lys			
TRP 84	NH_2	Thr	Ser	Asp	Thr	Val	Cys	Asp	Ser	Cys	Glu	Asp	Ser	Thr	Tyr	Thr	Gln	Leu	Trp	---				

(Von Heijne, 1986) lies between the glycine and isoleucine residues designated in Figure 1D as −1 and +1 respectively. The other major hydrophobic domain, located between residues 191 and 213, is flanked at both ends by several charged amino acids, characteristic of a membrane anchoring domain (Pidgeon *et al.*, 1989). As in several other transmembrane proteins, the amino acids confining the hydrophobic domain at its COOH-terminal are basic. The transmembrane domain bisects the predicted protein into almost equally sized extracellular and intracellular domains.

The extracellular domain has three putative sites for asparagine-linked glycosylation (overlined in Figure 1D). Assuming that the amount of oligosaccharides in the extracellular domain is similar to that reported in TBPI (Seckinger *et al.*, 1989b), the molecular size of the mature protein is very similar to that estimated for the type I receptor (~58 kd) (Hohmann *et al.*, 1989; Engelmann *et al.*, 1990a).

Features of the predicted amino acid sequence in the type I TNF-R and relationship to the structure of TBPI and TBPII

The amino acid sequence of the extracellular domain of the protein encoded by the E13 cDNA fully matches several determined TBPI amino acid sequences (Table I). It contains the NH_2-terminal amino acid sequence of TBPI at amino acids 20−32 (compare Figure 1D and 1A a), a sequence corresponding to the COOH-terminus of TBPI at amino acids 178−180, and also, adjacent to the first methionine located further downstream in the encoded protein, a sequence identical to the NH_2-terminal amino acid sequence of a cyanogen-bromide cleavage fragment of TBPI (broken lines in Figure 1D). There is also a marked similarity in amino acid composition between the extracellular domains of the receptor and TBPI (Table II). The most salient feature of this amino acid composition is a very high content of cysteine residues (shown boxed in Figure 1D). The positioning of the cysteine residues as well as of other amino acids within the extracellular domain displays a four-fold repetition pattern (Figure 2 and underlined in Figure 1D). As shown in Figure 2, there is a marked homology between this four-

Table II. Similarity of the amino acid compositions of the TNF binding protein TBPI and a corresponding region in the extracellular domain of the TNF-R (type I)

Amino acid	mol/100 mol of amino acids	
	TBPI[a]	Residues 20−180 in the extracellular domain[b]
Ala	1.7	1.2
Cys	12.8	14.9
Asp + Asn	10.9	11.1
Glu + Gln	13.9	12.4
Phe	3.2	3.1
Gly	6.3	5.6
His	4.4	4.3
Ile	2.8	2.5
Lys	6.2	6.2
Leu	8.0	6.8
Met	0.4	0.6
Pro	3.8	3.1
Arg	4.7	4.3
Ser	8.1	9.3
Thr	6.1	6.2
Val	4.2	4.3
Trp	−	0.6
Tyr	2.4	3.1

[a]According to Olsson *et al.*, 1989.
[b]Residue 20 corresponds to the NH_2-terminal amino acid of TBPI. Residue 180 is the COOH-terminal residue of TBPI.

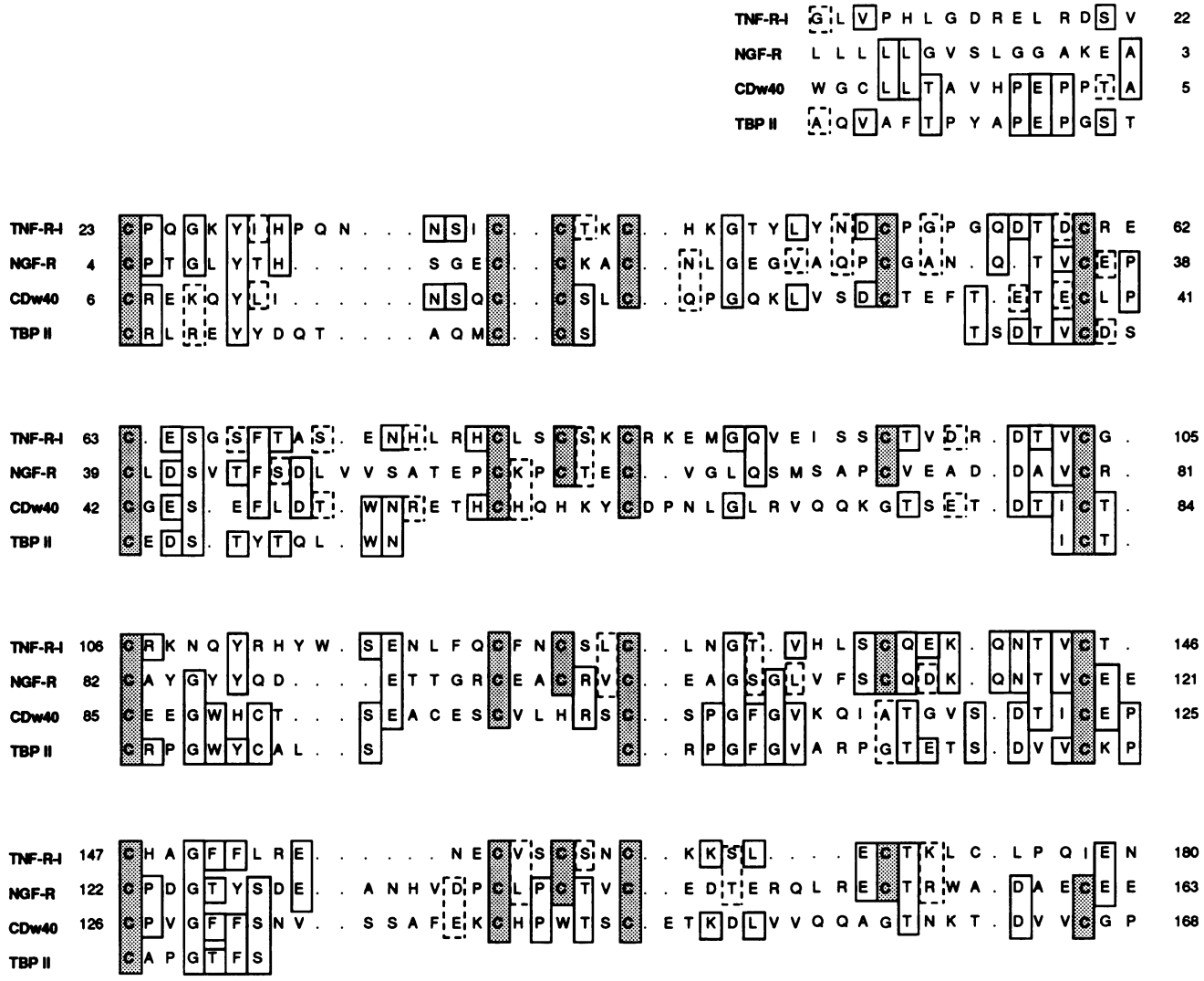

Fig. 2. Internal cysteine-rich repeats in the extracellular domain of the TNF-R and their alignment with the homologous repeats in the extracellular domains of human NGF-R and the CDw40 antigen and with sequences of amino acids present in TBPII. The amino acid sequences (one-letter symbols) are aligned for maximal homology. Dashes indicate gaps introduced to optimize the alignment. Identities in sequences are shown in boxes. Conservative substitutions (I = L = V; D = E; K = R = H; T = S; G = A; N = Q) are boxed with dotted lines. The positions of the amino acids within the receptors are denoted in the right and left hand margins. The sequences in TBPII (Table I and Materials and methods) were optimally aligned with the sequences of the other three proteins presented in this figure, using the 'Best Fit' alignment program in the UWGCG software package.

domain structure and sequences found in the extracellular domain of the receptor for the nerve growth factor (NGF-R) (Johnson *et al.*, 1986; Radeke *et al.*, 1987) and also, to a somewhat lower degree, to sequences in the extracellular domain of the recently cloned receptor-like B cell antigen CDw40 (Braesch-Andersen *et al.*, 1989; Stamenkovic *et al.*, 1989). The amino acid sequence within the extracellular domain of the TNF-R, which corresponds to the COOH-terminal sequence of TBPI (see Table I and Figure 7), is located at the COOH-terminus of the cysteine-rich repeat region. The sequence corresponding to the NH_2-terminal sequence of TBPI corresponds to a sequence located a few amino acids upstream of the NH_2-terminal end of this region (broken lines in Figure 1D) in the extracellular domain.

In contrast to the identity of amino acid sequences between TBPI and the extracellular domain of the type I receptor, sequences examined in the soluble form of the type II TNF-R (TBPII, Table I) were not identical to any sequence in the type I TNF-R. This finding is expected, considering the lack of immunological cross-reactivity between the two receptors (Engelmann *et al.*, 1990b). However, as demonstrated in Figure 2, the sequences in TBPII have a significant homology of structure with the four-fold cysteine-rich repeat region in the extracellular domains of the type I TNF-R, the NGF-R and the CDw40 protein. The similarity between TBPII and the CDw40 protein is particularly notable and seems to extend also to a region within the two proteins which protrudes from the cysteine-rich structures in the NH_2 direction (Figure 2).

In contrast to the very high content of cysteine residues in the putative extracellular domain of the type I TNF-R, there are only five cysteine residues in the intracellular domain. Between the two which are proximal to the transmembrane domain (positions 227 and 283) extends a stretch of 55 amino acids which is rich in proline residues (16% of the residues) and even richer in serine and threonine residues (36%), most located very close to or adjacent to each other. The consensus sequence Gly-x-Gly-x-x-Gly which characterizes protein kinases and various other

nucleotide binding proteins (Kamps *et al.*, 1984) is not present in the intracellular domain.

Expression of the type I TNF-R cDNA

To explore the relation between the protein encoded by the E13 cDNA and TBPI further, we expressed this protein in CHO cells. The E13 cDNA was introduced into an expression vector and was cotransfected with a recombinant vector containing the dihydrofolate reductase (DHFR) cDNA into DHFR-deficient cells. After selection by growth in a nucleotide-free medium, individual clones were amplified by growth in the presence of methotrexate. A number of clones which react with several monoclonal antibodies that bind to spatially distinct epitopes in TBPI were detected (Figure 3). Expression of the protein was correlated with an increase in specific binding of human TNF to the cells (Table III).

Applying a sensitive immunoassay for TBPI, in which polyclonal antibodies and a monoclonal antibody against this protein were employed, we could also detect a soluble form of the protein in the growth medium of CHO cells, which express the human TNF-R on their surface (Table III). All of five different CHO clones which expressed the TNF-R produced this soluble protein. Several other transfected clones which did not express the cell surface receptor did not produce its soluble form either (not shown). When analyzed by reversed phase HPLC, the CHO-produced soluble TNF-R eluted as a single peak, with a retention time identical to that of TBPI (Figure 4).

Northern blot analysis using the E13 cDNA as a probe

To gain information on the transcripts which encode the type I TNF-R, we tested mRNAs from cells of differing origin for their ability to hybridize with the E13 cDNA. As shown in Figure 5, in all the cell types, including the HT29 cells, which continuously secrete a soluble form of the type I TNF-R (Aderka,D., Nophar,Y., Engelmann,H. and Wallach,D., manuscript in preparation), only a single hybridizing transcript was detectable, in all cases of the same size i.e. ~2300 bp, corresponding to the full length of the cDNA. Interestingly, significant amounts of this type

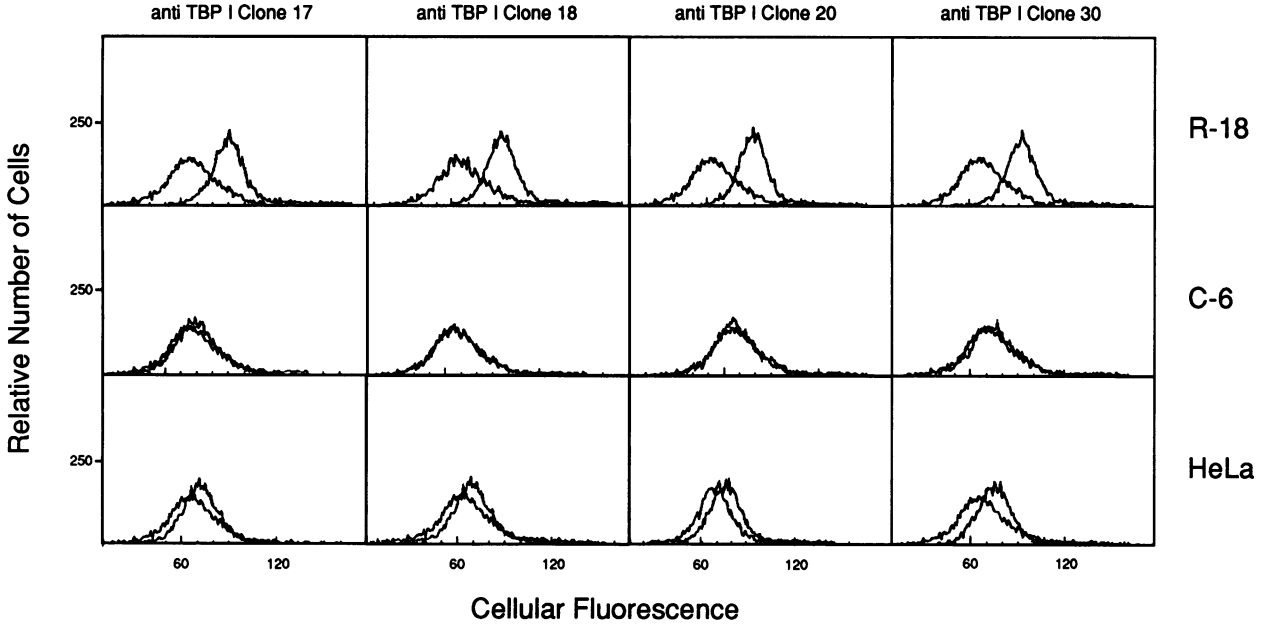

Fig. 3. Detection of type I TNF-R using monoclonal antibodies to TBPI in CHO cells transfected with E13 cDNA. CHO cells, clones R-18 (transfected with an expression vector in which the E13 cDNA was placed under the control of an SV40 promotor) and C-6 (control; a clone of cells transfected with an expression vector in which E13 was placed in the inverse orientation), and HeLa cells were stained with the anti-TBPI monoclonal antibodies 17, 18, 20 and 30 followed by incubation with FITC conjugated anti-mouse F(ab). Fluorescence intensity is compared with that observed when a mouse monoclonal antibody against TNF was used in the first step of the staining as a control.

Table III. Expression of the cell surface and soluble forms of human type I TNF-R in CHO cells

CHO cell clone	Specific binding of TNF (c.p.m./10^6 cells)	Cells expressing human cell surface TNF-R (% fluorescent cells)	Human soluble type I TNF receptors (pg/ml)
nontransfected	180 ± 45	<1%	<0.03
C6	175 ± 50	<1%	<0.03
R-16	550 ± 60	73%	30
R-18	610 ± 40	89%	49

The R-16 and R-18 clones consist of cells transfected with a recombinant expression vector containing E13 cDNA. C-6 cells were transfected with a control vector (see Figure 3). Binding of radiolabeled TNF to the cells was determined in quintuplicate samples. Detection of immunoreactive receptors on the surface of the cells was carried out using combined 17, 18 and 20 anti-TBPI monoclonal antibodies. Results are expressed as percentage of fluorescent cells (background values, obtained by staining the cells with an anti-TNF monoclonal antibody, are subtracted). For other details, see Materials and methods.

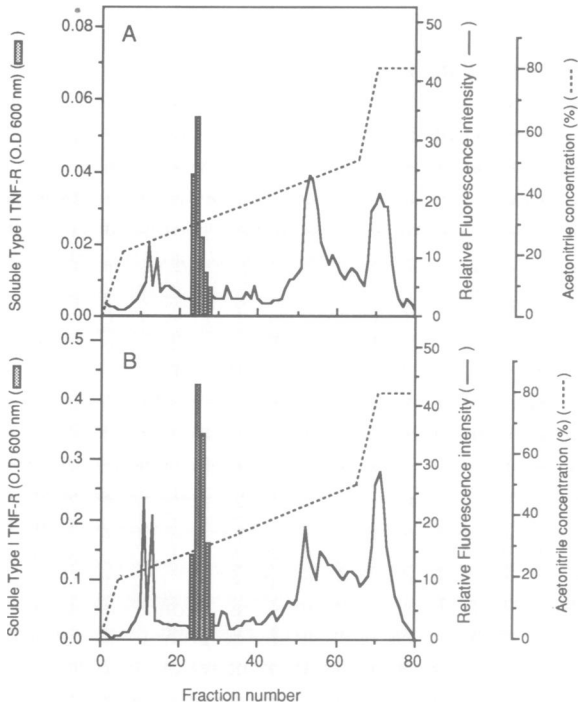

Fig. 4. Reversed phase HPLC of the CHO-produced, soluble form of the type I TNF-R. A concentrate of the conditioned medium of the CHO R-18 clones (see Figure 3) and a concentrate of the CHO C-6 clone to which 3 ng pure TBPI was added were applied to an Aquapore RP300 column. Elution was performed with a gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid (---). Fractions were examined for content of protein (——) and of the soluble form of the type I by ELISA (▩), as described in Materials and methods. None of the eluted fractions of a concentrate of the CHO C-6 clone without addition of TBPI was found to contain any detectable amounts of the soluble form of the receptor (not shown).

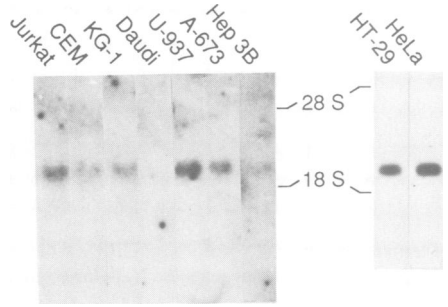

Fig. 5. Detection of the mRNA for the type I TNF-R by Northern blotting analysis. Hybridization of total RNA (25 μg/lane) from cells of the Jurkat (Gillis and Watson, 1980), CEM (Foley *et al.*, 1965), KG-1 (Koeffer and Golde, 1978), Daudi (Klein and Klein, 1968), K-562 (Lozzio and Lozzio, 1975), U-937 (Sundstroem and Nilsson, 1976), A-673 (Giard *et al.*, 1973), Hep 3B (Aden *et al.*, 1979), HT-29 (Fogh and Trempe, 1975), and HeLa (Gey *et al.*, 1952) lines with the ^{32}P-labeled E13 insert was carried out as described in Materials and methods. 28S and 18S refer to ribosomal RNA size markers.

I TNF-R mRNA could also be detected in the U937 cells, in which the prevalent TNF-R is type II (Engelmann *et al.*, 1990b), suggesting that post-transcriptional mechanisms take part in the control of the expression of the type I receptor.

Evidence for the involvement of type I TNF-R in stimulation of protein phosphorylation by TNF

Treating cells with TNF results in a rapid increase in the phosphorylation of certain specific cellular proteins including

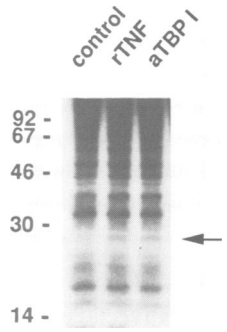

Fig. 6. Involvement of the type I TNF-R in stimulation of protein phosphorylation in cells. Effects of TNF-α (1000 U/ml) and rabbit antiserum to TBPI (1:1000) on the phosphorylation of proteins with a mol. wt of 27 kd in HeLa cells. Untreated cells served as a control. The 27 kd protein(s) are indicated with an arrow on the right and the migration of molecular weight markers (Amersham, UK) is shown on the left. Normal rabbit serum had no effect at a dilution of 1:1000 (not shown).

some with a mol. wt of ~27 kd (Hepburn *et al.*, 1988; Kaur and Saklatvala, 1988; Schutze *et al.*, 1989). Since it is apparent from the sequence data of the intracellular domain of the type I TNF-R that this receptor is devoid of intrinsic protein kinase activity, it was of interest to examine the extent to which this receptor is involved in TNF-mediated protein phosphorylation events. Antibodies to TBPI induce various effects in cells which are characteristic of TNF. This activity was shown to be correlated with the ability of the antibodies to cross-link the type I TNF-R molecules (Engelmann *et al.*, 1990a). As shown in Figure 6, treating HeLa cells which express the type I TNF-R (Engelmann *et al.*, 1990b) with antibodies to TBPI induced, as does TNF, a marked increase in the phosphorylation of protein(s) with a mol. wt of 27 kd, confirming that the type I TNF-R is involved in this effect.

Discussion

There is accumulating evidence for the natural occurrence of soluble forms of cell surface receptors. Such forms have been identified, for example, for the receptors to interleukin-2 (IL-2) (Rubin *et al.*, 1985; Osawa *et al.*, 1986), growth hormone (Leung *et al.*, 1987), NGF (DiStefano and Johnson, 1988), interleukin-6 (Novick *et al.*, 1989), interferon-γ (Novick *et al.*, 1989) and tumor necrosis factor (Engelmann *et al.*, 1989, 1990b; Olsson *et al.*, 1989; Seckinger *et al.*, 1989a). Yet knowledge of the exact structure of these soluble receptors and of the mechanisms of their formation is still limited. The most thoroughly characterized so far is the soluble form of the 55 kd receptor for IL-2. Based on detailed sequence analysis and studies of its mode of formation in cultured cells, it was suggested that it is derived from the cell surface form of the receptor by proteolytic cleavage (Robb and Kutny, 1987).

A different mechanism for the formation of soluble receptors was proposed in two recent studies describing the cloning of the cDNAs for the receptors to IL-4 and IL-7. Besides cDNA clones encoding the full length receptors, clones which encode truncated, soluble forms of these receptors were also isolated in these studies. It was suggested that these latter clones are derived from transcripts specifically encoding soluble forms of the receptors,

transcribed from the same genes which encode the cell surface forms, but differently spliced (Mosley *et al.*, 1989; Goodwin *et al.*, 1990).

Data presented in our study are consistent with the notion that TBPI—the soluble form for the type I TNF-R—constitutes a fragment of the cell surface form of this receptor corresponding to its extracellular domain. The receptor is recognized by several monoclonal antibodies to TBPI which interact with several spatially distinct epitopes in this protein (the present study and Engelmann *et al.*, 1990a). The amino acid sequence in the extracellular domain fully matches several sequences present in TBPI. Furthermore, the amino acid composition of the region within the extracellular domain which extends between those residues which correspond to the NH_2- and COOH-termini of TBPI is very similar to the amino acid composition reported for TBPI. There is also a similarity in size between TBPI and this part of the receptor [taking into account that about a third of the TBPI molecule consists of oligosaccharides (Seckinger *et al.*, 1989b)]. Particularly informative with regard to the mechanism of formation of TBPI is the finding that a soluble form of the type I TNF-R is produced by CHO cells transfected with the TNF-R cDNA. This finding implies that cells possess some mechanism(s) which allow(s) the formation of the soluble form of the TNF-R from the same transcript that encodes the cell surface form. There is no indication from the data of this study for the existence of transcripts which specifically encode soluble forms of TNF-R. Northern blot analysis did not reveal transcripts smaller than the full size of the TNF-R mRNA in any of the cells examined, not even in the HT29 cells, which continuously release significant amounts of a soluble form of the type I TNF-R into the culture medium. Furthermore, sequence and restriction mapping analyses of the various cDNA clones isolated in this study together with the C2 and E13 clones failed to reveal any difference in structure, besides differences in size, between these clones and the E13 cDNA (data not shown). The amino acid sequence data of TBPI also provide no indication of the existence of transcripts specific to this protein. Soluble receptors produced from alternatively spliced transcripts, as suggested for the IL-4 and IL-7 receptors, are expected to have unique COOH-terminal sequences (Mosley *et al.*, 1989; Goodwin *et al.*, 1990). The COOH-terminal sequence of TBPI was found to be identical to a sequence found in the cell surface receptor. Still, the existence of a minor population of transcripts which specifically code for soluble forms of TNF-Rs in amounts below the limit of detection of the techniques employed, although not supported by the data presented in this study, cannot be excluded.

The low rate of production of the soluble form of the type I TNF-R by the E13-transfected CHO cells does not necessarily reflect maximal activity. In HT29 cells, the spontaneous release of a soluble form of type I TNF-R occurs at about a 10-fold higher rate than that observed with the CHO-R-18 clone (data not shown). Furthermore, a recent study (Porteu and Nathan, 1990) indicates that the mechanism of formation of the soluble TNF-R can be effectively enhanced by certain specific stimuli. Stimulation of human neutrophils with N-formyl Met-Leu-Phe, or with several other physiological stimuli, was found to result, within a few minutes, in an extensive decrease of the cell-surface expressed TNF-R and an accompanying release of a soluble form of these receptors, similar in size to TBPI.

A likely mechanism whereby soluble forms of TNF receptors can be derived from the same transcripts which encode the cell surface forms is proteolytic cleavage. Indeed, flanking the amino acid residue which corresponds to the NH_2-terminus of TBPI there are, within the amino acid sequence of the receptor, two basic amino acid residues (Lys-Arg) which can serve as a site of cleavage by trypsin-like proteases. The identity of the proteases which might cause cleavage to take place at the COOH-terminus of TBPI is not known. In view of the marked structural homology between the extracellular domain of the type I TNF-R and the soluble form of the type II TNF-R (TBPII) as well as the homology with the extracellular domain of the NGF-R, for which existence of a soluble form has been also documented (DiStefano and Johnson, 1988; Zupan *et al.*, 1989), it is tempting to speculate that a common mechanism of cleavage and similar cleavage sites are involved in the formation of the soluble forms of those three receptors. Such a mechanism can have a dual effect on cell response to TNF. Its activation may result in the suppression of the response both in those cells in which it functions—as a consequence of the decrease in their intact cell surface receptors—as well as in other cells because of the ability of the released, soluble form of the receptor to sequestrate TNF. Detailed analysis of the biosynthesis of TNF-R, facilitated by use of cells transfected with TNF-R cDNA in vectors dictating its overexpression, should provide further information on the mechanism and functional implications of the formation of its soluble form.

Materials and methods

Determination of amino acid sequences within the TNF-binding proteins TBPI and TBPII

The TNF binding proteins TBPI and TBPII were isolated from concentrated preparations of urinary proteins, as described previously (Engelmann *et al.*, 1990b) by ligand (TNF) affinity chromatography followed by reversed phase HPLC. TBPI was cleaved with cyanogen bromide, yielding two peptides which, following reduction and alkylation (Andrews and Dixon, 1987), were isolated by reversed phase HPLC. The two peptides (CNBr-1 and CNBr-2 in Table I) were subjected to NH_2-terminal sequence analysis on a pulsed liquid gas phase protein microsequencer (Model 475A, Applied Biosystems Inc., Foster City, CA). The sequence found for one of the peptides (CNBr-1) was identical to the NH_2-terminal sequence of the intact TBPI protein (Engelmann *et al.*, 1989, 1990b).

The COOH-terminal amino acid sequence of TBPI was determined by digestion of the protein with carboxypeptidase Y followed by sequential analysis of the released amino acids. A sample of pure TBPI (32 μg) was mixed with 1 nmol norleucine, as an internal standard, dried thoroughly and resuspended in 8 μl 0.1 M sodium acetate buffer, pH 5.5, containing 0.8 μg carboxypeptidase Y (Sigma, St Louis, MO). Digestion was performed at room temperature. 2 μl aliquots withdrawn at various time points were acidified by adding 3 μl of 10% acetic acid to each, followed by addition of 15 μl 0.5% EDTA. They were then subjected to automated amino acid analysis (Applied Biosystems, UK, mod. 420A). The results (Figure 7) indicate the sequence Ile-Glu-Asn-COOH.

Sequences within TBPII were determined by generation of tryptic peptides of the protein. A sample of pure TBPII (200 μg) was reduced, alkylated and repurified on an Aquapore RP-300 reversed phase HPLC column. Fractions containing the modified protein were pooled and the pH was adjusted to 8.0 with $NaHCO_3$. Digestion with TPCK-trypsin (238 U/mg, Millipore Corp., Freehold, NJ) was performed for 16 h at room temperature at an enzyme to substrate ratio of 1:20 (w/w). The digest was loaded onto a C_{18} RP-P reversed phase HPLC column (SynCrom, Linden, IN) and the peptides separated by a linear 0–40% acetonitrile gradient in 0.3% aqueous trifluoroacetic acid. The NH_2-terminal amino acid sequences of the peptides and of the intact protein (N-terminus) are presented in Table I. The peptides were numbered according to their sequence of elution from the RP-P column. In the fractions denoted as 39, 44, 46, 53 and 54, where heterogeneity of sequences was observed, both the major and the secondary sequences are presented.

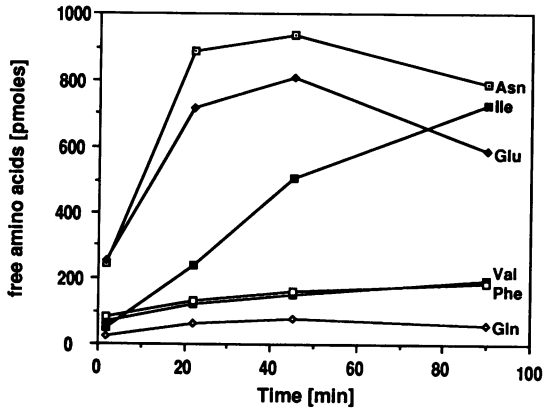

Fig. 7. Time course of the release of COOH-terminal amino acids from TBPI by carboxypeptidase Y.

Isolation of cDNA clones

Three mixtures of synthetic oligonucleotide probes generated from the nucleotide sequence deduced from the NH_2-terminal amino acid sequence of TBPI were used for the screening of cDNA libraries. Initial screenings were carried out with 48-fold degenerate, 26-mers into which deoxyinosine was introduced, wherever the codon ambiguity allowed for all four nucleotides (Figure 1A b). The validity of positive clones was examined by testing their hybridization to two mixed 17-mer nucleotide sequences, containing 96 and 128 degeneracies, corresponding to two overlapping amino acid sequences which constitute part of the sequences to which the 26 bp probes correspond (Figure 1A, c and d). An oligonucleotide probe corresponding to a sequence located close to the 5′ terminus of the longest of the partial cDNA clones isolated with the degenerated probes (nucleotides 478–458 in Figure 1D) was applied for further screening cDNA libraries for a full length cDNA clone. ^{32}P-labeling of the probes, using T4 polynucleotide kinase, plating of the phages in lawns of bacteria, then screening them with the radiolabeled probes, isolation of the positive clones and subcloning of their cDNA inserts were carried out using standard procedures (Sambrook *et al.*, 1989).

Nucleotide sequencing of the cDNA clones

cDNA inserts isolated from positive λGT11 recombinant phages were subcloned into the pBluescript KS(−) vector. Inserts found in λZAP phages were rescued by excising the plasmid pBluescript SK(−) in them, using the R408 helper phage (Short *et al.*, 1988). DNA sequencing in both directions was done by the dideoxy chain termination method (Sanger *et al.*, 1977). Overlapping deletion clones of the cDNAs were generated, in both orientations, by digestion of the cDNA with exonuclease III ('Erase a base' kit, Promega Biotec, Madison, WI). Single-stranded templates derived from these clones using the R408 phage were sequenced with a T7 DNA polymerase sequencing system (Promega.)

Constitutive expression of the type I human TNF-R in CHO cells

The E13 insert was introduced into a modified version of the pSVL expression vector (kindly made available to us by Dr H.Kahana). This construct was transfected, together with the pSV2-DHFR plasmid which contains the DHFR cDNA, into DHFR deficient CHO cells (Chernajovsky *et al.*, 1984), using the calcium phosphate precipitation method (Chen and Okayoma, 1987). Transfection with a recombinant pSVL vector which contained the E13 insert in the inverse orientation served as a control. Cells expressing the DHFR gene were selected by growth in nucleotide-free MEM α medium containing fetal calf serum which had been dialyzed against phosphate buffered saline. Individual clones were picked out and then further selected for amplification of the transfected cDNAs by growth in the presence of 500 nM sodium methotrexate.

Detection of surface-expressed type I TNF-R in the CHO cells

Binding of radiolabeled human rTNF to cells (seeded in 15 mm tissue culture plates at a density of 2.5×10^5 cells/plate) was quantified as described before (Holtmann and Wallach, 1987).

To examine the binding of monoclonal antibodies against TBPI to the CHO cells, the cells were detached by incubation in phosphate buffered saline (PBS: 140 mM NaCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4, 2.7 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$), containing 5 mM EDTA and then incubated for 45 min at 0°C with 50 μg/ml of the test monoclonal antibody in PBS containing 0.5% bovine serum albumin, and 15 mM sodium azide (PBS/BSA). After washing the cells with PBS/BSA they were incubated further for 30 min at 0°C with FITC labeled, affinity purified goat antibody to the F(ab) fragment of mouse IgG (1:120 in PBS/BSA) (Bio-Makor, Israel) and then analyzed by determining the intensity of fluorescence in samples of 10^4 cells using the Becton Dickinson fluorescence activated cell sorter 440. Three monoclonal antibodies to TBPI, clones 17, 18 and 20, shown by cross competition analysis to recognize four spatially distinct epitopes in the TBPI molecule (Engelmann *et al.*, 1990a) and, as a control, a monoclonal antibody against TNF-α (all purified from ascitic fluids by ammonium sulfate precipitation and of the IgG_2 isotype) were used.

Quantification of the soluble form of the type I TNF-R by ELISA

A sensitive enzyme linked immunosorbent assay was set up, using TBPI-specific monoclonal and polyclonal antibodies in a sandwich technique. Immunoglobulins of the anti-TBPI mAb clone 20 (Engelmann *et al.*, 1990a) were adsorbed to 96 well ELISA plates (maxisorp, Nunc, Denmark) by incubation of the plates for 2 h at 37°C with a solution of 25 μg/ml of the antibody in PBS. After incubating the wells further for 2 h at 37°C with a solution containing PBS, 1% BSA, 0.02% NaN_3 and 0.05% Tween 20 (blocking solution) to block nonspecific further binding of protein, tested samples were applied in aliquots of 50 μl/well. The plates were then incubated for 2 h at 37°C, rinsed 3 times with PBS supplemented with 0.05% Tween 20 (washing solution) and then rabbit polyclonal antiserum against TBPI, diluted 1:500 in blocking solution, was added to the wells. After further incubation for 12 h at 4°C, the plates were rinsed again and incubated for 2 h with horse radish peroxidase-conjugated purified goat anti rabbit IgG. The assay was developed using 2,2′-azino-bis (3-ethylbenzthiazoline-6 sulfonic acid) as a substrate (Sigma). The enzymatic product was determined colorimetrically at 600 nm. Pure TBPI served as a standard.

Detection of a soluble form of the type I TNF-R in the growth medium of the transfected CHO cells and its analysis by reversed phase HPLC

The amounts of the soluble form of the type I TNF-R in samples of the growth medium of the tested CHO cells, collected 48 h after medium replacement, were determined by the immunoassay described above. For analysis of the soluble receptor by reversed phase HPLC, the CHO cells were cultured for 48 h in serum-free medium (nucleotide-free MEM α). The medium samples were concentrated 100-fold by ultrafiltration on an Amicon PM10 membrane and 100 μl aliquots were then applied to an Aquapore RP300 column (4.6 × 30 mm, Brownlee Labs) pre-equilibrated with 0.3% aqueous trifluoroacetic acid. The column was washed with this solution at a flow rate of 0.5 ml/min until all unbound proteins were removed, and then eluted with a concentration gradient of acetonitrile in 0.3% aqueous trifluoroacetic acid, as described before (Engelmann *et al.*, 1989). Fractions of 0.5 ml were collected and, after concentration *in vacuo*, were neutralized with 1 M HEPES buffer, pH 9.0. Amounts of soluble type I TNF-R in the fractions were determined by ELISA and the concentration of protein by the fluorescamine method (Stein and Moschera, 1981).

RNA isolation and analysis

RNA was isolated by a modification of the procedure described by Feramisco *et al.* (Feramisco *et al.*, 1982; Queen and Baltimore, 1983) and analyzed by electrophoresis in 1.5% agarose/6% formaldehyde gels, followed by blotting to 'Genescreen plus' hybridization transfer membranes (NEN, Boston, MA). The E13 cDNA insert was ^{32}P-labeled by random oligomer priming, using the Amersham random primer labeling kit (Amersham, UK). The membranes were hybridized at 42°C in the presence of 50% formamide and then washed as prescribed by Sambrook *et al.* (1989) for the detection of low abundance sequences.

Determination of the effect of TNF and of antibodies to TBPI on protein phosphorylation

Confluent monolayers of HeLa cells (Gey *et al.*, 1952), in 9 mm microwells, were incubated for 100 min with 100 μCi/ml [^{32}P]orthophosphate (Nuclear Research Center, Beer Sheva, Israel) in phosphate-free DMEM containing 10% fetal calf serum which had been dialyzed against 0.9% NaCl. Recombinant human TNF-α (kindly provided by Dr G.Adolf, Boehringer Institute, Vienna, Austria) and rabbit antiserum to TBPI (1:1000) (Engelmann *et al.*, 1990b) or, for comparison, normal rabbit serum (1:1000) were then added to the cells for 20 min. The cells were rinsed, and immediately solubilized by boiling in SDS–PAGE sample buffer containing β-mercaptoethanol. The solubilized proteins were analyzed by SDS–PAGE (12%) followed by autoradiography.

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Since submission of this article, cloning of the cDNA for the type I TNF-R has been described in two publications [Loetscher,H., Pan,Y.-C.E., Lahm,H.W., Gentz,R., Brockhaus,M., Tabuchi,H. and Lesslauer,W. (1990) *Cell*, **61**, 351–359 and Schall,T.J. *et al.*, *ibid.*, 361–370.] In two other publications [Smith,C.A. *et al.* (1990) *Science*, **248**, 1019–1023 and Kohno,T. *et al.* (1990) *Proc. Natl. Acad. Sci. USA* (in press)] the cloning of the type II TNF-R was described. The predicted amino acid sequence in the extracellular domain of this receptor fully matches the sequences presented here of amino acids in TBP II indicating further that, like TBPI, this soluble TNF binding protein is also derived from its immunologically cross-reacting cell surface TNF-R.