Proc. Natl. Acad. Sci. USA
Vol. 87, pp. 6151–6155, August 1990
Biochemistry

Complementary DNA cloning of a receptor for tumor necrosis factor and demonstration of a shed form of the receptor

(tumor necrosis factor-binding protein/cachexia/inflammation)

RENU A. HELLER*†, KYUNG SONG*, MARTHA A. ONASCH*, WOLFGANG H. FISCHER‡, DAVID CHANG*, AND GORDON M. RINGOLD*

*Institute of Cancer and Developmental Biology, Syntex Research, Palo Alto, CA 94304; and ‡The Salk Institute, La Jolla, CA 92037

Communicated by Ronald W. Davis, May 16, 1990

ABSTRACT **Tumor necrosis factor (TNF) receptor (TNFR) was isolated as a 68-kDa glycoprotein from UC/HeLa 2-5 cells developed from a parental B-cell line (UC cells) to overexpress the receptor. Tryptic digests of two separate TNFR preparations provided amino acid sequences of four different peptides. Amino-terminal analysis indicated the presence of the amino-acid sequence Val-Ala-Phe-Thr-Pro, reported to be the amino-terminal sequence of a 30-kDa urinary TNF-binding protein II. Examination of the cultured medium of UC/HeLa 2-5 cells showed an abundance of a 40-kDa TNF-binding protein, indicating that the previously cited 30-kDa TNF-binding protein II is likely to be a shed form of the TNFR. Based on the peptide sequences, oligonucleotides were synthesized, and two of these were used as primers in the polymerase chain reaction to amplify cDNA sequences from poly(A)$^+$ RNA of UC/HeLa 2-5 cells. These PCR fragments were radiolabeled and used to screen a cDNA library made from UC/HeLa 2-5 mRNA. Further analysis identified cDNA sequences that encoded the amino acid sequences of all four TNFR peptides. RNA blot-hybridization analysis of UC/HeLa 2-5 mRNA revealed a 3.8-kilobase transcript of the same size as the mRNA in the parental UC cells. Genomic Southern blots indicated the presence of a single gene in parental cells and a second, amplified gene in TNFR-overexpressing cells, suggesting amplification of the transfected gene as a possible mechanism for the increase in TNFR numbers in UC/HeLa 2-5 cells.**

Tumor necrosis factor α (TNF-α) mediates a variety of responses by binding to specific high-affinity cell-surface receptors (1). The biological activities attributed to TNF are cytotoxicity toward certain tumors and tumor cells (2, 3), inhibition of adipose-specific genes (4–6), cachexia (1, 7), inflammation (8–10), and the mediation of endotoxin shock (11). Effects of TNF appear also to be mediated by inducing release of other cytokines such as interleukins 1 and 6 (1, 12) and arachidonic acid metabolites (ref. 1; G.M.R., unpublished results), some of which stimulate proliferation of a variety of cells (1, 13, 14).

TNF receptors (TNFRs) exhibit affinity constants for TNF in the nanomolar range. Receptor numbers vary from 1000 to 10,000 per cell (13, 15–17), and molecular size estimates of 54–138 kDa (17–20) have been deduced from complexes of cross-linked radiolabeled ligand to receptor. Because the number of the ligand molecules in these complexes is uncertain, these estimates have been tentative. Based on (*i*) the sizes of these receptor–ligand complexes, (*ii*) the analyses of tryptic fragments generated from these complexes, and (*iii*) the apparent absence of immunological cross-reactivity between receptors from different cell types, the existence of two different receptors has been proposed (21). In addition, the isolation of two distinct TNF-binding proteins (both ≈30 kDa in size) from human urine has been provided as added evidence for the existence of different types of receptors. These urinary proteins are believed to represent soluble forms of different receptors (22–24).

We have developed B-cell lines (among them UC/HeLa 2-5) that express 150,000–180,000 TNFRs per cell (25). Receptor protein purified from these cells has a molecular size of ≈68–70 kDa and, when covalently linked with TNF, forms complexes primarily of 87 kDa and to a lesser extent 104 kDa. These observations suggest that TNF associates predominantly in the monomeric form with the receptor (25). In this paper we report the cloning of the cDNA for the TNFR[§] from these receptor-amplified cells. The cDNA clone was isolated by using radiolabeled probes generated by the polymerase chain reaction (PCR) with oligonucleotide primers corresponding to amino acid sequences of the purified receptor. Amino-terminal analysis of the purified receptor protein indicates identity with one of the urinary TNF-binding proteins (24).

METHODS

Protein Purification, Peptide Analysis, and Protein Sequencing. TNFR was purified from ≈2 × 10^{11} UC/HeLa 2-5 human B cells (25). The protein was analyzed by NaDodSO$_4$/PAGE (26), electroblotted to nitrocellulose membrane (27), and stained with 0.1% amido black. The 68-kDa TNFR protein band was treated with polyvinylpyrrolidone (PVP-40) to block additional protein binding and was treated with trypsin (28, 29). Peptide fragments released into the supernatant were resolved by reversed-phase HPLC. Edman degradation of the selected fragments in a gas-phase protein sequencer revealed the amino acid sequences of the peptides. Amino-terminal sequence was determined by procedures described by Matsudaira (30).

Filter Binding Assay and Ligand Blot Assay. These assays were developed to identify soluble TNFR and were conducted as described (25).

RNA Isolation and Blot-Hybridization (Northern) Analyses. Cellular RNA was prepared by the guanidinium isothiocyanate procedure (31). Poly(A)$^+$ RNA selected by chromatography on oligo(dT)-cellulose (32) was denatured with glyoxal and dimethyl sulfoxide (33), fractionated by electrophoresis in 0.8% agarose gels in 10 mM sodium phosphate buffer (pH 7.0), and transferred to Nytran membrane (Schleicher & Schuell). Prehybridization and hybridization with random-hexamer primed [α-^{32}P]dCTP-labeled cDNA probe (34) was carried out at 42°C in 50% formamide containing 5× SSPE

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor; PCR, polymerase chain reaction.

†To whom reprint requests should be addressed.

§The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35857).

(1× = 0.18 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA), 5× Denhardt's solution (1× = 0.02% bovine serum albumin/0.02% Ficoll/0.02% polyvinylpyrrolidone), and 100 μg of carrier salmon sperm DNA per ml (35). The filters were washed at 58°C twice in 1× SSPE/0.1% $NaDodSO_4$ and twice with 0.1× SSPE/0.1% $NaDodSO_4$ and autoradiographed.

cDNA Cloning. cDNA libraries in the mammalian expression vector pCDM8 (36) were constructed by Invitrogen (San Diego, CA) from double-stranded oligo(dT)-primed cDNA synthesized from UC and UC/HeLa 2-5 cell poly(A)$^+$ RNA (37). The UC/HeLa 2-5 cDNA library contained $>1.3 \times 10^6$ recombinants, and the UC library contained $>0.7 \times 10^6$ recombinants. The UC/HeLa 2-5 library was screened with radiolabeled PCR-generated probes. Additional screening with the 21- or 23-base degenerate oligonucleotides (A, B, or C; see below) or the 51-base oligonucleotide (sequence D) was carried out by end-labeling with [γ-^{32}P]ATP and polynucleotide kinase. Filters hybridized with PCR probes were washed according to conditions described above. When oligonucleotides were used as probes, conditions that use tetramethylammonium chloride were used (38).

Oligonucleotide Synthesis. All oligonucleotides were synthesized by using a solid-phase phosphoramidite method on a Biosearch 8700-DNA synthesizer and, after deprotection, were purified by PAGE in 7 M urea. From peptide G523 (see Fig. 1), the 21-base oligonucleotide sequence corresponding to amino-acids 2 through 8 and called A in the sense orientation is 5′-ACN-CAR-GGN-CCN-GAR-CAR-CAR-CA-3′, in which N = A, C, G, or T and R = A or G. From peptide G522 an oligonucleotide C′ in the antisense orientation corresponding to amino-acids 2 through 8 is 5′-NGT-YTC-NGG-NGT-YTC-NAR-YTG-3′, in which N and R are as before and Y = T or C. Also from peptide G522, a second oligonucleotide B′ in the antisense orientation to amino acids 11 through 17 is 5′-NGG-YTT-YTC-YTC-NGT-NSW-NCC, in which Y and N are as before and S = G or C and W = A or T. Peptide G519-derived 51-base oligonucleotide sequence (D) based on human preferred codon usage is 5′-GAC-CAG-CCC-CAG-GCC-CCC-GGC-GTG-GAG-GCC-TCC-GGC-GCC-GGC-GAG-GCC-CGC-3′.

DNA Amplification. cDNA was prepared from poly(A)$^+$ RNA of UC/HeLa 2-5 cells and used as template (39). PCR-amplified specific DNA fragments were generated with the Perkin–Elmer/Cetus DNA thermal cycler according to conditions specified by the manufacturer. Briefly, 100 μl of the reaction mix contained 10 μl of 10× reaction buffer [670 mM Tris·HCl, pH 8.8/67 mM $MgCl_2$/1.7 mg of bovine serum albumin per ml/166 mM $(NH_4)_2SO_4$], 16 μl of each dNTP at 1.25 mM, 5 μl each of 20 μM primers, 10 μl of cDNA template (≈100 ng), 53.5 μl of H_2O, and 0.5 μl of *Thermus aquaticus* (*Taq*) polymerase (2.5 units per assay). Samples overlayed with 100 μl of mineral oil were subjected to 25 cycles of the following protocol: denaturation for 1 min at 95°C, annealing for 2 min at 55°C, elongation for 3 min at 72°C, and a final incubation for 7 min at 72°C. By using [^{32}P]dCTP and a mixture of oligonucleotides minus dCTP (40), high-specific-activity radiolabeled PCR probes were generated.

Southern Blot Analysis of cDNA Derived from pCDM8 Libraries and Genomic DNA from UC and UC/HeLa 2-5 Cells. cDNA inserts from the libraries were excised with *Xho* I or *Xba* I. Genomic DNA was isolated (35) and digested with different restriction enzymes, electrophoresed through 0.8% agarose in 89 mM Tris borate/2 mM EDTA, and transferred to Nytran membrane. Radiolabeled probes used are described in the figure legends. Hybridization and wash conditions were as described above for Northern blots.

DNA Sequence Analysis. The cDNA inserts from the selected pCDM8 clones were subcloned into M13mp19 (41) or pGemblue (Promega Biotec). The DNA was sequenced by the dideoxy chain-termination method using Sequenase (United States Biochemical) with ^{35}S-substituted deoxyadenosine 5′-[α-(^{35}S)thio] triphosphate (42).

RESULTS

TNFR Protein Analysis. Purified TNFR preparations contained a major polypeptide band of 68 kDa (Fig. 1*A*), which was judged to be the receptor by its high TNF-binding activity (25). From two separate purifications, 8 and 10 μg of the 68-kDa protein were subjected separately to trypsin treatment, and the released peptide fragments revealed the amino acid sequences shown in Fig. 1*B*. However, the amino acid sequence of the amino terminus was heterogeneous. The following residues were identified in the first five cycles of Edman degradation: (Ser or Val)-(Thr, Ala, or Pro)-(His, Pro, or Phe)-(Thr or Tyr)-(Ala, Pro, or Val); these residues are compatible with the sequence Val-Ala-Phe-Thr-Pro, reported to be the amino terminus of the urinary TNF-binding protein II (TNFBPII in ref. 24).

Presence of TNF-Binding Protein in the Culture Medium of Receptor-Amplified Cells. The heterogeneity in the amino-terminal sequence of the purified receptor plus the close identity of this sequence with the reported amino-terminal sequence of TNF-binding protein II (24) led us to analyze the growth medium of the cells for the presence of soluble forms of the receptor. The presence of TNF-binding protein was tested with the filter binding assay (25). Supernatants from suspension cultures of cells were slot-blotted to nitrocellulose membranes (Fig. 2*A*), incubated with ^{125}I-labeled TNF, washed, and autoradiographed (25). TNF-binding proteins were easily observed in the culture medium of UC/HeLa 2-5 cells but not of the parental UC cells. To estimate the size, TNF-binding proteins were enriched from the cultured medium by a TNF-Sepharose column, and the eluted fraction was used for a ligand blot assay (Fig. 2*B*). A major band of ≈40 kDa and a minor band of ≈30 kDa were identified.

Selection of Oligonucleotides as Primers and the Generation of Radiolabeled DNA Probes by PCR. Oligonucleotides A, B,

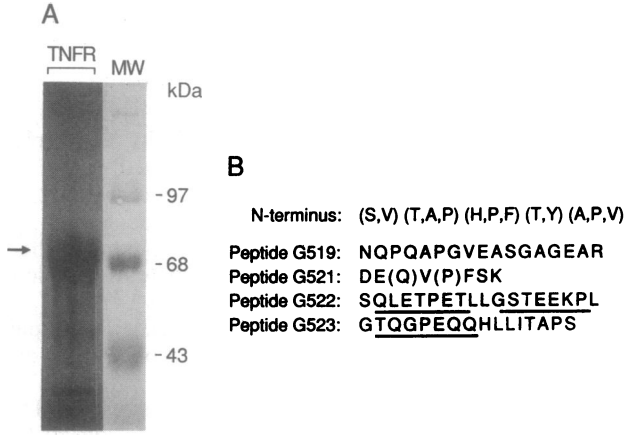

FIG. 1. (*A*) Amido-black stain of a purified preparation of TNFR from ≈2 × 10^{11} UC/HeLa 2-5 cells (25). The final protein fraction resolved by $NaDodSO_4$/PAGE in 10% acrylamide gels was electroblotted to nitrocellulose and stained with amido black. The arrow indicates the position of the TNFR polypeptide of 68 kDa. Tryptic fragments plus their amino acid sequences were obtained from this band. Lane MW shows molecular mass standards in kDa. (*B*) Amino acid sequences obtained are shown in single-letter code beginning at the amino terminus of each peptide. Underlined sequences in peptide G522 are the amino acid sequences against which oligonucleotides C and B were synthesized, respectively, while peptide G523 contains the amino acid sequence for oligonucleotide A. These oligonucleotides were used as primers in the PCR. A nucleotide sequence of 51 bases derived from peptide G519 (oligonucleotide D) was used as an oligonucleotide probe.

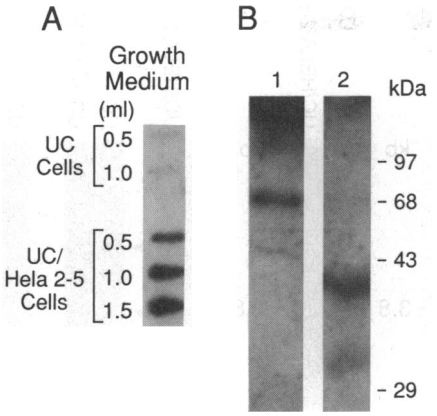

FIG. 2. (*A*) Filter binding assay to identify TNF-binding proteins in cultured media of UC and UC/HeLa 2-5 cells. Cells at a density of 1×10^6 per ml in RPMI 1640 medium containing 10% fetal bovine serum were centrifuged for 15 min at $1000 \times g$, and aliquots of the supernatant were slot-blotted to nitrocellulose membrane to immobilize TNFR. The filter was incubated with ^{125}I-labeled TNF to estimate relative amounts of TNFR as described (25). (*B*) Ligand blot analysis of purified TNFR and TNF-binding proteins shed into the growth medium. Growth medium from UC/HeLa 2-5 cells at a density of 1×10^6 cells per ml was collected and passed through a 3-ml bed volume of a TNF-Sepharose column. The column was washed, and the bound protein was eluted and subjected to ligand blot analysis for TNFR activity as described (25). Lane 1 contains the 68-kDa TNFR from UC/HeLa 2-5 cells as a control. Lane 2 contains the TNF-binding protein recovered from the growth medium of UC/HeLa 2-5 cells and shows a major band at 40 kDa.

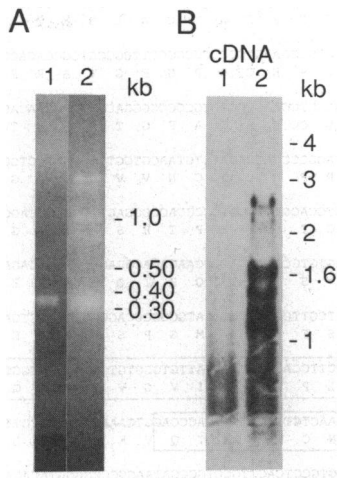

FIG. 3. (*A*) Gel electrophoresis of PCR-amplified DNA fragment. cDNA from UC/HeLa 2-5 cells was used as template with the primer pairs A in the sense orientation. 5′-ACN-CAR-GGN-CCN-GAR-CAR-CAR-CA●-3′, and C′ in the antisense orientation, 5′-NGT-YTC-NGG-NGT-YTC-NAR-YTG-3′ where N = A, C, G, or T; R = A or G; and Y = C or T. The PCR product was electrophoresed in a 1.5% agarose gel and stained with ethidium bromide. Lane 1 shows the 350-bp amplified product. Lane 2 shows the 350-bp product and other products mentioned in the text. (*B*) Southern blot analysis of pCDM8 cDNA libraries made from UC and UC/HeLa 2-5 poly(A)$^+$ RNA. DNA (10 μg) from UC cell cDNA library (lane 1) and UC/HeLa 2-5 cell cDNA library (lane 2) was digested with *Xba* I, electrophoresed, blotted, and probed with the [^{32}P]dCTP-labeled 350-bp PCR product shown in *A*, lane 1.

and C were selected as primers for PCR from the peptide sequences of TNFR. Since the amino- versus carboxyl-terminal orientation of peptides G522 and G523 to one another was not known, oligonucleotide A was used in the sense and antisense orientation with primers B′ or C′ in the opposite orientation. A PCR-amplified product of ≈350 base pairs (bp) in length was generated only when primer A was used in the sense orientation with primer B′ or C′ in the antisense orientation (Fig. 3*A*, lane 1). This productive combination of primers established that peptide G523 lies amino-terminal to peptide G522 within the TNFR protein. Upon occasion, by using the same A and C′ combination of primers, additional products of the PCR were also observed (Fig. 3*A*, lane 2). These spurious bands are possibly due to the high degeneracy (greater than 1:1000) of the oligonucleotide primer sequences. By including [^{32}P]dCTP during the PCR, a high-specific-activity radiolabeled DNA probe ($2–5 \times 10^9$ cpm/μg) was generated with primers A and C′, which was then used to analyze and screen the pCDM8 UC/HeLa 2-5 cDNA library.

Preferential Hybridization of Radiolabeled PCR Probe to pCDM8 cDNA Inserts from Receptor-Overexpressing Cells. cDNA inserts in the pCDM8 cDNA libraries of UC cells and UC/HeLa 2-5 cells, fractionated by gel electrophoresis, were hybridized to the radiolabeled PCR probe. There was significantly increased hybridization to the cDNA from receptor-amplified cells than from the parental cells, presumably reflecting increased abundance of TNFR mRNA in the UC/HeLa 2-5 cells (Fig. 3*B*). These results also indicate that cDNA clones for the receptor are well represented in this library.

TNFR cDNA Cloning. Of 5×10^5 recombinants screened from the UC/HeLa 2-5 library, a total of 30 clones were isolated by hybridization to the radiolabeled PCR probe. These clones were tested for binding to individual oligonucleotides, and 16 clones that hybridized to all four oligonucleotides (i.e., A, B, C, and the 51-mer D) were selected. Restriction analyses plus cross-hybridization of their cDNA inserts revealed overlapping sequences ranging in insert size from 1.0 to 2.9 kb.

TNFR clone 27 contained a 2.8-kb cDNA, and TNFR clone 16 contained a 2.5-kb insert; together these clones span a length of 3.3 kb. Clone 27 contains *Alu* repeats and the 3′ polyadenylylated end of the mRNA, and clone 16 extends ≈500 bases further than clone 27 towards the 5′ end of the mRNA. Clone 16 was sequenced and contained a single open reading frame encoding 344 amino acids and an in-frame termination codon, TAA. The 3′ untranslated sequence comprises ≈2.3 kb of the combined 3.3-kb length of clones 16 and 27. The deduced amino acid sequence of the open reading frame of clone 16 is presented here (Fig. 4) and contains the sequence for all four receptor peptides. Therefore, this cDNA clone obtained from the purified TNF-receptor appears to encode a partial sequence of the TNFR gene.

Size of the TNFR mRNA. Poly(A)$^+$ RNAs from parental UC cells and UC/HeLa 2-5 cells were analyzed with random hexamer-primed ^{32}P-labeled clone 16 cDNA. A single mRNA species 3.8 kb long was readily identified in UC/HeLa 2-5 RNA preparations, and a longer exposure of the blot to x-ray film revealed a mRNA of the same size in the parental unamplified cells (Fig. 5 *A* and *B*).

Analysis of Genomic DNA for the TNFR Gene. Southern blots of genomic DNA digested with several restriction enzymes showed that UC cells contained single *Kpn* I and *Sph* I restriction fragments hybridizing to the cDNA probe, whereas the *Xba* I digest revealed two bands, possibly caused by the presence of this restriction site within the parental gene. These results are compatible with the presence of a single TNFR gene in these cells. Restriction digests of UC/HeLa 2-5 DNA generate the same bands plus an additional more intense band that presumably reflects amplification of the introduced gene from the EBOpcD-HeLa cDNA library (25). Support for this interpretation comes from parallel experiments with *Eco*RI as the restriction enzyme in which radiolabeled vector sequences hybridized to the same

```
1    CGCATCTGCACCTGCAGGCCCGGCTGGTACTGCGCGCTGAGCAAGCAGGAGGGGTGCCGG   60
     R  I  C  T  C  R  P  G  W  Y  C  A  L  S  K  Q  E  G  C  R

61   CTGTGCGCGCCGCTGCCGAAGTGCCGCCCGGGCTTCGGCGTGGCCAGACCAGGAACTGAA  120
     L  C  A  P  L  P  K  C  R  P  G  F  G  V  A  R  P  G  T  E

121  ACATCAGACGTGGTGTGCAAGCCCTGTGCCCCGGGGACGTTCTCCAACACGACTTCATCC  180
     T  S  D  V  V  C  K  P  C  A  P  G  T  F  S  N  T  T  S  S

181  ACGGATATTTGCAGGCCCCACCAGATCTGTAACGTGGTGGCCATCCCTGGGAATGCAAGC  240
     T  D  I  C  R  P  H  Q  I  C  N  V  V  A  I  P  G  N  A  S

241  AGGGATGCAGTCTGCACGTCCACGTCCCCCACCCGGAGTATGGCCCCAGGGGCAGTACAC  300
     R  D  A  V  C  T  S  T  S  P  T  R  S  M  A  P  G  A  V  H

301  TTACCCCAGCCAGTGTCCACACGATCCCAACACACGCAGCCAACTCCAGAACCCAGCACT  360
     L  P  Q  P  V  S  T  R  S  Q  H  T  Q  P  T  P  E  P  S  T

361  GCTCCAAGCACCTCCTTCCTGCTCCCAATGGGCCCCAGCCCCCCAGCTGAAGGGAGCACT  420
     A  P  S  T  S  F  L  L  P  M  G  P  S  P  P  A  E  G  S  T

421  GGCGACTTCGCTCTTCCAGTTGGACTGATTGTGGGTGTGACAGCCTTGGGTCTACTAATA  480
     G  D  F  A  L  P  V  G  L  I  V  G  V  T  A  L  G  L  L  I

481  ATAGGAGTGGTGAACTGTGTCATCATGACCCAGGTGAAAAAGAAGCCCTTGTGCCTGCAG  540
     I  G  V  V  N  C  V  I  M  T  Q  V  K  K  K  P  L  C  L  Q

541  AGAGAAGCCAAGGTGCCTCACTTGCCTGCCGATAAGGCCCGGGGTACACAGGGCCCCGAG  600
     R  E  A  K  V  P  H  L  P  A  D  K  A  R  G  T  Q  G  P  E
                                                 G523

601  CAGCAGCACCTGCTGATCACAGCGCCGAGCTCCAGCAGCAGCTCCCTGGAGAGCTCGGCC  660
     Q  Q  H  L  L  I  T  A  P  S  S  S  S  S  S  L  E  S  S  A

661  AGTGCGTTGGACAGAAGGGCGCCCACTCGGAACCAGCCACAGGCACCAGGCGTGGAGGCC  720
     S  A  L  D  R  R  A  P  T  R  N  Q  P  Q  A  P  G  V  E  A
                                   G519

721  AGTGGGGCCGGGGAGGCGAGGACCAGCACCGGGAGCTCAGATTCTTCCCCTGGTGGCCAT  780
     S  G  A  G  E  A  R  T  S  T  G  S  S  D  S  S  P  G  G  H

781  GGGACCCAGGTCAATGTCACCTGCATCGTGAACGTCTGTAGCAGCTCTGACCACAGCTCA  840
     G  T  Q  V  N  V  T  C  I  V  N  V  C  S  S  S  D  H  S  S

841  CAGTGCTCCTCCCAAGCCAGCTCCACAATGGGAGACACAGATTCCAGCCCCTCGGAGTCC  900
     Q  C  S  S  Q  A  S  S  T  M  G  D  T  D  S  S  P  S  E  S

901  CCGAAGGACGAGCAGGTCCCCTTCTCCAAGGAGGAATGTGCCTTTCGGTCACAGCTGGAG  960
     P  K  D  E  Q  V  P  F  S  K  E  E  C  A  F  R  S  Q  L  E
          G521                                       G522

961  ACGCCAGAGACCCTGCTGGGGAGCACCGAAGAGAAGCCCCTGCCCCTTGGAGTGCCTGAT 1020
     T  P  E  T  L  L  G  S  T  E  E  K  P  L  P  L  G  V  P  D

1021 GCTGGGATGAAGCCCAGTTAACCAGGCCGGTGTGGGCTGTGTCGTAGCCAAGGTGGGCTG 1080
     A  G  M  K  P  S  *
```

FIG. 4. Coding sequence from the 2.5-kb *Xba* I insert of TNFR clone 16 cDNA. The predicted amino acids encoded by the DNA sequence are shown in single-letter code, and the positions of the peptide sequences derived from the protein are underlined and are consistent with PCR amplification and ordering. The sequence shown indicates the stop codon, TAA, with a star.

intense band as the cDNA probe (data not shown). We surmise that this amplified sequence confers high-level TNFR expression to these cells.

DISCUSSION

Recently, we described the purification of TNFR protein (25). Four peptide sequences—8, 16, 17, and 18 amino acids long—were derived from tryptic digests of this protein (Fig. 1). This report describes the cloning of a TNFR cDNA based on these partial protein sequences. The cDNAs were isolated with PCR probes prepared with oligonucleotide primers corresponding to the amino acid sequence of two tryptic peptides. Of the 16 positive clones isolated, further analysis by restriction mapping and DNA sequencing of two cDNAs, clones 16 and 27 provided a total of 3.3 kb of DNA sequence. This sequence contains the 3′ end of the mRNA as evidenced by the presence of a poly(A) tail and also contains *Alu* repeated sequences (data not shown). A single open reading frame (encompassing 344 amino acids) followed by an in-frame termination codon is presented in this report. The deduced amino acid sequence from the open reading frame (Fig. 4) contains the sequence of all four receptor peptides derived from the purified protein. Therefore, this sequence was verified as a partial cDNA sequence encoding the TNFR. A hydropathy plot (43) of the coding sequence reveals a major hydrophobic domain of 30 amino acids (residues 141–170), presumably the transmembrane sequence, that separates a

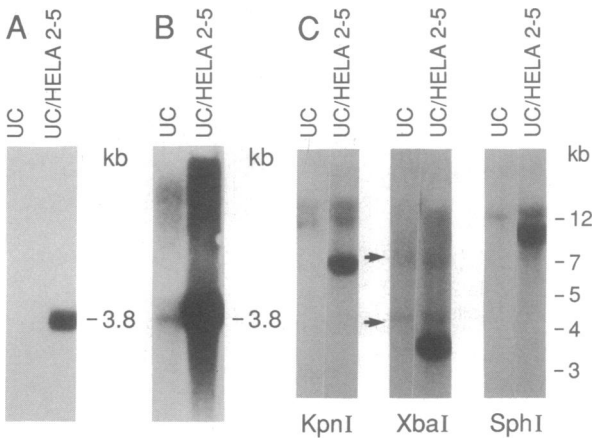

FIG. 5. Analyses of mRNA and genomic DNA encoding the TNFR. Northern blot analysis of poly(A)$^+$ RNA (10 μg per lane) from parental UC cells and UC/HeLa 2-5 cells was conducted as described with [^{32}P]dCTP-labeled TNFR clone 16 cDNA as a probe. Autoradiograph exposure times were 1 hr (*A*) and 16 hr (*B*). (*C*) Genomic Southern blot analysis. DNA (10 μg) was digested separately with restriction endonucleases (*Kpn* I, *Xba* I, and *Sph* I), electrophoresed, blotted, and probed with a [^{32}P]dCTP-labeled 270-bp fragment from the 5′ end clone 16 cDNA. Arrows indicate the positions of the hybridizing bands.

174-amino acid intracellular carboxyl-terminal signaling domain from an extracellular amino-terminal ligand-binding domain. Northern blot analysis showed an ≈3.8-kb mRNA present in UC/HeLa 2-5 and the parental UC cells. This observation implies that the cDNA sequence we have obtained is ≈500 bp short (at the 5′ end) of a full-length clone. Not accounting for the lengths of the 5′ untranslated and leader sequence, the receptor contains a maximum of 170 amino acids in addition to the 344 residues represented in clone 16. The mature TNFR would then be no more than 514 amino acids with a molecular mass of 57 kDa. This is a smaller size than the 68-kDa estimate obtained by PAGE, but the apparent discrepancy is most likely due to glycosylation of the protein.

The successful use of primers A and C in generating a PCR fragment allowed certain predictions about the TNFR protein sequence: (*i*) that peptide G523 is located amino-terminally with respect to peptide G522, and (*ii*) that ≈350 bp lie between their respective coding sequence. These predictions held true, and 387 nucleotides were found to cover the distance from A to C (Fig. 4). Generation of radiolabeled probes with PCR (39), as used here, has provided distinct advantages over screening libraries with oligonucleotides. The increase in the length of the probe by the added amplified DNA fragment and the generation of high-specific-activity DNA (2–5 × 10^9 cpm/μg) by incorporation of radioisotope during the amplification reaction have allowed stringent hybridization and wash conditions to be performed that eliminate high backgrounds and false positives as seen with the unstable hybrids made by short oligonucleotides.

Amino-terminal sequence analysis revealed heterogeneities, since several amino acids were detectable in each cycle. The result is compatible with the sequence Val-Ala-Phe-Thr-Pro found in the recently published (24) urinary TNF-binding protein II, which was also reported to be variable at the amino terminus. These comparisons implied a possible relatedness of the TNFR with TNF-binding protein II and the likelihood that the soluble protein was derived from the receptor. Upon analysis of the growth medium from TNFR-overexpressing cells, TNF-binding activity was readily observed, and after ligand blot analysis, a major and minor form of ≈40 kDa and 30 kDa, respectively, were seen. The size of the predominant form is larger than the 30-kDa estimate reported for the

urinary protein and suggests that further proteolysis of the 40-kDa form may occur during its passage through the serum or urine *in vivo* to generate the smaller molecule. If the extracellular domain of the TNFR comprised a maximum of 310 amino acids (≈34 kDa), then the soluble 40-kDa form either could be a product of a different mRNA or could be glycosylated, with the linked carbohydrates accounting for the difference in size. A comparative sequence analysis of the two proteins should provide further information regarding their structural relationships.

Soluble forms of cell-associated receptors are generated both by differential splicing of the receptor mRNA (44–46) and by the action of proteases on extracellular domains of the receptor (47, 48). The mechanism(s) for the production of the shed form of TNFR is presently not known; however, the degree of overexpression of mRNA and the abundant production of the TNFR by these cells should facilitate analysis of this phenomenon. The physiological significance of soluble receptors can at best be only surmised. They may provide a protective mechanism against systemic activity of these potent cytokines or a mechanism for their elimination or both. Possibly the low abundance of cytokine receptors may be explained in part by their shedding from cell surfaces (49).

UC/HeLa 2-5 cells were developed by transfection of HeLa cDNA libraries into UC cells (25). We have assumed that increased receptor production is associated with an introduced copy of the TNFR coding sequence. Results from genomic Southern blots presented here show that while the parental gene in UC cells is unaltered, a second very intense band of TNFR is seen only in UC/HeLa 2-5 cells. This may represent the introduced cDNA that upon amplification confers high-level TNFR expression.

After this paper was submitted for publication, two reports on the cloning of a 50- to 55-kDa TNFR were published (50, 51), the sequence of which is different from the 68-kDa TNFR reported here. In addition to the difference in size of the mature proteins and the mRNA transcripts encoding them, the calculated identity based on protein sequences and the BestFit alignment of Smith and Waterman (52) is 21.7%. Therefore, these proteins appear to represent two unique TNFRs that give rise to the two reported (24) urinary TNF-binding proteins.

We are grateful to José Freire-Moar for his advice with the PCR technique and many helpful discussions, to Chinh Bach for synthesis of oligonucleotides used in this work, to Cetus Corporation for providing recombinant TNF, to Pat Lowry for growing UC cells, to Richard Hyman and David Webb for critical reading of the manuscript, and to Linda Miencier for preparing the manuscript.

1. Beutler, B. & Cerami, A. (1988) *Annu. Rev. Biochem.* **57**, 505–518.
2. Carswell, E. A., Old, L. J., Kassel, R. L., Green, S., Fiore, N. & Williamson, B. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3666–3670.
3. Old, L. J. (1987) *Nature (London)* **326**, 330–331.
4. Kawakami, M., Pekala, P. H., Lane, M. D. & Cerami, A. (1972) *Proc. Natl. Acad. Sci. USA* **79**, 12–16.
5. Pekala, P. H., Kawakami, M., Angus, C. W., Lane, M. D. & Cerami, A. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 2743–2747.
6. Torti, F. M., Diekmann, M., Beutler, B., Cerami, A. & Ringold, G. M. (1985) *Science* **229**, 867–869.
7. Sherry, B. & Cerami, A. (1988) *J. Cell Biol.* **107**, 1269–1277.
8. Dayer, J. M., Beutler, B. & Cerami, A. (1985) *J. Exp. Med.* **162**, 2163–2168.
9. Saklatvala, J. (1986) *Nature (London)* **322**, 547–549.
10. Bertolini, D. R., Nedwin, G. E., Bringman, T. S., Smith, D. D. & Mundy, G. R. (1986) *Nature (London)* **319**, 516–518.
11. Beutler, B., Milsark, I. W. & Cerami, A. (1985) *Science* **229**, 860–871.
12. Kohase, M., Henriksen-Distefano, D., May, L. T., Vilcek, J. & Sehgal, P. K. (1986) *Cell* **45**, 659–666.
13. Shalaby, M. R., Palladino, M. A., Hirabayashi, S. E., Eessalu, T., Lewis, G. D., Shepard, H. M. & Aggarwal, B. B. (1987) *J. Leukocyte Biol.* **41**, 196–204.
14. Kehrl, J. H., Alvarez-Mon, M., Delsing, G. A. & Fauci, A. S. (1987) *Science* **238**, 1144–1146.
15. Kull, F. C., Jr., Jacobs, S. & Cuatrecasas, P. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 5756–5760.
16. Baglioni, C., McCandless, S., Tavernier, J. & Fiers, W. (1985) *J. Biol. Chem.* **260**, 13395–13397.
17. Yoshie, O., Tada, K. & Ishida, N. (1986) *J. Biochem. (Tokyo)* **100**, 531–541.
18. Stauber, G. B., Aiyer, R. A. & Aggarwal, B. B. (1988) *J. Biol. Chem.* **263**, 19098–19104.
19. Kull, F. C., Jr., & Cuatrecasas, P. (1981) *Cancer Res.* **41**, 4885–4890.
20. Creasey, A. A., Yamamoto, R. & Vitt, C. R. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3293–3297.
21. Hohmann, H. P., Remy, R., Brockhaus, M. & van Loon, A. P. G. M. (1989) *J. Biol. Chem.* **264**, 14927–14934.
22. Seckinger, P., Isaaz, S. & Dayer, J. M. (1989) *J. Biol. Chem.* **264**, 11966–11973.
23. Engelmann, H., Aderka, D., Rubinstein, M., Rotman, D. & Wallach, D. (1989) *J. Biol. Chem.* **264**, 11974–11980.
24. Engelmann, H., Novick, D. & Wallach, D. (1990) *J. Biol. Chem.* **265**, 1531–1536.
25. Heller, R. A., Song, K., Villaret, D., Margolskee, R., Dunne, J., Hayakawa, H. & Ringold, G. M. (1990) *J. Biol. Chem.* **265**, 5708–5717.
26. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
27. Burnetts, W. N. (1981) *Anal. Biochem.* **112**, 195–203.
28. Aebersold, R. H., Levitt, J., Saavedra, R. A., Hood, L. E. & Kent, S. B. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 6970–6974.
29. Fischer, W. H., Gonzalez, G., Behan, D., Potter, E., Montminy, M. & Vale, W. (1990) *J. Protein Chem.*, in press.
30. Matsudaira, P. (1987) *J. Biol. Chem.* **262**, 10035–10038.
31. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18**, 5294–5299.
32. Aviv, H. & Leder, P. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 1408–1412.
33. McMaster, G. K. & Carmichael, G. G. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 4835–4838.
34. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13.
35. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
36. Seed, B. & Aruffo, A. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 8573–8577.
37. Gubler, U. & Hoffman, B. J. (1983) *Gene* **25**, 263–269.
38. Wood, W. I., Gitschier, J., Laskey, L. A. & Lawn, R. M. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 1585–1588.
39. Frohman, M. A., Dush, M. K. & Martin, G. R. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8998–9002.
40. Schowalter, D. B. & Somner, S. S. (1989) *Anal. Biochem.* **177**, 90–94.
41. Vieira, J. & Messing, J. (1982) *Gene* **19**, 259–268.
42. Sanger, F., Nicklen S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
43. Kyte, J. & Doolittle, R. F. (1982) *J. Mol. Biol.* **157**, 105–132.
44. Mosley, B., Beckmann, M. P., March, C. J., Idzerda, R. L., Gimpel, S. D., VandenBos, T., Friend, D., Alpert, A., Anderson, D., Jackson, J., Wignall, J. M., Smith, C., Gallis, B., Sims, J. E., Urdal, D., Widmer, M. B., Cosman, D. & Park, L. S. (1989) *Cell* **59**, 335–348.
45. Loosfelt, H., Misrahi, M., Atger, M., Salesse, R., Thi, M., Jolivet, A., Gocochon-Mantel, A., Sar, S., Jallal, B., Garnier, J. & Milgnom, E. (1989) *Science* **245**, 525–528.
46. Goodwin, R. G., Friend, D., Ziegler, S., Jerzy, R., Falk, B. A., Gimpel, S., Cosman, D., Dower, S. K., March, C. J., Namen, A. E. & Park, L. S. (1990) *Cell* **60**, 941–951.
47. Leung, D. W., Spencer, S. A., Cachiones, G., Hammonds, G., Collins, C., Henzel, W. J., Barnard, R., Waters, M. J. & Wood, W. I. (1987) *Nature (London)* **330**, 537–543.
48. Robb, R. J. & Kutny, R. M. (1987) *J. Immunol.* **139**, 855–862.
49. Novick, D., Engelmann, H., Wallach, D. & Rubinstein, M. (1989) *J. Exp. Med.* **170**, 1409–1414.
50. Loetscher, H., Pan, Y.-C. E., Lahm, H.-W., Gentz, R., Brockhaus, M., Tabuchi, H. & Lesslauer, W. (1990) *Cell* **61**, 351–359.
51. Schall, T. J., Lewis, M., Koller, K. J., Lee, A., Rice, G. C., Wong, G. H. W., Gatanaga, T., Granger, G. A., Lentz, R., Raab, H., Kohr, W. J. & Goeddel, D. V. (1990) *Cell* **61**, 361–370.
52. Devereux, J., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395.