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(57) Abstract

The invention provides methods and compositions relating to a novel tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide, which transcriptionally activates Nuclear Factor κB. The invention provides isolated TRAF6 hybridization probes and primers capable of hybridizing with the disclosed TRAF6 gene, nucleic acids encoding the subject TRAF6 polypeptides, methods of making the subject TRAF6 polypeptides, and methods of using the subject compositions in diagnosis and drug screening.

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NOVEL PROTEIN - TRAF6

INTRODUCTION

Field of the Invention

The field of this invention is a class of human proteins involved in transcription and immuno-regulation.

Background

Nuclear factor κB (NF-κB) is a homo- or heterodimer of members of the Rel family of transcriptional activators that is involved in the inducible expression of a wide variety of important cellular genes including numerous cytokines, cytokine receptors, major histocompatibility antigens, serum amyloid A protein, etc. as well as many viral genes including genes of HIV, SV40, cytomegalovirus, etc.

Several tumor necrosis factor receptor-associated factor (TRAF) proteins have been identified and shown to be involved in the signaling of various cellular responses including cytotoxicity, anti-viral activity, immuno-regulatory activities and the transcriptional regulation of a number of genes.

Accordingly, the ability to exogenously modulate the activity of NF-κB and/or TRAF proteins would yield therapeutic application for numerous clinical indications. In addition, components of such pathways would provide valuable target reagents for automated, cost-effective, high throughput drug screening assays and hence would have immediate application in domestic and international pharmaceutical and biotechnology drug development programs. The present invention provides novel TRAF proteins which regulate NF-κB expression, their use in drug screens, and nucleic acids encoding the same.

Relevant Literature

Rothe *et al.* (1994) Cell 78, 681-692 report that NF-κB expression can be mediated by the 75-80 kDa TNF receptor (TNF-R2), that a short region of the 78 amino acids at the C-terminus of the cytoplasmic domain of TNF-R2 is required for signaling NF-κB activation, and that this region binds to closely related putative effectors, TRAF1 and TRAF2, see also, Hsu *et al.* (1995) Cell 81, 495 and Rothe *et al.*, pending US patent application Serial No: 08/446,915. A third distinct TRAF, TRAF3, has been reported by Huet al. (1994) J. Biol Chem 269, 30069; Cheng *et al.* (1995) Science 267, 1494-1498; Mosialos *et al.* (1995) Cell

80, 389; and Sato *et al.* (1995) FEBS Letters, 358, 113. Rothe *et al.* (1995) Science 269, 1424-1427 report TRAF2- (but not TRAF1- or 3-) mediated activation of NF-κB by TNF-R2 and CD40.

SUMMARY OF THE INVENTION

The invention provides methods and compositions relating to a novel tumor necrosis factor receptor associated factor number six (TRAF6) protein and gene. The subject TRAF6 proteins are encoded by cDNAs which hybridizes with SEQ ID NO:01 under high stringency conditions; specifically bind a natural intracellular TRAF6 binding target and comprise the amino sequence of SEQ ID NO:2 or fragment thereof sufficient to specifically bind a natural intracellular TRAF6 binding target.

The invention also provides isolated TRAF6 hybridization probes and primers capable of hybridizing with the disclosed TRAF6 cDNA, nucleic acids encoding the subject TRAF6 proteins, methods of making the subject TRAF6 proteins, and methods of using the subject compositions in diagnosis (e.g. genetic hybridization screens for TRAF6 gene mutations), and in the biopharmaceutical industry (e.g. reagents for screening chemical libraries for lead compounds for a pharmacological agent useful in the diagnosis or treatment of disease associated with immune regulation).

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the effect of coexpression of a TRAF6 deletion mutant on NF-κB-dependent reporter gene activation by TRAF6.

Figure 2 shows NF-κB-dependent reporter gene activation by TRAF proteins.

DETAILED DESCRIPTION OF THE INVENTION

The nucleotide sequence of a natural cDNA encoding human TRAF6 is shown as SEQ ID NO:1 and the full conceptual translate shown as SEQ ID NO:2. The TRAF6 proteins of the invention include incomplete translates of SEQ ID NO:1 and deletion mutants of SEQ ID NO: 2, which translates and deletions mutants have TRAF6-specific activity.

The nucleotide sequence of a natural cDNA encoding a human TRAF6 polypeptide is shown as SEQ ID NO:1, and the full conceptual translate is shown as SEQ ID NO:2. The TRAF6 polypeptides of the invention include incomplete translates of SEQ ID NO:1 and

deletion mutants of SEQ ID NO:2, which translates and deletion mutants have TRAF6-specific amino acid sequence and binding specificity or function. Hence, the TRAF6 polypeptides of the invention include polypeptides comprising SEQ ID NO:2 and TRAF6 domains thereof. Such TRAF6 domains have at least 10, preferably at least about 12, more preferably at least about 14 consecutive residues of SEQ ID NO:2 and provide TRAF6 specific activity or function, such as specifically binding TRAF6, TRAF2 and/or TRAF3 distinguishably from TRAF1-5.

TRAF6-specific activity or function may be determined by convenient *in vitro*, cell-based, or *in vivo* assays: e.g. *in vitro* binding assays, cell culture assays, in animals (e.g. gene therapy, transgenics, etc.), etc. Binding assays encompass any assay where the molecular interaction of a TRAF6 polypeptide with a binding target is evaluated. The binding target may be a natural intracellular binding target such as TRAF6 (dimerization) or other TRAF6 regulator protein that directly modulates TRAF6 activity or its localization; or non-natural binding target such a specific immune protein such as an antibody, or a TRAF6 specific agent such as those identified in screening assays such as described below. TRAF6-binding specificity may assayed by binding equilibrium constants (usually at least about $10^7 M^{-1}$, preferably at least about $10^8 M^{-1}$, more preferably at least about $10^9 M^{-1}$), by the ability of the subject polypeptide to function as negative mutants in TRAF6-expressing cells, to elicit TRAF6 specific antibody in a heterologous host (e.g a rodent or rabbit), etc. In any event, the TRAF6 binding specificity of the subject TRAF6 polypeptides necessarily distinguishes TRAF1-5.

The claimed TRAF6 polypeptides are isolated or pure: an "isolated" polypeptide is unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, and more preferably at least about 5% by weight of the total polypeptide in a given sample and a pure polypeptide constitutes at least about 90%, and preferably at least about 99% by weight of the total polypeptide in a given sample. The TRAF6 polypeptides and polypeptide domains may be synthesized, produced by recombinant technology, or purified from mammalian, preferably human cells. A wide variety of molecular and biochemical methods are available for biochemical synthesis, molecular expression and purification of the subject compositions, see e.g. Molecular Cloning, A Laboratory Manual (Sambrook, *et al.* Cold Spring Harbor Laboratory), Current

Protocols in Molecular Biology (Eds. Ausubel, *et al.*, Greene Publ. Assoc., Wiley-Interscience, NY) or that are otherwise known in the art.

The invention provides natural and non-natural TRAF6-specific binding agents, methods of identifying and making such agents, and their use in diagnosis, therapy and pharmaceutical development. For example, TRAF6-specific agents are useful in a variety of diagnostic and therapeutic applications, especially where disease or disease prognosis is associated with improper utilization of a pathway involving TRAF6, e.g. NF-κB activation. Novel TRAF6-specific binding agents include TRAF6-specific receptors, such as somatically recombined polypeptide receptors like specific antibodies or T-cell antigen receptors (see, e.g Harlow and Lane (1988) Antibodies, A Laboratory Manual, Cold Spring Harbor Laboratory) and other natural intracellular binding agents identified with assays such as one-, two- and three-hybrid screens, non-natural intracellular binding agents identified in screens of chemical libraries such as described below, etc. For diagnostic uses, the binding agents are frequently labeled, such as with fluorescent, radioactive, chemiluminescent, or other easily detectable molecules, either conjugated directly to the binding agent or conjugated to a probe specific for the binding agent. Agents of particular interest modulate TRAF6 function, e.g. TRAF6-dependent signal transduction; for example, isolated cells, whole tissues, or individuals may be treated with a TRAF6 binding agent to activate, inhibit, or alter TRAF6-dependent intracellular signalling processes.

The amino acid sequences of the disclosed TRAF6 polypeptides are used to back-translate TRAF6 polypeptide-encoding nucleic acids optimized for selected expression systems (Holler et al. (1993) Gene 136, 323-328; Martin et al. (1995) Gene 154, 150-166) or used to generate degenerate oligonucleotide primers and probes for use in the isolation of natural TRAF6-encoding nucleic acid sequences ("GCG" software, Genetics Computer Group, Inc, Madison WI). TRAF6-encoding nucleic acids used in TRAF6-expression vectors and incorporated into recombinant host cells, e.g. for expression and screening, transgenic animals, e.g. for functional studies such as the efficacy of candidate drugs for disease associated with TRAF6-modulated signal transduction, etc.

The invention also provides nucleic acid hybridization probes and replication / amplification primers having a TRAF6 cDNA specific sequence contained in SEQ ID NO:1 and sufficient to effect specific hybridization thereto (i.e. specifically hybridize with SEQ ID NO:1 in the presence of human umbilical vein endothelial cell cDNA, preferably in the

presence of TRAF1-5 cDNA). Such primers or probes are at least 12, preferably at least 24, more preferably at least 36 and most preferably at least 96 bases in length. Demonstrating specific hybridization generally requires stringent conditions, for example, hybridizing in a buffer comprising 30% formamide in 5 x SSPE (0.18 M NaCl, 0.01 M $NaPO_4$, pH7.7, 0.001 M EDTA) buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2 x SSPE; preferably hybridizing in a buffer comprising 50% formamide in 5 x SSPE buffer at a temperature of 42°C and remaining bound when subject to washing at 42°C with 0.2 x SSPE buffer at 42°C. TRAF6 cDNA homologs can also be distinguished from other polypeptide using alignment algorithms, such as BLASTX (Altschul *et al.* (1990) Basic Local Alignment Search Tool, J Mol Biol 215, 403-410).

The subject nucleic acids are of synthetic/non-natural sequences and/or are isolated, i.e. unaccompanied by at least some of the material with which it is associated in its natural state, preferably constituting at least about 0.5%, preferably at least about 5% by weight of total nucleic acid present in a given fraction, and usually recombinant, meaning they comprise a non-natural sequence or a natural sequence joined to nucleotide(s) other than that which it is joined to on a natural chromosome. Nucleic acids comprising the nucleotide sequence of SEQ ID NO:1 or fragments thereof, contain such sequence or fragment at a terminus, immediately flanked by a sequence other than that which it is joined to on a natural chromosome, or flanked by a native flanking region fewer than 10 kb, preferably fewer than 2 kb, which is at a terminus or is immediately flanked by a sequence other than that which it is joined to on a natural chromosome. While the nucleic acids are usually RNA or DNA, it is often advantageous to use nucleic acids comprising other bases or nucleotide analogs to provide modified stability, etc.

The subject nucleic acids find a wide variety of applications including use as translatable transcripts, hybridization probes, PCR primers, diagnostic nucleic acids, etc.; use in detecting the presence of TRAF6 genes and gene transcripts and in detecting or amplifying nucleic acids encoding additional TRAF6 homologs and structural analogs. In diagnosis, TRAF6 hybridization probes find use in identifying wild-type and mutant TRAF6 alleles in clinical and laboratory samples. Mutant alleles are used to generate allele-specific oligonucleotide (ASO) probes for high-throughput clinical diagnoses. In therapy, therapeutic TRAF6 nucleic acids are used to modulate cellular expression or intracellular concentration or availability of active TRAF6.

The invention provides efficient methods of identifying agents, compounds or lead compounds for agents active at the level of a TRAF6 modulatable cellular function. Generally, these screening methods involve assaying for compounds which modulate TRAF6 interaction with a natural TRAF6 binding target. A wide variety of assays for binding agents are provided including labeled *in vitro* protein-protein binding assays, immunoassays, cell based assays, etc. The methods are amenable to automated, cost-effective high throughput screening of chemical libraries for lead compounds. Identified reagents find use in the pharmaceutical industries for animal and human trials; for example, the reagents may be derivatized and rescreened in *in vitro* and *in vivo* assays to optimize activity and minimize toxicity for pharmaceutical development. Target indications may include infection, genetic disease, cell growth and regulatory disfunction, such as neoplasia, inflammation, hypersensitivity, etc.

In vitro binding assays employ a mixture of components including a TRAF6 polypeptide, which may be part of a fusion product with another peptide or polypeptide, e.g. a tag for detection or anchoring, etc. The assay mixtures comprise a natural intracellular TRAF6 binding target, such as a TRAF2, TRAF3 or TRAF6 polypeptide. While native binding targets may be used, it is frequently preferred to use portions (e.g. peptides) thereof so long as the portion provides binding affinity and avidity to the subject TRAF6 polypeptide conveniently measurable in the assay. The assay mixture also comprises a candidate pharmacological agent. Candidate agents encompass numerous chemical classes, though typically they are organic compounds; preferably small organic compounds and are obtained from a wide variety of sources including libraries of synthetic or natural compounds. A variety of other reagents may also be included in the mixture. These include reagents like salts, buffers, neutral proteins, e.g. albumin, detergents, protease inhibitors, nuclease inhibitors, antimicrobial agents, etc. may be used.

The resultant mixture is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the TRAF6 polypeptide specifically binds the cellular binding target, portion or analog with a reference binding affinity. The mixture components can be added in any order that provides for the requisite bindings and incubations may be performed at any temperature which facilitates optimal binding. Incubation periods are likewise selected for optimal binding but also minimized to facilitate rapid, high-throughput screening.

After incubation, the agent-biased binding between the TRAF6 polypeptide and one or more binding targets is detected by any convenient way. For cell-free binding type assays, a separation step is often used to separate bound from unbound components. Separation may be effected by precipitation (e.g. TCA precipitation, immunoprecipitation, etc.), immobilization (e.g on a solid substrate), etc., followed by washing by, for examples, membrane filtration, gel chromatography (e.g. gel filtration, affinity, etc.).

Detection may be effected in any convenient way. For cell-free binding assays, one of the components usually comprises or is coupled to a label. The label may provide for direct detection as radioactivity, luminescence, optical or electron density, etc. or indirect detection such as an epitope tag, an enzyme, etc. A variety of methods may be used to detect the label depending on the nature of the label and other assay components, e.g. through optical or electron density, radiative emissions, nonradiative energy transfers, etc. or indirectly detected with antibody conjugates, etc.

A difference in the binding affinity of the TRAF6 polypeptide to the target in the absence of the agent as compared with the binding affinity in the presence of the agent indicates that the agent modulates the binding of the TRAF6 polypeptide to the TRAF6 binding target. Analogously, in the cell-based assay also described below, a difference in the TRAF6-dependent chromatin degredation or instability in the presence and absence of an agent indicates the agent modulates TRAF6 function. A difference, as used herein, is statistically significant and preferably represents at least a 50%, more preferably at least a 90% difference.

The following experiments and examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

The human TRAF6 cDNA of SEQ ID NO:1 was cloned from human spleen cell and human umbilical vein endothelial cell cDNA libraries by high stringency hybridization: hybridization in 40% formamide, 5% Dextran sulfate, 0.5% SDS, 5x SSPE at 42°C followed by washes in 2x SSPE, 0.1% SDS at 25°C and in 0.1x SSPE, 0.1% SDS at 42°C; using TRAF oligonucleotide probes.

The resultant cDNA (SEQ ID NO:1) encodes human TRAF6 (SEQ ID NO:2). In cotransfection experiments, TRAF6 was shown to activate an NF-κB-dependent reporter gene, see Fig 1.

A yeast two-hybrid system was used to identify TRAF2, TRAF3 and TRAF6 as an intracellular binding targets of the TRAF6 protein of SEQ ID NO: 2. A transfection based co-immunoprecipitation assay was also used to identify intracellular binding targets of the TRAF6 protein of SEQ ID NO: 2. Briefly, FLAG-tagged TRAF1, 2, 3, 4 and 6 were cotransfected with HA-tagged TRAF6. Lysates were immunoprecipitated with anti-HA antibody and proteinA glass beads. Western blot analysis using an anti-FLAG antibody revealed TRAF6-TRAF6 and TRAF6-TRAF3 complexes.

Deletion mutagenesis of TRAF6 indicate that residues 115-522 are sufficient to mediate activation of NF-κB, see Fig 2. In contrast, continuing the 5' deletion to the second Zn finger domain abolished activity; as did 5' deletions through all five Zn finger domains, and deletions through the C domain. Similarly, an internal delection of the Zn finger domains abolished activity.

EXAMPLES

1. Protocol for High-Throughput Human TRAF6 - TRAF6 Binding Assay.

A. Reagents:

- Neutralite Avidin: 20 µg/ml in PBS.

- Blocking buffer: 5% BSA, 0.5% Tween 20 in PBS; 1 hour at room temperature.

- Assay Buffer: 100 mM KCl, 20 mM HEPES pH 7.6, 0.25 mM EDTA, 1% glycerol, 0.5% NP-40, 50 mM β-mercaptoethanol, 1 mg/ml BSA, cocktail of protease inhibitors.

- ^{33}P human TRAF6 (SEQUENCE ID NO:2, residues 1-522) 10x stock: 10^{-8} - 10^{-6} M unlabeled human TRAF6 supplemented with 200,000-250,000 cpm of labeled human TRAF6 (Beckman counter). Place in the 4°C microfridge during screening.

- Protease inhibitor cocktail (1000X): 10 mg Trypsin Inhibitor (BMB # 109894), 10 mg Aprotinin (BMB # 236624), 25 mg Benzamidine (Sigma # B-6506), 25 mg Leupeptin (BMB # 1017128), 10 mg APMSF (BMB # 917575), and 2mM $NaVo_3$ (Sigma # S-6508) in 10 ml of PBS.

- ΔTRAF6: 10^{-8} - 10^{-5} M biotinylated truncated ΔTRAF6 (SEQUENCE ID NO:2, residues 115-522) in PBS.

B. Preparation of assay plates:

- Coat with 120 µl of stock N-Avidin per well overnight at 4°C.
- Wash 2 times with 200 µl PBS.
- Block with 150 µl of blocking buffer.
- Wash 2 times with 200 µl PBS.

C. Assay:

- Add 40 µl assay buffer/well.
- Add 10 µl compound or extract.
- Add 10 µl ^{33}P-human TRAF6 (20,000-25,000 cpm/0.1-10 pmoles/well =10^{-9}- 10^{-7} M final concentration).
- Shake at 25°C for 15 minutes.
- Incubate additional 45 minutes at 25°C.
- Add 40 µl biotinylated truncated ΔTRAF6 (0.1-10 pmoles/40 ul in assay buffer)
- Incubate 1 hour at room temperature.
- Stop the reaction by washing 4 times with 200 µl PBS.
- Add 150 µl scintillation cocktail.
- Count in Topcount.

D. Controls for all assays (located on each plate):

a. Non-specific binding

b. Soluble (non-biotinylated truncated ΔTRAF6) at 80% inhibition.

2. Protocol for NF-κB-Dependent Reporter Gene Assay

- 293 cells are transiently co-transfected with an E-selectin-luciferase reporter gene plasmid (Schindler *et al.* (1994) Mol Cell Biol 14, 5820) and TRAF6 expression vectors containing the TRAF6 coding region (see, SEQUENCE ID NO:1) produced as described for TRAF1, 2 and 3 in Rothe *et al.* (1995) Science 269, 1424.

-Control cells are transiently co-transfected with a CMV promoter luciferase reporter gene plasmid and/or TRAF2 expression vectors as described *supra*.

- The transfected cells are incubated 24 hours in the presence of the candidate compound or extract and then the cells harvested and luciferase activities determined and normalized on the basis of β-galactosidase expression, as described in Fig 3B of Rothe *et al.* (1995) Science 269, 1424.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent application were specifically and individually indicated to be incorporated by reference. Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: TULARIK, INC.

(ii) TITLE OF INVENTION: Novel Protein - TRAF6

(iii) NUMBER OF SEQUENCES: 2

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(v) COMPUTER READABLE FORM:
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(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
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(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:
(I) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 2248 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 230..1795

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGCACGAGCC GCAGCTGGGG CTTGGCCTGC GGGCGGCCAG CGAAGGTGGC GAAGGCTCCC 60

ACTGGATCCA GAGTTTGCCG TCCAAGCAGC CTCGTCTCGG CGCGCAGTGT CTGTGTCCGT 120

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CCTCTACCAG CGCCTTGGCT GAGCGGAGTC GTGCGGTTGG TGGGGGAGCC CTGCCCTCCT      180

GGTTCGGCCT CCCCGCGCAC TAGAACGAGC AAGTGATAAT CAAGTTACT ATG AGT          235
                                                        Met Ser
                                                          1

CTG CTA AAC TGT GAA AAC AGC TGT GGA TCC AGC CAG TCT GAA AGT GAC         283
Leu Leu Asn Cys Glu Asn Ser Cys Gly Ser Ser Gln Ser Glu Ser Asp
          5                  10                  15

TGC TGT GTG GCC ATG GCC AGC TCC TGT AGC GCT GTA ACA AAA GAT GAT         331
Cys Cys Val Ala Met Ala Ser Ser Cys Ser Ala Val Thr Lys Asp Asp
     20                  25                  30

AGT GTG GGT GGA ACT GCC AGC ACG GGG AAC CTC TCC AGC TCA TTT ATG         379
Ser Val Gly Gly Thr Ala Ser Thr Gly Asn Leu Ser Ser Ser Phe Met
 35                  40                  45                  50

GAG GAG ATC CAG GGA TAT GAT GTA GAG TTT GAC CCA CCC CTG GAA AGC         427
Glu Glu Ile Gln Gly Tyr Asp Val Glu Phe Asp Pro Pro Leu Glu Ser
                55                  60                  65

AAG TAT GAA TGC CCC ATC TGC TTG ATG GCA TTA CGA GAA GCA GTG CAA         475
Lys Tyr Glu Cys Pro Ile Cys Leu Met Ala Leu Arg Glu Ala Val Gln
            70                  75                  80

ACG CCA TGC GGC CAT AGG TTC TGC AAA GCC TGC ATC ATA AAA TCA ATA         523
Thr Pro Cys Gly His Arg Phe Cys Lys Ala Cys Ile Ile Lys Ser Ile
          85                  90                  95

AGG GAT GCA GGT CAC AAA TGT CCA GTT GAC AAT GAA ATA CTG CTG GAA         571
Arg Asp Ala Gly His Lys Cys Pro Val Asp Asn Glu Ile Leu Leu Glu
     100                 105                 110

AAT CAA CTA TTT CCA GAC AAT TTT GCA AAA CGT GAG ATT CTT TCT CTG         619
Asn Gln Leu Phe Pro Asp Asn Phe Ala Lys Arg Glu Ile Leu Ser Leu
115                 120                 125                 130

ATG GTG AAA TGT CCA AAT GAA GGT TGT TTG CAC AAG ATG GAA CTG AGA         667
Met Val Lys Cys Pro Asn Glu Gly Cys Leu His Lys Met Glu Leu Arg
                135                 140                 145

CAT CTT GAG GAT CAT CAA GCA CAT TGT GAG TTT GCT CTT ATG GAT TGT         715
His Leu Glu Asp His Gln Ala His Cys Glu Phe Ala Leu Met Asp Cys
            150                 155                 160

CCC CAA TGC CAG CGT CCC TTC CAA AAA TTC CAT ATT AAT ATT CAC ATT         763
Pro Gln Cys Gln Arg Pro Phe Gln Lys Phe His Ile Asn Ile His Ile
          165                 170                 175

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TCA ATG GCA TTT GAA GAT AAA GAG ATC CAT GAC CAG AAC TGT CCT TTG         859
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195                 200                 205                 210

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                215                 220                 225

ATG CCT AAT CAT TAT GAT CTA GAC TGC CCT ACA GCC CCA ATT CCA TGC      955
Met Pro Asn His Tyr Asp Leu Asp Cys Pro Thr Ala Pro Ile Pro Cys
            230                 235                 240

ACA TTC AGT ACT TTT GGT TGC CAT GAA AAG ATG CAG AGG AAT CAC TTG     1003
Thr Phe Ser Thr Phe Gly Cys His Glu Lys Met Gln Arg Asn His Leu
        245                 250                 255

GCA CGC CAC CTA CAA GAG AAC ACC CAG TCA CAC ATG AGA ATG TTG GCC     1051
Ala Arg His Leu Gln Glu Asn Thr Gln Ser His Met Arg Met Leu Ala
    260                 265                 270

CAG GCT GTT CAT AGT TTG AGC GTT ATA CCC GAC TCT GGG TAT ATC TCA     1099
Gln Ala Val His Ser Leu Ser Val Ile Pro Asp Ser Gly Tyr Ile Ser
275                 280                 285                 290

GAG GTC CGG AAT TTC CAG GAA ACT ATT CAC CAG TTA GAG GGT CGC CTT     1147
Glu Val Arg Asn Phe Gln Glu Thr Ile His Gln Leu Glu Gly Arg Leu
                295                 300                 305

GTA AGA CAA GAC CAT CAA ATC CGG GAG CTG ACT GCT AAA ATG GAA ACT     1195
Val Arg Gln Asp His Gln Ile Arg Glu Leu Thr Ala Lys Met Glu Thr
            310                 315                 320

CAG AGT ATG TAT GTA AGT GAG CTC AAA CGA ACC ATT CGA ACC CTT GAG     1243
Gln Ser Met Tyr Val Ser Glu Leu Lys Arg Thr Ile Arg Thr Leu Glu
        325                 330                 335

GAC AAA GTT GCT GAA ATC GAA GCA CAG CAG TGC AAT GGA ATT TAT ATT     1291
Asp Lys Val Ala Glu Ile Glu Ala Gln Gln Cys Asn Gly Ile Tyr Ile
    340                 345                 350

TGG AAG ATT GGC AAC TTT GGA ATG CAT TTG AAA TGT CAA GAA GAG GAG     1339
Trp Lys Ile Gly Asn Phe Gly Met His Leu Lys Cys Gln Glu Glu Glu
355                 360                 365                 370

AAA CCT GTT GTG ATT CAT AGC CCT GGA TTC TAC ACT GGC AAA CCC GGG     1387
Lys Pro Val Val Ile His Ser Pro Gly Phe Tyr Thr Gly Lys Pro Gly
                375                 380                 385

TAC AAA CTG TGC ATG CGC TTG CAC CTT CAG TTA CCG ACT GCT CAG CGC     1435
Tyr Lys Leu Cys Met Arg Leu His Leu Gln Leu Pro Thr Ala Gln Arg
            390                 395                 400

TGT GCA AAC TAT ATA TCC CTT TTT GTC CAC ACA ATG CAA GGA GAA TAT     1483
Cys Ala Asn Tyr Ile Ser Leu Phe Val His Thr Met Gln Gly Glu Tyr
        405                 410                 415

GAC AGC CAC CTC CCT TGG CCC TTC CAG GGT ACA ATA CGC CTT ACA ATT     1531
Asp Ser His Leu Pro Trp Pro Phe Gln Gly Thr Ile Arg Leu Thr Ile
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     420                 425                 430

CTT GAT CAG TCT GAA GCA CCT GTA AGG CAA AAC CAC GAA GAG ATA ATG      1579
Leu Asp Gln Ser Glu Ala Pro Val Arg Gln Asn His Glu Glu Ile Met
435                 440                 445                 450

GAT GCC AAA CCA GAG CTG CTT GCT TTC CAG CGA CCC ACA ATC CCA CGG      1627
Asp Ala Lys Pro Glu Leu Leu Ala Phe Gln Arg Pro Thr Ile Pro Arg
                455                 460                 465

AAC CCA AAA GGT TTT GGC TAT GTA ACT TTT ATG CAT CTG GAA GCC CTA      1675
Asn Pro Lys Gly Phe Gly Tyr Val Thr Phe Met His Leu Glu Ala Leu
            470                 475                 480

AGA CAA AGA ACT TTC ATT AAG GAT GAC ACA TTA TTA GTG CGC TGT GAG      1723
Arg Gln Arg Thr Phe Ile Lys Asp Asp Thr Leu Leu Val Arg Cys Glu
        485                 490                 495

GTC TCC ACC CGC TTT GAC ATG GGT AGC CTT CGG AGG GAG GGT TTT CAG      1771
Val Ser Thr Arg Phe Asp Met Gly Ser Leu Arg Arg Glu Gly Phe Gln
    500                 505                 510

CCA CGA AGT ACT GAT GCA GGG GTA TAGCTTGCCC TCACTTGCTC AAAAACAACT     1825
Pro Arg Ser Thr Asp Ala Gly Val
515                 520

ACCTGGAGAA AACAGTGCCT TTCCTTGCCC TGTTCTCAAT AACATGCAAA CAAACAAGCC    1885

ACGGGAAATA TGTAATATCT ACTAGTGAGT GTTGTTAGAG AGGTCACTTA CTATTTCTTC    1945

CTGTTACAAA TGATCTGAGG CAGTTTTTTC CTGGGAATCC ACACGTTCCA TGCTTTTTCA    2005

GAAATGTTAG GCCTGAAGTG CCTGTGGCAT GTTGCAGCAG CTATTTTGCC AGTTAGTATA    2065

CCTCTTTGTT GTACTTTCTT GGGCTTTTGC TCTGGTGTAT TTTATTGTCA GAAAGTCCAG    2125

ACTCAAGAGT ACTAAACTTT TAATAATAAT GGATTTTCCT TAAAACTTCA GTCTTTTTGT    2185

AGTATTATAT GTAATATATT AAAAGTGAAA ATCACTACCG CCTTGAAAAA AAAAAAAAAA    2245

AAA                                                                  2248
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 522 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Ser Leu Leu Asn Cys Glu Asn Ser Cys Gly Ser Ser Gln Ser Glu
 1               5                  10                  15
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Ser Asp Cys Cys Val Ala Met Ala Ser Ser Cys Ser Ala Val Thr Lys
            20                  25                  30

Asp Asp Ser Val Gly Gly Thr Ala Ser Thr Gly Asn Leu Ser Ser Ser
        35                  40                  45

Phe Met Glu Glu Ile Gln Gly Tyr Asp Val Glu Phe Asp Pro Pro Leu
    50                  55                  60

Glu Ser Lys Tyr Glu Cys Pro Ile Cys Leu Met Ala Leu Arg Glu Ala
65                  70                  75                  80

Val Gln Thr Pro Cys Gly His Arg Phe Cys Lys Ala Cys Ile Ile Lys
                85                  90                  95

Ser Ile Arg Asp Ala Gly His Lys Cys Pro Val Asp Asn Glu Ile Leu
            100                 105                 110

Leu Glu Asn Gln Leu Phe Pro Asp Asn Phe Ala Lys Arg Glu Ile Leu
        115                 120                 125

Ser Leu Met Val Lys Cys Pro Asn Glu Gly Cys Leu His Lys Met Glu
    130                 135                 140

Leu Arg His Leu Glu Asp His Gln Ala His Cys Glu Phe Ala Leu Met
145                 150                 155                 160

Asp Cys Pro Gln Cys Gln Arg Pro Phe Gln Lys Phe His Ile Asn Ile
                165                 170                 175

His Ile Leu Lys Asp Cys Pro Arg Arg Gln Val Ser Cys Asp Asn Cys
            180                 185                 190

Ala Ala Ser Met Ala Phe Glu Asp Lys Glu Ile His Asp Gln Asn Cys
        195                 200                 205

Pro Leu Ala Asn Val Ile Cys Glu Tyr Cys Asn Thr Ile Leu Ile Arg
    210                 215                 220

Glu Gln Met Pro Asn His Tyr Asp Leu Asp Cys Pro Thr Ala Pro Ile
225                 230                 235                 240

Pro Cys Thr Phe Ser Thr Phe Gly Cys His Glu Lys Met Gln Arg Asn
                245                 250                 255

His Leu Ala Arg His Leu Gln Glu Asn Thr Gln Ser His Met Arg Met
            260                 265                 270

Leu Ala Gln Ala Val His Ser Leu Ser Val Ile Pro Asp Ser Gly Tyr
        275                 280                 285

Ile Ser Glu Val Arg Asn Phe Gln Glu Thr Ile His Gln Leu Glu Gly
    290                 295                 300

Arg Leu Val Arg Gln Asp His Gln Ile Arg Glu Leu Thr Ala Lys Met
305                 310                 315                 320
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Glu Thr Gln Ser Met Tyr Val Ser Glu Leu Lys Arg Thr Ile Arg Thr
325 330 335

Leu Glu Asp Lys Val Ala Glu Ile Glu Ala Gln Gln Cys Asn Gly Ile
340 345 350

Tyr Ile Trp Lys Ile Gly Asn Phe Gly Met His Leu Lys Cys Gln Glu
355 360 365

Glu Glu Lys Pro Val Val Ile His Ser Pro Gly Phe Tyr Thr Gly Lys
370 375 380

Pro Gly Tyr Lys Leu Cys Met Arg Leu His Leu Gln Leu Pro Thr Ala
385 390 395 400

Gln Arg Cys Ala Asn Tyr Ile Ser Leu Phe Val His Thr Met Gln Gly
405 410 415

Glu Tyr Asp Ser His Leu Pro Trp Pro Phe Gln Gly Thr Ile Arg Leu
420 425 430

Thr Ile Leu Asp Gln Ser Glu Ala Pro Val Arg Gln Asn His Glu Glu
435 440 445

Ile Met Asp Ala Lys Pro Glu Leu Leu Ala Phe Gln Arg Pro Thr Ile
450 455 460

Pro Arg Asn Pro Lys Gly Phe Gly Tyr Val Thr Phe Met His Leu Glu
465 470 475 480

Ala Leu Arg Gln Arg Thr Phe Ile Lys Asp Asp Thr Leu Leu Val Arg
485 490 495

Cys Glu Val Ser Thr Arg Phe Asp Met Gly Ser Leu Arg Arg Glu Gly
500 505 510

Phe Gln Pro Arg Ser Thr Asp Ala Gly Val
515 520

WHAT IS CLAIMED IS:

1. An isolated tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide wherein said polypeptide comprises the amino acid sequence of SEQ ID NO:2 or a deletion mutant of SEQ ID NO:2 sufficient to specifically bind a natural intracellular TRAF6 binding target.

2. An isolated tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide according to claim 1, wherein said polypeptide comprises SEQ ID NO:2, residues 1-114.

3. An isolated tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide according to claim 1, wherein said polypeptide comprises SEQ ID NO:2, residues 115-522.

4. An isolated nucleic acid encoding a tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide according to claim 1.

5. A recombinant nucleic acid comprising an open reading frame encoding a tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide according to claim 1, said open reading frame joined directly to a nucleotide other than that which said open reading frame is joined to on a natural chromosome.

6. An isolated hybridization probe or primer comprising a portion of SEQ ID NO: 1 sufficient to specifically hybridize with a nucleic acid having the sequence defined by SEQ ID NO:1 in the presence of human umbilical vein endothelial cell cDNA.

7. A method of making an isolated tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide, comprising steps: introducing a nucleic acid according to claim 5 into a host cell, growing said host cell under conditions whereby said nucleic acid is expressed as a transcript and said transcript is expressed as a translation product comprising said polypeptide, and isolating said translation product.

8. A method of screening for an agent which modulates the binding of a tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide to a natural intracellular TRAF6 binding target, said method comprising the steps of:

incubating a mixture comprising:

an isolated TRAF6 polypeptide according to claim 1,

a natural intracellular binding target of said polypeptide, wherein said binding target is capable of specifically binding said polypeptide, and

a candidate agent;

under conditions whereby, but for the presence of said agent, said polypeptide specifically binds said binding target at a reference affinity;

detecting the binding affinity of said polypeptide to said binding target to determine an agent-biased affinity,

wherein a difference between the agent-biased affinity and the reference affinity indicates that said agent modulates the binding of said polypeptide to said binding target.

9. A method according to claim 8, wherein said binding target comprises a tumor necrosis factor receptor associated factor or fragment thereof sufficient to specifically bind said TRAF6 polypeptide.

10. A method of identifying an agent which modulates the transcription of a tumor necrosis factor receptor associated factor number six (TRAF6) polypeptide-inducible gene, said method comprising the steps of:

contacting a cell comprising a reporter gene operatively linked to an NF-κB inducible promoter, and a TRAF6 gene comprising a nucleic acid according to claims 5, wherein said TRAF6 gene is expressed as a TRAF6 polypeptide, with a candidate agent;

incubating said cell under conditions whereby, but for the presence of said agent, said TRAF6 polypeptide specifically induces said promoter at a reference induction;

detecting the induction of said promoter by said TRAF6 polypeptide by measuring the expression of said reporter gene to determine an agent-biased induction,

wherein a difference between said agent-biased induction and said reference affinity indicates that said agent modulates the transcription of a TRAF6 polypeptide-inducible gene.

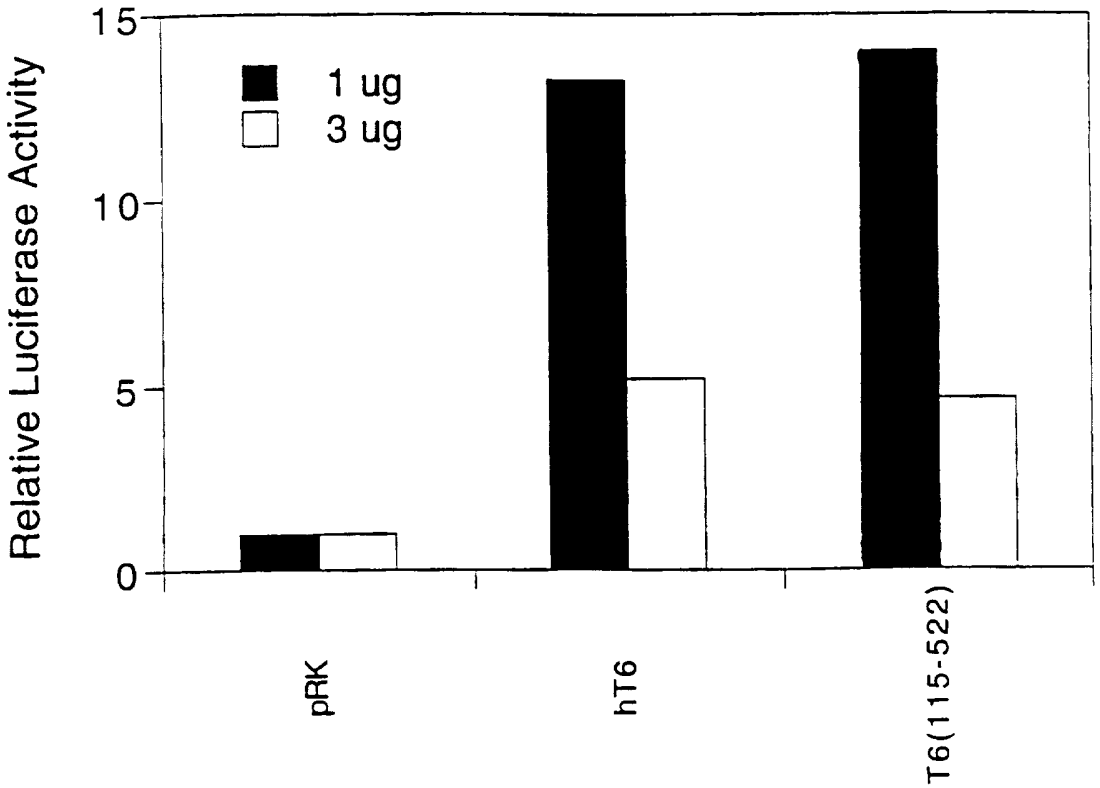
Relative Luciferase Activity
15
10
5
0
1 ug
3 ug
pRK
hT6
T6(115-522)

Fig. 1

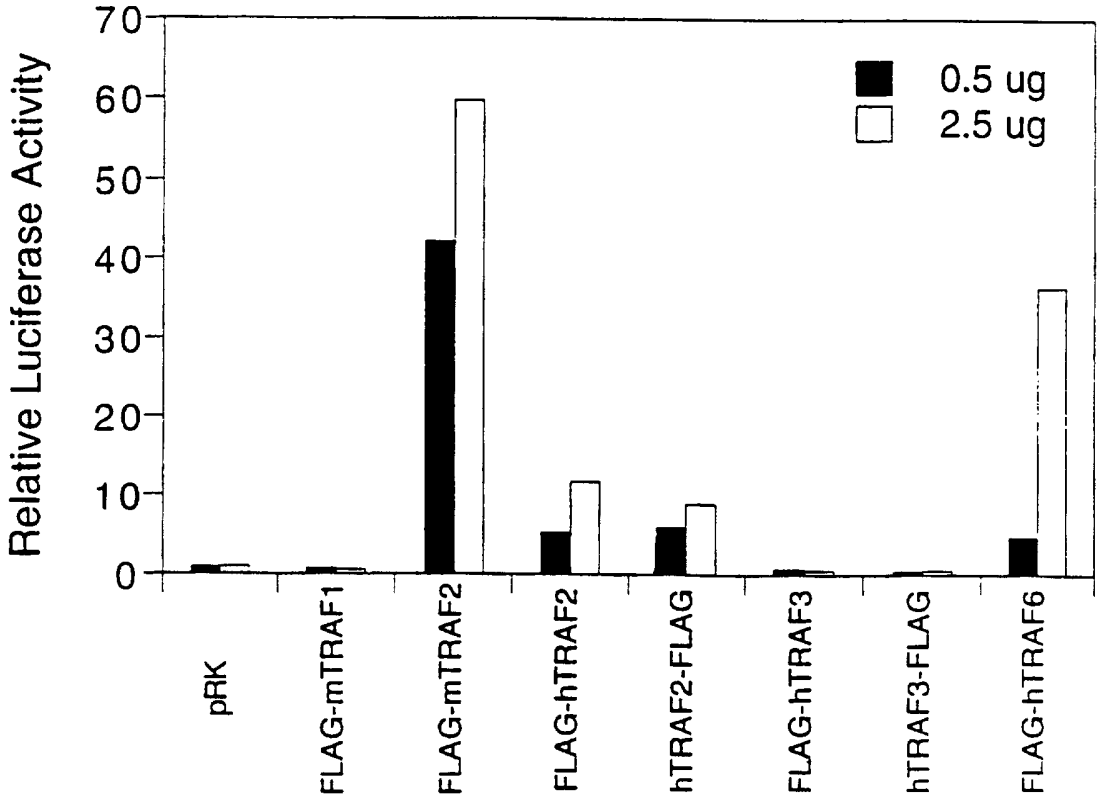
Relative Luciferase Activity
70
60
50
40
30
20
10
0
0.5 ug
2.5 ug
pRK
FLAG-mTRAF1
FLAG-mTRAF2
FLAG-hTRAF2
hTRAF2-FLAG
FLAG-hTRAF3
hTRAF3-FLAG
FLAG-hTRAF6

Fig. 2

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06451

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12Q 1/68; C12N 15/09; C07H 21/04; C07K 14/475; G01N 33/68
US CL : 435/6, 7.1, 69.1; 530/350; 536/23.1, 23.5

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.1, 69.1; 530/350; 536/23.1, 23.5

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, GENBANK, EMBL, SWISS-PROT, EST-STS, GENESEQ
search terms: tumor necrosis factor receptor associated, traf#

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	FIELDS et al. The two-hybrid system: an assay for protein-protein interactions. Trends in Genetics. August 1994. Vol. 10, No. 8., pages 286-292.	1-10
A	NGO et al. 'Computational complexity, protein structure prediction, and the Levinthal paradox. The Protein Folding Problem and Tertiary Structure Prediction. Birkhauser, 1994, p. 492-495.	1-10
A	ROTHE et al. TRAF2-mediated activation of NF-kappaB by TNF receptor 2 and CD40. Science. 08 September 1995, Vol. 269, pages 1424-1427.	1-10

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
12 JUNE 1997	25 JUL 1997

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	SCOTT D. PRIEBE
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/06451

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database on EST-STS-TWO, National Center for Biological Information (Bethesda, MD, USA), Accession No. T89991, yd38c04.s1 Homo sapiens cDNA clone 110502 3', HILLIER et al. 'The WashU-EST Project', 20 March 1995.	6
X	Database on EST-STS-TWO, National Center for Biological Information (Bethesda, MD, USA), Accession No. T82812, yd38c04.r1 Homo sapiens cDNA clone 110502 5', HILLIER et al. 'The WashU-EST Project', 16 March 1995.	6