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Molecular Cloning and Expression of a Receptor for Human Tumor Necrosis Factor

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Summary

A human tumor necrosis factor (TNF) binding protein from serum of cancer patients was purified to homogeneity and partially sequenced. Synthetic DNA probes based on amino acid sequence information were used to isolate cDNA clones encoding a receptor for TNF. The TNF receptor (TNF-R) is a 415 amino acid polypeptide with a single membrane-spanning region. The extracellular cysteine-rich domain of the TNF-R is homologous to the nerve growth factor receptor and the B cell activation protein Bp50. Human embryonic kidney cells transfected with a TNF-R expression vector specifically bind both ¹²⁵I-labeled and biotinylated TNF- α . Unlabeled TNF- α and TNF- β were equally effective at displacing the binding of labeled TNF- α to TNF-R expressing cells. Northern analysis indicates a single species of mRNA for the TNF-R in a variety of cell types. Therefore, the soluble TNF binding protein found in human serum is probably proteolytically derived from the TNF-R.

Introduction

Tumor necrosis factor- α (TNF- α) is a multipotent cytokine produced mainly by activated macrophages. TNF- α was originally identified as a tumoricidal protein effecting hemorrhagic necrosis of transplanted solid tumors in mice (Carswell et al., 1975) but has since been implicated in diverse biologic processes including inflammation and immunoregulation, antiviral defense, endotoxic shock, cachexia, angiogenesis, and mitogenesis (Goeddel et al., 1986; Beutler and Cerami, 1988; Old, 1988). The related cytokine lymphotoxin (TNF- β) is synthesized by activated lymphocytes and shares many of the biological activities of TNF- α (Goeddel et al., 1986).

The mechanisms through which the TNFs mediate their multiple activities are largely unknown, but like most polypeptide hormones, binding to specific cell surface receptors is an initial event. Stable trimers comprised of identical TNF- α polypeptides of 17,350 daltons bind to sites on a variety of cell types, with dissociation constants (K_d)

ranging from 1.3×10^{-9} M to 7.1×10^{-11} M (Aggarwal et al., 1985; Kull et al., 1985; Tsujimoto et al., 1985; Baglioni et al., 1985; Watanabe et al., 1986; Tsujimoto and Vileck, 1987; Stauber et al., 1988; Hohmann et al., 1989; Ding et al., 1989). While most investigators report a single class of cell surface binding sites, others report the presence of both high ($K_d = 2.6 \times 10^{-13}$ M) and low ($K_d = 1.5 \times 10^{-10}$ M) affinity sites on the same cell (Imamura et al., 1987). TNF- β and TNF- α have been shown to compete for binding to the same receptor on the human cervical carcinoma cell line ME-180 (Aggarwal et al., 1985) and the histiocytic lymphoma cell line U-937 (Stauber and Aggarwal, 1989). Estimates of the size of the TNF receptor (TNF-R) determined by affinity labeling studies range from 54 to 175 kd (Creasey et al., 1987; Stauber et al., 1988; Hohmann et al., 1989; Smith and Baglioni, 1989). A recent report suggests the existence of two major receptor types for TNF- α : a myeloid cell type receptor with a K_d of 7.1×10^{-11} M and an epithelial cell type receptor with a K_d of 3.2×10^{-10} M. These two receptor types differ in size, glycosylation, and in their peptide maps (Hohmann et al., 1989).

In addition to the cell surface receptors for TNFs, several groups have identified soluble proteins in human urine (Peetre et al., 1988; Seckinger et al., 1988, 1989; Engelman et al., 1989, 1990) and in the serum of human cancer patients (Gatanaga et al., 1990) capable of specifically binding TNFs. In one instance two immunologically distinct TNF binding proteins (TNF BPs) were isolated from human urine (Engelman et al., 1990). Antibodies raised against those two proteins (TBP I and TBP II) had an inhibitory effect on the binding of TNF- α to its cell surface receptor, suggesting a structural similarity between the cell surface TNF-R and the soluble TBPs.

Soluble cytokine binding proteins in biological fluids have been shown in some cases to represent "shed" forms of cell surface cytokine receptors (Rubin et al., 1985; Novick et al., 1989; Zupan et al., 1989). To ascertain whether this was the case for the TNF-R, we purified a soluble TNF BP from human serum and isolated a corresponding cDNA by molecular cloning. This cDNA encodes a cell surface receptor for TNF that can presumably be processed to yield a soluble TNF BP.

Results

Purification and Characterization of TNF BP

A protein that inhibits the activity of both TNF- α and TNF- β has been detected in the serum of cancer patients but not healthy individuals (Gatanaga et al., 1990). This protein was purified by TNF- α affinity chromatography. Proteins eluted from the TNF- α affinity column were separated by reverse-phase HPLC, and several residues of N-terminal amino acid sequence were determined for each. Only one sequence was obtained, DSV(C/H)PQGKYIH, that did not correspond to a known serum protein. This protein, with an apparent M_r of 28,000, showed N-terminal sequence homology with a TNF BP isolated from human urine (Ols-

son et al., 1989; Engelmann et al., 1989, 1990). To obtain the amino acid sequence of internal peptides, the HPLC-purified TNF BP was subject to proteolysis using lysine C in the presence of SDS and the resulting proteolytic fragments were separated. The sequences of the two major eluting peaks were determined: GTYLYNDCPGFGQDENE for PF I and EMGQVEISSCTVDNDTVCG for PF II.

cDNA Cloning and Characterization Reveal a Receptor Structure

Two synthetic DNA probes were synthesized based on the amino acid sequence of PF I and PF II, using human codon bias (Lathe, 1985). The two probes were used to screen cDNA libraries made from placental tissue and from the promyelocytic cell line HL-60. Several positive clones were obtained from both libraries. The DNA sequence was determined for four overlapping cDNA clones from the HL-60 library. The composite sequence contained a single long open reading frame. The sequence of a 2.1 kb cDNA from the placental library was also determined and found to overlap the combined sequences of the HL-60 clones. The placental cDNA clone contains all of the presumed coding region as well as some of the 5' and 3' untranslated regions. The composite nucleotide sequence of the cDNAs and the deduced amino acid sequence of the predicted protein is shown in Figure 1A. There are three nucleotide differences between the placental and HL-60 clones (A at nucleotide position 75 in the placental vs. G in the HL-60, G vs. A at position 219, and G vs. A at position 1342), none of which results in an amino acid change. The open reading frame defines a protein of 455 amino acids starting at nucleotide position 182 and terminating at nucleotide position 1545.

The encoded protein exhibits a predicted domain structure typical of a cell surface receptor: the hydrophathy profile indicates a signal peptide at the beginning of the protein and a potential transmembrane domain in the middle that separates the presumed extra- and intracellular domains (Figure 1B). The 11 amino acids designated +1 through 11 match the N-terminal amino acids of the soluble TNF BP isolated from serum. Therefore, we assigned Asp+1 as the N-terminal residue, although it is not known whether the N-terminus of the cell surface form of this molecule is the same as the N-terminus of the soluble form that was sequenced. Residues -40 to -10 are largely hydrophobic and probably serve as a signal peptide. Although the precise cleavage point is not known, the Gly (-12)-Leu (-11) peptide bond is a possible site (von Heijne, 1986). The mature protein may result from further proteolytic processing at the basic Lys (-2)-Arg (-1) dipeptide. Residues +1 through 172 probably constitute a cysteine-rich extracellular domain, with 24 cysteine residues and 3 potential sites for N-glycosylation (Asn-X-Ser/Thr) at residues 14, 105, and 111. In addition to the identity to the N-terminal sequence of soluble TNF BP, this domain contains sequences corresponding to the lysine C-generated proteolytic fragments PF I (residues 24-41) and PF II (residues 67-86). The 23 amino acid hydrophobic region in the middle of the molecule, which is flanked on its amino-terminal side by Thr 171 and on its carboxy-

terminal side by Arg 195, is characteristic of a transmembrane-spanning domain. The putative cytoplasmic domain would be comprised of the remaining 220 amino acids.

Cysteine Repeats in the Extracellular Domain of the TNF-R

In the presumed mature extracellular domain of the predicted TNF-R protein, 24 of the 171 total amino acids are cysteines and the spacing of the cysteine residues is periodic. In contrast, the remaining 265 residues contain only 6 cysteines. Dot matrix analysis of these regions using the ALIGN score and the Dayhoff matrix (Dayhoff et al., 1978, 1983) reveals significant diagonal patterns of homology, indicating internal homologies (Figure 2A). Inspection of the extracellular domain sequence reveals it can be roughly divided into four related subdomains (Figure 2B). This 4-fold symmetry may represent duplication events of an ancestral subdomain in the evolution of the TNF-R.

TNF-R Is Related to Nerve Growth Factor Receptor and the B Cell Activation Molecule Bp50

Cysteine-rich repeats have been detected in the extracellular domains of the EGF precursor and EGF receptor (Doolittle et al., 1984; Ullrich et al., 1984) and LDL receptor (Yamamoto et al., 1984), but the TNF-R sequence reveals no significant amino acid homologies with these molecules. However, cysteine-rich extracellular domains have also been reported in the nerve growth factor receptor (NGF-R) (Johnson et al., 1986) and the B lymphocyte activation molecule Bp50 (CDw40) (Stamenkovic et al., 1989). These molecules have significant homology to the TNF-R in their extracellular domains (Figure 3). All three molecules share a similar set of cysteine-rich subdomains. Optimal alignment of the sequences for the extracellular domains of TNF-R, NGF-R, and Bp50 shows the close conservation of the cysteine residues and an overall identity of 29% between TNF-R and NGF-R and 24% between TNF-R and Bp50 in 167 residues (Figure 3). No significant homology is seen in the transmembrane or intracellular domains of these molecules.

TNF-R Transcript Is Expressed in a Variety of Cell Types

A panel of human cells and tissues was examined for the presence of TNF-R mRNA. Figure 4A shows expression of TNF-R mRNA in human term placenta and adult liver, as well as the breast carcinoma MB436, the nontumorigenic transformed breast epithelial cell line HBL100, the glioblastoma A172, and a primary squamous carcinoma, FG. In addition, cell lines resistant and sensitive to TNF cytotoxicity were tested for TNF-R mRNA. TNF-resistant cell lines (T-24 bladder carcinoma, A549 lung carcinoma) and TNF-sensitive cell lines (MCF-7 breast carcinoma and ME-180 cervical carcinoma) all exhibit TNF-R mRNA (Figure 4B).

The increased expression of TNF-Rs on cells after treatment with interferon- γ has been reported (Aggarwal et al., 1985). To test whether the levels of transcript for the TNF-R are affected by interferon- γ or by treatment with TNF- α or

Figure 1. Human TNF-R cDNA and Amino Acid Sequences and Hydropathy Plot

(A) Composite nucleotide and deduced amino acid sequence of TNF-R cDNA. Amino acids are numbered in the sequence. The composite of HL-60 clones begins at the first nucleotide and ends at nucleotide 1607 (▼). The placental clone begins at nucleotide 67 (●) and ends at the last nucleotide. Three nucleotide differences at positions 75, 217, and 1342 are noted, with the top nucleotide representing that found in the placental clone and the bottom representing the HL-60 sequence. Aspartic acid (Asp) is the initial residue in the N-terminal sequence obtained from native TNF BP in human serum and is labeled as position 1. Amino acids -40 to -1 comprise presumed signal peptide and proteolytically processed N-terminal residues. Overlines represent three potential N-linked glycosylation sites (residues 14, 105, and 111). The predicted transmembrane region (residues 172-194) is boxed.

(B) Hydropathy profile of protein sequence in (A).

- β , we treated A549 cells with these cytokines for 24 hr prior to harvest of the mRNA. Levels of TNF-R mRNA are relatively constant after treatment with these cytokines alone or in combination (Figure 4C). Thus, if interferon- γ or TNF regulation of receptor does occur in A549 cells, it does not appear to be transcriptional or it is mediated

through another class of receptor. We do not know whether TNF-R mRNA is affected by these cytokines in other cell types.

TNF-R message was also found in several hematopoietic cell lines. The cultured T cell lines CEM, HSB, and HuT 78, the functional cytotoxic T cell line PW, and the

Figure 2. Internal Repeats in the TNF-R

(A) Dot matrix plot of internal homologies in the TNF-R extracellular domains. Dots are placed where Dayhoff mutation matrix alignment scores >20 are obtained (Dayhoff et al., 1983). The plot is necessarily symmetrical around the diagonal line of identity; other lines at 45° angles that are off of the main diagonal represent areas of internal homology. Numbers denote amino acid positions in the predicted mature extracellular domain.

(B) Internal cysteine repeats in the TNF-R extracellular domain. Alignment of amino acid sequences in the extracellular portion of the TNF-R represent four internal subdomains. Identical residues are boxed; amino acids are numbered in the left margin.

erythroleukemia line K562 have a single detectable species of TNF-R mRNA (data not shown). The cultured B cell line RPMI-1788, but not the EBV-transformed B cell lines JY, LB, and BOC, have TNF-R mRNA (data not shown). Uninduced U-937 cells were found to have relatively high levels of TNF-R mRNA, and uninduced HL-60 cells contained markedly less (data not shown). Thus, message for the cloned TNF-R seems widely though not ubiquitously expressed. In all cells that express TNF-R, a single species of mRNA of about 3.0 kb is detected. This suggests

that the cDNA clones we obtained do not represent complete copies of the TNF-R mRNA; some nucleotide sequences in the untranslated regions are missing.

Transfection and Expression of the Human TNF-R cDNA

The 2.1 kb placental cDNA clone was inserted into the mammalian expression vector pRK5. This cDNA starts at nucleotide position 64 (Figure 1A), with the initiating me-

Figure 3. Homology in the Extracellular Domains of NGF-R, TNF-R, and Bp50

Optimized alignment of the protein sequence of the extracellular domains of NGF-R, TNF-R, and Bp50 (CDw40) is shown with gaps introduced to optimize matches. Identical amino acids are boxed. Cysteine residues not conserved among all three sequences are circled. Residues are numbered in relation to their position in TNF-R.

Figure 4. Northern Blot Analysis of TNF-R mRNA

(A) Northern analysis of TNF-R mRNA in placenta, liver, and transformed cell lines. Northern blot analysis of 4 μ g of poly(A)⁺ RNA from normal human term placental and adult liver tissues, the human transformed cell lines MB436, HBL100, and A172, and the primary squamous carcinoma FG. (B) Northern blot analysis of both TNF-resistant and TNF-sensitive cell lines. TNF-resistant cell lines: A549 and T-24. TNF-sensitive cell lines: MCF-7 and ME-180. There is 3 μ g of poly(A)⁺ RNA per lane. The positions of ribosomal RNA bands are denoted as 28S and 18S. (C) Levels of TNF-R mRNA in A549 cells after IFN- γ or TNF treatment. There is 7 μ g of poly(A)⁺ RNA per lane from A549 cells after treatment with either TNF- α , TNF- β , or interferon γ , or both (0.1 μ g/ml each) for 24 hr prior to harvest of mRNA. Control lane: untreated cells.

thionine 118 bp downstream (position 182, Figure 1A). The cDNA is under the transcriptional control of the cytomegalovirus immediate-early promoter and is followed by the SV40 termination and polyadenylation signals. The TSA 201 cell line, a subclone of the human embryonic kidney cell line 293s (Graham et al., 1977), which constitutively expresses large T antigen, was selected for transient transfection experiments because of its high transfection efficiency and low numbers of endogenous TNF-Rs. Following transfection with the TNF-R expression construct, cells were tested for the ability to bind specifically either biotinylated or ¹²⁵I-labeled TNF- α .

An increase in the relative capacity of pRK-TNF-R-transfected cells to bind biotinylated TNF- α can be seen using fluorescence-activated cell sorting (FACS). Mock-transfected TSA 201 cells display a low level of binding of biotinylated TNF- α , presumably to endogenous receptors (Figures 5A and 5B). The levels of TNF- α binding are substantially increased on cells transfected with the TNF-R construct, as shown by the shift to the right of the fluorescence histogram (Figure 5F). The intensity of fluorescent staining is reduced to background levels in both populations by preincubation of cells with either nonbiotinylated TNF- α or TNF- β (Figures 5C, 5D, 5G, and 5H), demonstrating that the observed binding is specific.

A saturation isotherm for the specific binding of ¹²⁵I-TNF- α was performed by sequential dilution of the spe-

cific activity of the radioligand with unlabeled TNF- α at concentrations ranging from 67 pM to 33 nM (Figure 6A). The specific binding of ¹²⁵I-TNF- α is saturable, and the Scatchard analysis of these data using nonlinear least-squared regression reveals two binding sites ($p < 0.05$; Figure 6A, inset) with high ($K_d = 0.66$ nM) and low ($K_d = 19.6$ nM) affinity. The number of binding sites for these two receptor subtypes on the transiently expressing TSA 201 cells is $\sim 50,000$ and $630,000$ sites per cell, respectively. ¹²⁵I-TNF- α binding to mock-transfected control cells was at least 10-fold lower than pRK-TNF-R-transfected cells, suggesting low numbers of endogenous receptors. Increasing concentrations of unlabeled TNF- β cause a dose-dependent decrease in specific ¹²⁵I-TNF- α binding in a manner very similar to that seen with unlabeled TNF- α (Figure 6B). This suggests that the TNF-R expressed on the transfected cells recognizes TNF- α and TNF- β with approximately equal affinity.

Discussion

The data presented here describe the cloning and expression of a receptor for human TNF- α and - β . The deduced amino acid sequence of the TNF-R reveals a structure typical of cell surface receptors for polypeptides: it contains a signal peptide and extracellular, transmembrane, and intracellular regions. There is a significant degree of homol-

Figure 5. FACS Analysis of pRK-TNF-R-Transfected TSA 201 Cells

The left column contains histograms from mock pRK5-transfected cells; the right column contains histograms from pRK-TNF-R-transfected cells. Cells were stained with PE-conjugated streptavidin after pretreatment with or without biotinylated TNF- α (b-TNF- α). (A) and (E), PE-streptavidin without b-TNF- α ; (B) and (F), PE-streptavidin plus b-TNF- α ; (C) and (G), preincubation of the cells with a 5-fold excess of unlabeled TNF- α prior to staining with PE-streptavidin plus b-TNF- α ; (D) and (G), preincubation with a 2-fold excess of unlabeled TNF- β prior to staining with PE-streptavidin plus b-TNF- α .

ogy between the TNF-R and the NGF-R, as well as the B lymphocyte activation molecule Bp50, suggesting that they comprise a family of related growth factor receptors. The four 40 amino acid repeats of the extracellular portions of these receptors may form relatively independent folded subdomains, each tightly cross-linked by disulfide bonds. Because these cysteine-rich domains form virtually all of the extracellular portion of the TNF-R, it is likely that they contain the ligand binding site(s), although it is not immediately clear how the biologically active TNF trimer binds the receptor. In this regard it is interesting to note that the net charge of the extracellular domain of the TNF-R is positive, while that of its ligand is negative. The converse is true for NGF-R and its ligand, where the extracellular domain is strongly acidic and the ligand is basic. This would suggest that electrostatic attractions play an important role in the interactions of these related receptor molecules with their respective ligands. One possibility is that the cysteine repeat units form a general structural framework that, depending upon specific amino acid substitutions, could be varied to accommodate a variety of ligands.

The mechanisms of signal transduction by TNF and its receptor are obscure. The intracellular domain, though large enough to possess an enzymatic activity or to interact with other proteins that may mediate signal transduction, has no apparent sequence homology with any proteins in the available data bases. The cytoplasmic domain is rich in serine, threonine, and tyrosine but shows no homology to the catalytic domain of the tyrosine or ser-

ine/threonine-specific protein kinases. However, there is sequence similarity to the canonical phosphorylation sites (Ser/Thr-X-Arg/Lys) that can be acted upon by protein kinase C (Woodget et al., 1986) at amino acid positions 223, 366, and 371. Also present are a potential cyclic nucleotide-dependent protein kinase phosphorylation site at amino acid 368 (Feramisco et al., 1980) and a consensus tyrosine kinase phosphorylation site at residue 354 (Patchinsky et al., 1982). The binding of TNF to its receptor has been shown to increase both GTP binding and GTPase activity in HL-60 membrane preparations, leading to the suggestion that a GTP binding protein might couple TNF-induced signaling to biological effects (Imamura et al., 1988). However, the TNF-R has no homology to other receptors that are known to interact with G proteins.

Internalization of the native TNF-R in the absence of ligand has been shown (Smith et al., 1990; Ding et al., 1989) and shown to occur more rapidly in the presence of ligand (Smith et al., 1990; Imamura et al., 1987). While a majority of reports indicates the degradation of receptor after internalization (Watanabe et al., 1988; Smith et al., 1990), one suggests that the receptor is continuously recycled (Vuk-Pavlovic and Kovach, 1989). These differences could represent cell type-specific differences or differences in processing for distinct types of TNF-R. Regions of the intracellular domain of the cloned TNF-R reported here are extremely rich in proline, glutamic acid, serine, and threonine. The presence of these so-called PEST sequences has been shown to correlate with rapid intracellu-

Figure 6. Binding Characteristics of Recombinant Human TNF-R Expressed in TSA 201 Cells

(A) Saturation isotherm of specific binding of ^{125}I -labeled TNF- α on transfected cells. Replicate samples of TSA 201 cells transiently expressing pRK-TNF-R were incubated with ^{125}I -TNF- α (16.7 pM) alone or in the presence of increasing amounts of unlabeled TNF- α , and the specific binding of ^{125}I -TNF- α was determined at each concentration. The inset presents the data transformed by Scatchard analysis. These results were from a single experiment that had been repeated three times with either triplicate or duplicate determinations.

(B) Displacement curves showing inhibition of the specific binding of ^{125}I -TNF- α by unlabeled TNF- α (□) or TNF- β (●). The experiment was performed as described in (A), with ^{125}I -TNF- α displaced with increasing amounts of TNF- α or - β .

lar protein degradation (Rogers et al., 1986). This leads to the speculation that the receptor encoded by this gene has a high turnover rate in its native state. This speculation is consistent with our observations that A549 cells have relatively high levels of mRNA for the TNF-R yet relatively low numbers of cell surface receptors (unpublished data).

It is not clear that the effector functions of TNF are achieved by transduction of a signal via the receptor after ligand binding. A direct intracellular role for TNF in cytotoxicity has been proposed based on TNF microinjection experiments (Smith et al., 1990), although using other cell lines we have not been able to demonstrate activity for microinjected TNF (D. Pennica and D. V. G., unpublished data). If internalization of the ligand is the important step

in the biochemical action of TNF, the receptor may play no more a role than transporting TNF to the inside of the cell. However, the fact that antibodies generated against soluble TNF BPs will cross-react with cell surface molecules and act as TNF agonists suggests that TNF-R signal induction can occur without internalization of the TNF (Engelmann et al., 1990).

Shedding the extracellular domain of the TNF-R might be used as a protective mechanism by cells to avoid the cytotoxic effects of TNF. The presence of soluble TNF BP in the serum of cancer patients may represent a mechanism by which tumors evade host anti-tumor defenses by modulating systemic levels of TNF. Soluble forms of the receptor for IL-2 are released from activated human lymphoid cells (Rubin et al., 1985), and its levels are found increased in bodily fluids in disease states (Marcon et al., 1988). Soluble receptors for IL-6 and IFN- γ have recently been detected in human urine (Novick et al., 1989), soluble truncated forms of the NGF receptor have been seen in human urine and amniotic fluid (Zupan et al., 1989), and a cDNA that encodes a soluble form of murine IL-4 has been reported (Mosley et al., 1989). The prevalence of such soluble receptors suggests a normal regulatory role for these molecules. Their mode of action could be to limit the amount of available cytokine by binding it in solution, thus preventing the cytokine from reaching its cell surface target. TBP I, the soluble TNF-BP that shares N-terminal homology to the TNF-R reported here, binds TNF- α with greater affinity than TNF- β (Engelmann et al., 1990). Our studies show that TNF-R binds TNF- α and - β with approximately equal affinities. This suggests that solubilization of the extracellular domain of the receptor may induce a change in the relative affinities of the binding component for TNF- α and TNF- β .

Most if not all mammalian cells appear to have receptors for TNF. However, the number of TNF-Rs per cell is relatively low. In the 5' untranslated region of TNF-R mRNA two short open reading frames are seen, one of 43 codons and one of 3 codons. Such short open reading frames are not uncommon in growth factor receptor RNAs. They are found 5' of the main open reading frame in the GM-CSF R (Gearing et al., 1989), the human IL-6 R (Yamasaki et al., 1988), the murine IL-1 R (Sims et al., 1988), and the human IL-2 R (Nikaido et al., 1984; Hatakeyama et al., 1989). It has been postulated that these short open reading frames might act, if translated, to dampen the translation of the main receptor coding regions (Gearing et al., 1989). Such a mechanism might partly explain the low numbers of these receptors, in relation to the levels of mRNA, on normal cell types.

Interpretation of published data from affinity labeling cross-linking studies is made difficult because cross-linked ligand is itself resolved into monomers, dimers, and trimers by SDS-PAGE, but estimates of the size of cell surface TNF-Rs is generally between 55 and 138 kd (Creasey et al., 1987; Stauber et al., 1988; Hohmann et al., 1989). Receptors of considerably larger size, up to 310 kd, have also been reported (Smith and Baglioni, 1989), but these forms might reflect receptors cross-linked to associated regulatory proteins, or even complexes of cross-

linked receptors. Association with other proteins or "adaptor subunits" has been shown for other receptors, most notably IL-2 R (Teshigawara et al., 1987; Hatekeyama et al., 1989) and IL-6 R (Yamasaki et al., 1988). In addition, NGF-R is thought to require association with another molecule for high-affinity binding of its ligand (Radeke et al., 1987). Association with an as yet unknown protein may be required for the TNF-R reported here to bind ligand with high affinity. One possible explanation for the two binding sites observed in the cells transfected with pRK-TNF-R is that a small number of the transiently expressed receptor proteins interacts with an endogenous protein present in a limited quantity within the TSA 201 cell. This subpopulation of receptors might then bind ^{125}I -TNF- α with a higher relative affinity ($K_d = 0.66$ nM), while the bulk of the expressed receptors (92% based on B_{max} predictions) binds ligand with ~ 30 -fold lower affinity.

The relationship between the TNF-R described here and other potential TNF-Rs is not clear. The predicted 415 amino acid molecule we identified by cDNA cloning encodes a protein with a predicted M_r of 50,578. Since there are three potential N-linked glycosylation sites, and since protein biochemical studies consistently reveal carbohydrate content on the TNF-R molecules analyzed, it is likely that the apparent M_r of the TNF-R reported here is greater than 50,000 in its native state. The recent report of two immunologically distinct forms of TNF BPs in urine, one of which has N-terminal sequence homology to the sequence reported here (Engelmann et al., 1990), argues for the existence of at least two cell surface TNF-Rs that shed their extracellular domains. This is consistent with the report of two major types of TNF-R on different cell types (Hohmann et al., 1989), suggesting that the molecule reported probably represents one of the two types of cell surface TNF-R. The mechanisms by which the extracellular domains of the TNF-R are shed are not known. Soluble IL-4 receptor is thought to be the result of alternative splicing of IL-4 mRNA (Mosley et al., 1989). In contrast, the presence of only one detected species of mRNA for this TNF-R suggests that the soluble form is generated proteolytically by cleavage of the extracellular domain from the cell surface receptor. Soluble TNF BP may be resistant to subsequent proteolysis after release as a result of a compact disulfide-bonded structure. The physiological significance of this process remains unknown. The availability of this and other cloned TNF-R cDNAs should allow for the resolution of these issues as well as the elucidation of many of the complexities of the multiple activities of TNF.

Experimental Procedures

Purification of Serum TNF BP and Amino Acid Sequencing of Proteolytic Fragments

TNF- α affinity chromatography fractions of serum proteins from human cancer patients were shown to inhibit the activity of TNF- α and TNF- β (Gatanaga et al., 1990). These fractions were electrophoresed on SDS-PAGE and found to contain several components. Samples from the affinity column were loaded directly onto a small glass column (1.5 mm $\times$ 50 mm) packed with 15 μm of C-18 packing material (J. T. Baker) and eluted on a HP 1090 HPLC with a linear gradient of 1%–60% acetonitrile in 0.1% TFA and water at a flow rate of 0.2 ml/min. Eluted

peaks were sequenced directly on a prototype sequencer (U.S. patent number EP0257735), and sequences obtained were compared to the protein sequences in the available data bases using the DFASTP program. An early eluting peak, $M_r = 28,000$, produced the only unknown sequence. Further internal sequence of this protein was obtained by digesting the purified material with a 1:10 ratio (enzyme to substrate) of lysyl endopeptidase (Wako Chemicals) at pH 8.0 in the presence of 0.05% SDS, 0.1 M Tris-HCl at 37°C for 18 hr. The digested peptides were then separated on HPLC as above. The two major eluting peaks, PF I and PF II, were sequenced as above.

cDNA Cloning

Two λ gt10 libraries, a placental cDNA library prepared as described (Ullrich et al., 1985), and a random primed cDNA library made from the promyelomonocytic cell line HL-60 (provided by Karen Fisher) were probed with two oligonucleotide probes derived from the protein sequences of PF I and PF II using human codon bias (Lathe, 1985). The probes, 5'-AAGGGCACCTACCTGTACAATGACTGCCCTGGCTTTGGC-CAGGATGAGAATGA from PF I and 5'-AAGGAGATGGGCCAGGTGGGATCTCCTCCTGCACAGTGGAC AATGACACAGTGTGTGG from PF II, were labeled with [γ - ^{32}P] ATP using T4 polynucleotide kinase and used to screen replicate sets of nitrocellulose filters containing $\sim 1 \times 10^6$ independent clones from each of the two libraries. Filters were probed at 42°C in a hybridization solution that was 20% formamide, 0.1% SDS, 5 $\times$ Denhardt's solution, 50 $\mu\text{g}/\text{ml}$ salmon sperm DNA, 50 mM NaPO $_4$, 0.1% sodium pyrophosphate. Filters were washed twice in 0.5 $\times$ SSC, 0.1% SDS at 42°C. Hybridizing phage were plaque purified, DNA was prepared, and cDNA inserts were isolated and subcloned using standard techniques (Maniatis et al., 1982). Four clones from the HL-60 library, HL-60-2, -3, -10, and -14, and one clone from the placental library were sequenced on both strands using the chain termination procedure (Sanger et al., 1977).

Northern Analysis of TNF-R mRNA

Northern hybridization was performed as previously described (Thomas, 1980; Wong et al., 1988). Briefly, total cytoplasmic RNA was extracted from cells, enriched for poly(A) $^+$ mRNA, electrophoresed on a formaldehyde-agarose (1.2%) gel, and transferred to nitrocellulose. The filters were baked for 30 min at 80°C under vacuum and hybridized to a ^{32}P -labeled TNF-R probe for 16 hr. The probe consisted of the cDNA insert isolated from the placental clone (2.1 kb EcoRI fragment) labeled with [α - ^{32}P]dATP and [α - ^{32}P]dCTP by the random priming method. Filters were washed at 60°C in 0.1 $\times$ SSC, 0.1% SDS for 30 min. Autoradiography was carried out for 24 hr using Kodak XAR-5 film and an intensifying screen.

TNF-R Expression Plasmid and Transfection of TSA 201 Cells

A 2.1 kb TNF-R cDNA fragment was isolated by a partial EcoRI digest from the placental phage TNF-R clone. This fragment was ligated into the EcoRI site of the expression plasmid pRK5 (R. Klein and D. V. G., unpublished data). The cDNA in the expression construct, pRK-TNF-R, is downstream of the cytomegalovirus promoter/enhancer and under its transcriptional control. Downstream of the cDNA insert are SV40 termination and polyadenylation signals. Human TSA 201 cells (obtained from R. DuBridge) are a derivative of the human embryonic kidney cell line 293s (Graham et al., 1977), which constitutively expresses large T antigen. These were transfected with either the pRK-TNF-R expression plasmid or mock transfected with the pRK plasmid without a cDNA insert. Transfections were performed in 100 mm plates using 7.5 μg of plasmid DNA per plate by the calcium phosphate precipitation method essentially as described (Gorman, 1985), except that the precipitates were left on the cells for 16–18 hr, and the cells were not shocked with DMSO in PBS. The transfected cells, transiently expressing TNF-R, were assayed 48 hr after transfection.

Analysis of Transfected TSA 201 Cells

For FACS analysis, TNF- α was biotinylated using biotin-N-hydroxy-succinimide ester at a 1:2.5 ratio of biotin ester:protein as described (Ranges et al., 1989). Forty-eight hours after transfection, cells (10^5) were treated with 50 nM biotinylated TNF- α by incubation for 2 hr at 4°C in PBS + 2% fetal calf serum (FCS). After washing twice the cells were stained for 30 min at 4°C with phycoerythrin (PE)-conjugated streptavidin and then washed twice and resuspended in PBS + 2%

FCS containing 0.5 µg/ml propidium iodide. For competition binding with unlabeled TNF-α and TNF-β, cells were preincubated with either 250 nM nonbiotinylated TNF-α or 100 nM nonbiotinylated TNF-β prior to staining with PE-conjugated streptavidin plus 50 nM biotinylated TNF-α. The cells were analyzed on a Coulter Elite Flow Cytometer using the 488 nm line of an argon ion laser, gating only on propidium iodide-excluding cells. PE emission was detected using a 575 nm (± 25 nm) band pass filter, and propidium iodide was detected with a 680 nm long pass filter. Electronic compensation was used to subtract the spectra overlap of PE into the propidium iodide detector.

For analysis of binding of ¹²⁵I-TNF to transfected cells, pRK-TNF-R or pRK mock-transfected cells were harvested with PBS containing 1 mM EDTA and washed with PBS containing 0.1% BSA and 0.02% sodium azide (PBSA buffer). Duplicate or triplicate samples of 2–2.5 × 10⁶ cells in 0.5 ml of PBSA were incubated at 4°C for 2 hr while shaking with 16.7 pM ¹²⁵I-TNF-α (New England Nuclear; 88.6 mCi/mg) alone or in the presence of increasing concentrations of unlabeled recombinant human TNF-α or TNF-β (rHuTNF-α and -β, Genentech). Nonspecific binding was determined by the addition of 0.33 mM unlabeled TNF-α. Cells were centrifuged at 14,000 × g for 15 min, and unbound ¹²⁵I-TNF-α was aspirated. The cell pellet was washed once with 1 ml of ice-cold PBSA. The amount of ¹²⁵I bound was determined by counting the cell pellets in a gamma counter. The data were fit using nonlinear least-squared regression analyses according to Marquardt algorithms (GraphPAD Inplot version 3.0, GraphPAD Software, San Diego, CA).

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