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(54) Title: LIGAND THAT BINDS FAS ANTIGEN

(57) Abstract

Novel human and murine proteins designated Fas ligand (Fas-L) bind to the cell surface protein known as Fas antigen. DNA sequences, expression vectors and transformed host cells useful in producing Fas-L polypeptides are provided, along with antibodies immunoreactive with Fas-L.

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TITLE

LIGAND THAT BINDS FAS ANTIGEN

BACKGROUND OF THE INVENTION

The programmed cell death known as apoptosis occurs in embryogenesis, metamorphosis, endocrine-dependent tissue atrophy, normal tissue turnover, and death of immune thymocytes (induced through their antigen-receptor complex or by glucocorticoids) (Itoh et al., *Cell* 66:233, 1991). During maturation of T-cells in the thymus, T-cells that recognize self-antigens are destroyed through the apoptotic process, whereas others are positively selected. The possibility that some T-cells recognizing certain self epitopes (e.g., inefficiently processed and presented antigenic determinants of a given self protein) escape this elimination process and subsequently may play a role in autoimmune diseases has been suggested (Gammon et al., *Immunology Today* 12:193, 1991).

A cell surface antigen known as Fas has been reported to mediate apoptosis and is believed to play a role in clonal deletion of self-reactive T-cells (Itoh et al., *Cell* 66:233, 1991; Watanabe-Fukunage et al., *Nature* 356:314, 1992). Fas is a 45kDa molecule expressed on activated T cells, B cells, and neutrophils (Itoh, *supra*). High levels of Fas mRNA have been detected in thymus, liver, heart, lung and ovary of mice (Watanabe-Fukunaga et al., *J. Immunol.*, 148:1274, 1992). Cross-linking a specific monoclonal antibody to Fas has been reported to induce various cell lines to undergo apoptosis (Yonehara et al., *J. Exp. Med.*, 169:1747, 1989; Trauth et al., *Science*, 245:301, 1989). However, under certain conditions, binding of a specific monoclonal antibody to Fas can be stimulatory for freshly isolated T cells (Alderson et al., *J. Exp. Med.* 178:2231, 1993).

Cloning of cDNA encoding murine and human Fas antigen has been reported by Watanage-Fukunage et al. (*J. Immunol., supra*) and Itoh et al., *supra*, respectively. The amino acid sequences encoded by the cloned cDNAs indicate that both murine and human Fas antigens are transmembrane proteins exhibiting structural homology with members of the nerve growth factor receptor/tumor necrosis factor receptor (NGFR/TNFR) family of cell surface receptors.

Mice carrying the autosomal recessive mutation known as the lymphoproliferation (*lpr*) mutation have defects in the Fas antigen gene and do not express normal functional Fas protein capable of transducing the apoptotic signal (Watanabe-Fukunage et al., *Nature*

356:314, 1992). Mice carrying an *lpr* mutation develop lymphoproliferative disorders characterized by the accumulation of $CD4^-$ $CD8^-$ thymocytes in lymph nodes and the spleen, hypergammaglobulinaemia, autoantibody production, rheumatoid factor, arthritis and glomerulonephritis. (Watanabe-Fukunage et al., *Nature, supra*).

A clinical syndrome indistinguishable from that found in *lpr* mice is exhibited by mice carrying a mutant gene known as the generalized lymphoproliferative disease (*gld*) mutation (Watanabe-Fukunage et al., *Nature, supra*). The *gld* and *lpr* mutations involve different genes which map to different chromosomes (Seldin et al., *J. Exp. Med* 167:688, 1988; Watson et al., *Mammalian Genome* 2:158, 1992; Watanabe-Fukunage et al., *Biochem. Genet.* 29:325, 1991; and Watanabe-Fukunage et al., *J. Immunol, supra*).

Investigation into the existence and identity of molecule(s) that interact with the Fas antigen is desirable in order to provide further insight into the development of self-tolerance by the immune system, and provide a means for studying the etiology of autoimmune diseases. cDNA encoding a rat protein that binds Fas has been cloned (Suda et al., *Cell*, 75:1169, 1993). A human Fas ligand would be preferred for such uses as research involving the human immune system and development of diagnostic or therapeutic agents for use in humans. Prior to the present invention, however, no human protein that binds to the Fas antigen was known.

SUMMARY OF THE INVENTION

The present invention provides novel Fas ligand (Fas-L) proteins, as well as isolated DNA encoding the Fas-L proteins, expression vectors comprising the isolated DNA, and a method for producing Fas-L by cultivating host cells containing the expression vectors under conditions appropriate for expression of the Fas-L protein. Fas-L is a protein that binds to the antigen known as Fas (a cell surface protein). In particular embodiments, the Fas-L is a human or murine protein. Antibodies directed against the Fas-L protein or an immunogenic fragment thereof are also provided. Among the uses of the antibodies is blocking the binding of Fas-L to Fas. Biological activities mediated by such binding thus may be inhibited.

DETAILED DESCRIPTION OF THE INVENTION

DNA encoding a novel human polypeptide that can act as a ligand for the cell surface antigen known as Fas has been isolated in accordance with the present invention. Murine Fas-L DNA is provided as well. Also provided are expression vectors comprising the Fas ligand (Fas-L) DNA and methods for producing recombinant Fas-L polypeptides by cultivating host cells containing the expression vectors under conditions appropriate for expression of Fas-L, and recovering the expressed Fas-L. Substantially homogeneous purified Fas-L protein is also encompassed by the present invention.

The present invention also provides human or murine Fas-L or antigenic fragments thereof that can act as immunogens to generate antibodies specific to the Fas-L immunogens. Monoclonal antibodies specific for Fas-L or antigenic fragments thereof thus can be prepared.

The novel protein disclosed herein is a ligand for Fas, a cell surface protein that is a member of the TNF/NGF receptor superfamily. Fas is believed to play a role in clonal deletion of self-reactive T-cells. Mice carrying mutations that cause production of defective Fas antigen are afflicted with an autoimmune syndrome. One use of the Fas ligand of the present invention is as a research tool for studying autoimmune disorders and investigating the roles that Fas and Fas-L may play in inducing self-tolerance. The Fas-L polypeptides of the present invention also may be employed in *in vitro* assays for detection of Fas or Fas-L or the interactions thereof.

The Fas-L protein may be employed as a protein purification reagent. Fas-L attached to a solid support material is useful for purifying Fas protein by affinity chromatography. Fas-L also finds use in monitoring the biological activity of Fas proteins, as described in more detail below.

Isolation of a cDNA encoding human Fas-L is described in example 2 below. *E. coli* strain DH5α transformed with the cloning vector pBLUESCRIPT®SK containing this Fas-L cDNA was deposited with the American Type Culture Collection on January 5, 1994, and assigned accession no. 69527. The deposit was made under the terms of the Budapest Treaty.

The nucleotide sequence of the human Fas-L DNA isolated in example 2 is presented in SEQ ID NO:1. The amino acid sequence encoded thereby is presented in SEQ ID NO:2. This human Fas-L protein comprises an N-terminal cytoplasmic domain (amino acids 1-80), a transmembrane region (amino acids 81-105), and an extracellular domain (amino acids 106-281). The extracellular domain contains the receptor-binding region.

As used herein, the term "Fas-L" includes membrane-bound proteins (comprising a cytoplasmic domain, a transmembrane region, and an extracellular domain) as well as truncated proteins that retain the Fas-binding property. In one embodiment, soluble Fas-L polypeptides comprise all or part of the extracellular domain of a Fas-L protein, but lack the transmembrane region that would cause retention of the polypeptide on a cell membrane. Advantageously, a heterologous signal peptide is fused to the N-terminus such that the soluble Fas-L is secreted upon expression. The soluble Fas-L polypeptides that may be employed retain the ability to bind the Fas antigen. Soluble Fas-L may also include part of the transmembrane region or part of the cytoplasmic domain or other sequences, provided that the soluble Fas-L protein is capable of being secreted.

Soluble Fas-L may be identified (and distinguished from its non-soluble membrane-bound counterparts) by separating intact cells which express the desired protein from the

culture medium, e.g., by centrifugation, and assaying the medium (supernatant) for the presence of the desired protein. The culture medium may be assayed using procedures which are similar or identical to those described in the examples below. The presence of Fas-L in the medium indicates that the protein was secreted from the cells and thus is a soluble form of the desired protein. Soluble Fas-L may be a naturally-occurring form of this protein.

The use of soluble forms of Fas-L is advantageous for certain applications. Purification of the proteins from recombinant host cells is facilitated, since the soluble proteins are secreted from the cells. Further, soluble proteins are generally more suitable for intravenous administration.

Truncated Fas-L, including soluble polypeptides, may be prepared by any of a number of conventional techniques. In the case of recombinant proteins, a DNA fragment encoding a desired fragment may be subcloned into an expression vector. Alternatively, a desired DNA sequence may be chemically synthesized using known techniques. DNA fragments also may be produced by restriction endonuclease digestion of a full length cloned DNA sequence, and isolated by electrophoresis on agarose gels. Linkers containing restriction endonuclease cleavage site(s) may be employed to insert the desired DNA fragment into an expression vector, or the fragment may be digested at cleavage sites naturally present therein. The well known polymerase chain reaction procedure also may be employed to isolate a DNA sequence encoding a desired protein fragment.

In another approach, enzymatic treatment (e.g., using Bal 31 exonuclease) may be employed to delete terminal nucleotides from a DNA fragment to obtain a fragment having a particular desired terminus. Among the commercially available linkers are those that can be ligated to the blunt ends produced by Bal 31 digestion, and which contain restriction endonuclease cleavage site(s). Alternatively, oligonucleotides that reconstruct the N- or C-terminus of a DNA fragment to a desired point may be synthesized. The oligonucleotide may contain a restriction endonuclease cleavage site upstream of the desired coding sequence and position an initiation codon (ATG) at the N-terminus of the coding sequence.

The Fas-L DNA of the present invention includes cDNA, chemically synthesized DNA, DNA isolated by PCR, genomic DNA, and combinations thereof. Genomic Fas-L DNA may be isolated by hybridization to the Fas-L cDNA disclosed herein using standard techniques. RNA transcribed from the Fas-L DNA is also encompassed by the present invention.

Certain embodiments of the present invention provide isolated DNA comprising a nucleotide sequence selected from the group consisting of nucleotides 93-938 (coding region) or 408-938 (encoding the extracellular domain) of SEQ ID NO:1. DNA encoding biologically active fragments of the protein of SEQ ID NO:2 are also provided.

Also provided herein are purified Fas-L polypeptides, both recombinant and non-recombinant. Variants and derivatives of native Fas-L proteins that retain the desired biological activity are also within the scope of the present invention. Fas-L variants may be obtained by mutations of nucleotide sequences coding for native Fas-L polypeptides, for example. A Fas-L variant, as referred to herein, is a polypeptide substantially homologous to a native Fas-L, but which has an amino acid sequence different from that of native Fas-L because of one or a plurality of deletions, insertions or substitutions. Fas-L-encoding DNA sequences of the present invention encompass sequences that comprise one or more additions, deletions, or substitutions of nucleotides when compared to a native Fas-L DNA sequence, but that encode a Fas-L protein that is essentially bioequivalent to a native Fas-L protein.

The variant amino acid or DNA sequence preferably is at least 90% identical to a native Fas-L sequence. The degree of homology (percent identity) between a native and a mutant sequence may be determined, for example, by comparing the two sequences using the GAP computer program, version 6.0, described by Devereux et al. (*Nucl. Acids Res.* 12:387, 1984) and available from the University of Wisconsin Genetics Computer Group (UWGCG). The GAP program utilizes the alignment method of Needleman and Wunsch (*J. Mol. Biol.* 48:443, 1970), as revised by Smith and Waterman (*Adv. Appl. Math* 2:482, 1981). Briefly, the GAP program defines similarity as the number of aligned symbols (i.e., nucleotides or amino acids) which are similar, divided by the total number of symbols in the shorter of the two sequences. The preferred default parameters for the GAP program include: (1) a unary comparison matrix (containing a value of 1 for identities and 0 for non-identities) for nucleotides, and the weighted comparison matrix of Gribskov and Burgess, *Nucl. Acids Res.* 14:6745, 1986, as described by Schwartz and Dayhoff, eds., *Atlas of Protein Sequence and Structure*, National Biomedical Research Foundation, pp. 353-358, 1979; (2) a penalty of 3.0 for each gap and an additional 0.10 penalty for each symbol in each gap; and (3) no penalty for end gaps.

Alterations of the native amino acid sequence may be accomplished by any of a number of known techniques. Mutations can be introduced at particular loci by synthesizing oligonucleotides containing a mutant sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes an analog having the desired amino acid insertion, substitution, or deletion.

Alternatively, oligonucleotide-directed site-specific mutagenesis procedures can be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Exemplary methods of making the alterations set forth above are disclosed by Walder et al. (*Gene* 42:133, 1986); Bauer et al. (*Gene* 37:73, 1985); Craik (*BioTechniques*, January 1985, 12-19); Smith et al. (*Genetic*

Engineering: Principles and Methods, Plenum Press, 1981); and U.S. Patent Nos. 4,518,584 and 4,737,462, which are incorporated by reference herein.

Variants may comprise conservatively substituted sequences, meaning that a given amino acid residue is replaced by a residue having similar physiochemical characteristics. Examples of conservative substitutions include substitution of one aliphatic residue for another, such as Ile, Val, Leu, or Ala for one another, or substitutions of one polar residue for another, such as between Lys and Arg; Glu and Asp; or Gln and Asn. Other such conservative substitutions, for example, substitutions of entire regions having similar hydrophobicity characteristics, are well known.

Fas-L also may be modified to create Fas-L derivatives by forming covalent or aggregative conjugates with other chemical moieties, such as glycosyl groups, lipids, phosphate, acetyl groups and the like. Covalent derivatives of Fas-L may be prepared by linking the chemical moieties to functional groups on Fas-L amino acid side chains or at the N-terminus or C-terminus of a Fas-L polypeptide or the extracellular domain thereof. Other derivatives of Fas-L within the scope of this invention include covalent or aggregative conjugates of Fas-L or its fragments with other proteins or polypeptides, such as by synthesis in recombinant culture as N-terminal or C-terminal fusions. For example, the conjugate may comprise a signal or leader polypeptide sequence (e.g. the α-factor leader of *Saccharomyces*) at the N-terminus of a Fas-L polypeptide. The signal or leader peptide co-translationally or post-translationally directs transfer of the conjugate from its side of synthesis to a site inside or outside of the cell membrane or cell wall.

Fas-L polypeptide fusions can comprise peptides added to facilitate purification and identification of Fas-L. Such peptides include, for example, poly-His or the antigenic identification peptides described in U.S. Patent No. 5,011,912 and in Hopp et al., *Bio/Technology* 6:1204, 1988. One such peptide is the FLAG® peptide, Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys (DYKDDDDK) (SEQ ID NO:3), which is highly antigenic and provides an epitope reversibly bound by a specific monoclonal antibody enabling rapid assay and facile purification of expressed recombinant protein. This sequence is also specifically cleaved by bovine mucosal enterokinase at the residue immediately following the Asp-Lys pairing. Fusion proteins capped with this peptide may also be resistant to intracellular degradation in *E. coli*. A murine hybridoma designated 4E11 produces a monoclonal antibody that binds the peptide DYKDDDDK (SEQ ID NO:3) in the presence of certain divalent metal cations (as described in U.S. Patent 5,011,912) and has been deposited with the American Type Culture Collection under accession no HB 9259. Monoclonal antibodies that bind the Flag® peptide are available from Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut.

The present invention further includes Fas-L polypeptides with or without associated native-pattern glycosylation. Fas-L expressed in yeast or mammalian expression

systems (e.g., COS-7 cells) may be similar to or significantly different from a native Fas-L polypeptide in molecular weight and glycosylation pattern, depending upon the choice of expression system. Expression of Fas-L polypeptides in bacterial expression systems, such as *E. coli*, provides non-glycosylated molecules.

DNA constructs that encode various additions or substitutions of amino acid residues or sequences, or deletions of terminal or internal residues or sequences not needed for biological activity or binding can be prepared. For example, N-glycosylation sites in the Fas-L extracellular domain can be modified to preclude glycosylation while allowing expression of a homogeneous, reduced carbohydrate analog using yeast expression systems. N-glycosylation sites in eukaryotic polypeptides are characterized by an amino acid triplet Asn-X-Y, wherein X is any amino acid except Pro and Y is Ser or Thr. Such sites are found in human Fas-L at amino acids 76-78, 184-186, 250-252, and 260-262 of SEQ ID NO: 2. Appropriate modifications to the nucleotide sequence encoding this triplet will result in substitutions, additions or deletions that prevent attachment of carbohydrate residues at the Asn side chain. Known procedures for inactivating N-glycosylation sites in proteins include those described in U.S. Patent 5,071,972 and EP 276,846.

In another example, sequences encoding Cys residues that are not essential for biological activity can be altered to cause the Cys residues to be deleted or replaced with other amino acids, preventing formation of incorrect intramolecular disulfide bridges upon renaturation. Other variants are prepared by modification of adjacent dibasic amino acid residues to enhance expression in yeast systems in which KEX2 protease activity is present. EP 212,914 discloses the use of site-specific mutagenesis to inactivate KEX2 protease processing sites in a protein. KEX2 protease processing sites are inactivated by deleting, adding or substituting residues to alter Arg-Arg, Arg-Lys, and Lys-Arg pairs to eliminate the occurrence of these adjacent basic residues. Such KEX2 protease processing sites are found at residues 38-39, 43-44, 73-74, and 144-145 of SEQ ID NO:2. Lys-Lys pairings are considerably less susceptible to KEX2 cleavage, and conversion of Arg-Lys or Lys-Arg to Lys-Lys represents a conservative and preferred approach to inactivating KEX2 sites.

Naturally occurring Fas-L variants are also encompassed by the present invention. Examples of such variants are proteins that result from alternative mRNA splicing events (since Fas-L is encoded by a multi-exon gene) or from proteolytic cleavage of the Fas-L protein, wherein the Fas-binding property is retained. Alternative splicing of mRNA may yield a truncated but biologically active Fas-L protein, such as a naturally occurring soluble form of the protein, for example. Variations attributable to proteolysis include, for example, differences in the N- or C-termini upon expression in different types of host cells, due to proteolytic removal of one or more terminal amino acids from the Fas-L protein. Fas-L proteins having amino acid sequences that are truncated at the N- or C- terminus by

from one to five amino acids are encompassed by the present invention. In addition, proteolytic cleavage may release a soluble form of Fas-L from a membrane-bound form of the protein.

Due to degeneracy of the genetic code, two DNA sequences may differ, yet encode the same amino acid sequence. The present invention thus provides isolated DNA sequences encoding biologically active Fas-L, selected from DNA comprising the coding region of a native human or murine Fas-L cDNA, or fragments thereof, and DNA which is degenerate as a result of the genetic code to the native Fas-L DNA sequence. One embodiment of the present invention provides DNA comprising the coding region of the nucleotide sequence of SEQ ID NO:1, as well as DNA sequences degenerate thereto.

Variants possessing the requisite ability to bind Fas may be identified by any suitable assay. Biological activity of Fas-L may be determined, for example, by competition for binding to the ligand binding domain of Fas (i.e. competitive binding assays).

Assays

One type of a competitive binding assay for Fas-L polypeptide uses a radiolabeled, soluble human Fas-L and intact cells expressing cell surface Fas. Instead of intact cells, one could substitute soluble Fas (such as a Fas/Fc fusion protein) bound to a solid phase through a Protein A or Protein G interaction with the Fc region of the fusion protein. Another type of competitive binding assay utilizes radiolabeled soluble Fas such as a Fas/Fc fusion protein, and intact cells expressing Fas-L. Alternatively, soluble Fas-L could be bound to a solid phase.

Competitive binding assays can be performed using standard methodology. Qualitative results can be obtained by competitive autoradiographic plate binding assays, or Scatchard plots may be utilized to generate quantitative results.

Competitive binding assays with intact cells expressing Fas can be performed by two methods. In a first method, cells expressing endogenous cell surface Fas are grown either in suspension or by adherence to tissue culture plates. Adherent cells can be removed by treatment with 5 mM EDTA treatment for ten minutes at 37° C. In a second method, transfected cells expressing membrane-bound recombinant Fas can be used. COS cells or another mammalian cell can be transfected with human Fas cDNA in an appropriate vector to express full length Fas with an extracellular region.

Alternatively, soluble Fas can be bound to a solid phase such as a column chromatography matrix or a similar substrate. Binding to a solid phase can be accomplished, for example, by preparing a Fas/Fc fusion protein and binding it to a protein A or protein G-containing matrix.

Another means to measure the biological activity of Fas-L (including variants) is to utilize conjugated, soluble Fas (for example, ^{125}I-Fas/Fc) in competition assays similar to those described above. In this case, however, intact cells expressing Fas-L, or soluble Fas-L bound to a solid substrate, are used to measure competition for binding of conjugated, soluble Fas to Fas-L by a sample containing a putative Fas-L variant.

Oligomers

The present invention encompasses Fas-L polypeptides in the form of oligomers, such as dimers or trimers. Oligomers may be formed by disulfide bonds between cysteine residues on different Fas-L polypeptides. In one embodiment of the invention, a Fas-L dimer is created by fusing Fas-L to the Fc region of an antibody (IgG1). The term "Fc polypeptide" includes native and mutein forms, as well as truncated Fc polypeptides containing the hinge region that promotes dimerization. The Fc polypeptide preferably is fused to a soluble Fas-L (comprising only the extracellular domain). Preparation of fusion proteins comprising heterologous polypeptides fused to various portions of antibody-derived polypeptides (including the Fc domain) has been described, e.g., by Ashkenazi et al. (*PNAS USA* 88:10535, 1991) and Byrn et al. (*Nature* 344:667, 1990), hereby incorporated by reference.

A gene fusion encoding the Fas-L/Fc fusion protein is inserted into an appropriate expression vector. The Fas-L/Fc fusion proteins are allowed to assemble much like antibody molecules, whereupon interchain disulfide bonds form between Fc polypeptides, yielding divalent Fas-L. In other embodiments, Fas-L may be substituted for the variable portion of an antibody heavy or light chain. If fusion proteins are made with both heavy and light chains of an antibody, it is possible to form a Fas-L oligomer with as many as four Fas-L extracellular regions. Alternatively, one can link two soluble Fas-L polypeptides with a peptide linker such as those described in United States Patent 5,073,627 (hereby incorporated by reference).

The present invention provides oligomers of Fas-L extracellular domains or fragments thereof, linked by disulfide interactions, or expressed as fusion polymers with or without spacer amino acid linking groups. For example, a dimer Fas-L molecule can be linked by an IgG Fc region linking group.

Expression Systems

The present invention provides recombinant expression vectors for expression of Fas-L, and host cells transformed with the expression vectors. Any suitable expression system may be employed. The vectors include a DNA encoding a Fas-L polypeptide, operably linked to suitable transcriptional or translational regulatory nucleotide sequences, such as those derived from a mammalian, microbial, viral, or insect gene. Examples of

regulatory sequences include transcriptional promoters, operators, or enhancers, an mRNA ribosomal binding site, and appropriate sequences which control transcription and translation initiation and termination. Nucleotide sequences are operably linked when the regulatory sequence functionally relates to the Fas-L DNA sequence. Thus, a promoter nucleotide sequence is operably linked to a Fas-L DNA sequence if the promoter nucleotide sequence controls the transcription of the Fas-L DNA sequence. The ability to replicate in the desired host cells, usually conferred by an origin of replication, and a selection gene by which transformants are identified, may additionally be incorporated into the expression vector.

In addition, sequences encoding appropriate signal peptides that are not native to the Fas-L gene can be incorporated into expression vectors. For example, a DNA sequence for a signal peptide (secretory leader) may be fused in frame to the Fas-L sequence so that the Fas-L is initially translated as a fusion protein comprising the signal peptide. A signal peptide that is functional in the intended host cells enhances extracellular secretion of the Fas-L polypeptide. The signal peptide is cleaved from the Fas-L polypeptide upon secretion of Fas-L from the cell.

Suitable host cells for expression of Fas-L polypeptides include prokaryotes, yeast or higher eukaryotic cells. Appropriate cloning and expression vectors for use with bacterial, fungal, yeast, and mammalian cellular hosts are described, for example, in Pouwels et al. *Cloning Vectors: A Laboratory Manual,* Elsevier, New York, (1985). Cell-free translation systems could also be employed to produce Fas-L polypeptides using RNAs derived from DNA constructs disclosed herein.

Prokaryotes include gram negative or gram positive organisms, for example, *E. coli* or *Bacilli*. Suitable prokaryotic host cells for transformation include, for example, *E. coli, Bacillus subtilis, Salmonella typhimurium,* and various other species within the genera *Pseudomonas, Streptomyces,* and *Staphylococcus*. In a prokaryotic host cell, such as *E. coli*, a Fas-L polypeptide may include an N-terminal methionine residue to facilitate expression of the recombinant polypeptide in the prokaryotic host cell. The N-terminal Met may be cleaved from the expressed recombinant Fas-L polypeptide.

Expression vectors for use in prokaryotic host cells generally comprise one or more phenotypic selectable marker genes. A phenotypic selectable marker gene is, for example, a gene encoding a protein that confers antibiotic resistance or that supplies an autotrophic requirement. Examples of useful expression vectors for prokaryotic host cells include those derived from commercially available plasmids such as the cloning vector pBR322 (ATCC 37017). pBR322 contains genes for ampicillin and tetracycline resistance and thus provides simple means for identifying transformed cells. An appropriate promoter and a Fas-L DNA sequence are inserted into the pBR322 vector. Other commercially available

vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and pGEM1 (Promega Biotec, Madison, WI, USA).

Promoter sequences commonly used for recombinant prokaryotic host cell expression vectors include β-lactamase (penicillinase), lactose promoter system (Chang et al., *Nature 275*:615, 1978; and Goeddel et al., *Nature 281*:544, 1979), tryptophan (trp) promoter system (Goeddel et al., *Nucl. Acids Res. 8*:4057, 1980; and EP-A-36776) and tac promoter (Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, p. 412, 1982). A particularly useful prokaryotic host cell expression system employs a phage λ P_L promoter and a cI857ts thermolabile repressor sequence. Plasmid vectors available from the American Type Culture Collection which incorporate derivatives of the λ P_L promoter include plasmid pHUB2 (resident in *E. coli* strain JMB9 (ATCC 37092)) and pPLc28 (resident in *E. coli* RR1 (ATCC 53082)).

Fas-L alternatively may be expressed in yeast host cells, preferably from the *Saccharomyces* genus (e.g., *S. cerevisiae*). Other genera of yeast, such as *Pichia* or *Kluyveromyces*, may also be employed. Yeast vectors will often contain an origin of replication sequence from a 2μ yeast plasmid, an autonomously replicating sequence (ARS), a promoter region, sequences for polyadenylation, sequences for transcription termination, and a selectable marker gene. Suitable promoter sequences for yeast vectors include, among others, promoters for metallothionein, 3-phosphoglycerate kinase (Hitzeman et al., *J. Biol. Chem. 255*:2073, 1980) or other glycolytic enzymes (Hess et al., *J. Adv. Enzyme Reg. 7*:149, 1968; and Holland et al., *Biochem. 17*:4900, 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Other suitable vectors and promoters for use in yeast expression are further described in Hitzeman, EPA-73,657. Another alternative is the glucose-repressible ADH2 promoter described by Russell et al. (*J. Biol. Chem. 258*:2674, 1982) and Beier et al. (*Nature 300*:724, 1982). Shuttle vectors replicable in both yeast and *E. coli* may be constructed by inserting DNA sequences from pBR322 for selection and replication in *E. coli* (Amp^r gene and origin of replication) into the above-described yeast vectors.

The yeast α-factor leader sequence may be employed to direct secretion of the Fas-L polypeptide. The α-factor leader sequence is often inserted between the promoter sequence and the structural gene sequence. *See*, e.g., Kurjan et al., *Cell 30*:933, 1982 and Bitter et al., *Proc. Natl. Acad. Sci. USA 81*:5330, 1984. Other leader sequences suitable for facilitating secretion of recombinant polypeptides from yeast hosts are known to those of skill in the art. A leader sequence may be modified near its 3' end to contain one or more restriction sites. This will facilitate fusion of the leader sequence to the structural gene.

Yeast transformation protocols are known to those of skill in the art. One such protocol is described by Hinnen et al., *Proc. Natl. Acad. Sci. USA 75*:1929, 1978. The Hinnen et al. protocol selects for Trp^+ transformants in a selective medium, wherein the selective medium consists of 0.67% yeast nitrogen base, 0.5% casamino acids, 2% glucose, 10 μg/ml adenine and 20 μg/ml uracil.

Yeast host cells transformed by vectors containing ADH2 promoter sequence may be grown for inducing expression in a "rich" medium. An example of a rich medium is one consisting of 1% yeast extract, 2% peptone, and 1% glucose supplemented with 80 μg/ml adenine and 80 μg/ml uracil. Derepression of the ADH2 promoter occurs when glucose is exhausted from the medium.

Mammalian or insect host cell culture systems could also be employed to express recombinant Fas-L polypeptides. Bacculovirus systems for production of heterologous proteins in insect cells are reviewed by Luckow and Summers, *Bio/Technology* 6:47 (1988). Established cell lines of mammalian origin also may be employed. Examples of suitable mammalian host cell lines include the COS-7 line of monkey kidney cells (ATCC CRL 1651) (Gluzman et al., *Cell 23*:175, 1981), L cells, C127 cells, 3T3 cells (ATCC CCL 163), Chinese hamster ovary (CHO) cells, HeLa cells, and BHK (ATCC CRL 10) cell lines, and the CVI/EBNA cell line derived from the African green monkey kidney cell line CVI (ATCC CCL 70) as described by McMahan et al. (*EMBO J.* 10: 2821, 1991).

Transcriptional and translational control sequences for mammalian host cell expression vectors may be excised from viral genomes. Commonly used promoter sequences and enhancer sequences are derived from Polyoma virus, Adenovirus 2, Simian Virus 40 (SV40), and human cytomegalovirus. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early and late promoter, enhancer, splice, and polyadenylation sites may be used to provide other genetic elements for expression of a structural gene sequence in a mammalian host cell. Viral early and late promoters are particularly useful because both are easily obtained from a viral genome as a fragment which may also contain a viral origin of replication (Fiers et al., *Nature 273*:113, 1978). Smaller or larger SV40 fragments may also be used, provided the approximately 250 bp sequence extending from the *Hind* III site toward the *Bgl* I site located in the SV40 viral origin of replication site is included.

Exemplary expression vectors for use in mammalian host cells can be constructed as disclosed by Okayama and Berg (*Mol. Cell. Biol. 3*:280, 1983). A useful system for stable high level expression of mammalian cDNAs in C127 murine mammary epithelial cells can be constructed substantially as described by Cosman et al. (*Mol. Immunol.* 23:935, 1986). A useful high expression vector, PMLSV N1/N4, described by Cosman et al., *Nature 312*:768, 1984 has been deposited as ATCC 39890. Additional useful mammalian expression vectors are described in EP-A-0367566, and in PCT application

WO 91/18982, incorporated by reference herein. The vectors may be derived from retroviruses. For expression of Fas-L, a type II protein lacking a native signal sequence, a heterologous signal sequence may be added, such as the signal sequence for interleukin-7 (IL-7) described in United States Patent 4,965,195, the signal sequence for interleukin-2 receptor described in Cosman et al., *Nature* 312:768 (1984); the interleukin-4 receptor signal peptide described in EP 367,566; the type I interleukin-1 receptor signal peptide described in U.S. Patent 4,968,607; and the type II interleukin-1 receptor signal peptide described in EP 460,846.

Purified Fas-L Protein

The present invention provides purified human and murine Fas-L proteins, which may be produced by recombinant expression systems as described above or purified from naturally occurring cells. The desired degree of purity depends on the intended use of the protein. A relatively high degree of purity is desired when the protein is to be administered *in vivo*, for example. Advantageously, Fas-L polypeptides are purified such that no protein bands corresponding to other proteins are detectable upon analysis by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). It will be recognized by one skilled in the pertinent field that multiple bands corresponding to Fas-L protein may be visualized by SDS-PAGE, due to differential glycosylation, differential post-translational processing, and the like, as discussed above. A preparation of Fas-L protein is considered to be purified as long as no bands corresponding to different (non-Fas-L) proteins are visualized. Most preferably the Fas-L is purified to substantial homogeneity, as indicated by a single protein band upon analysis by SDS-PAGE. The protein band may be visualized by silver staining or (if the protein is radiolabeled) autoradiography.

In one embodiment, the Fas-L protein is a human protein characterized by the N-terminal amino acid sequence MQQPFNYPYPQI (amino acids 1-12 of SEQ ID NO:2). Such protein is encoded by DNA characterized by the 5'-terminal sequence ATGCAGCAGCCCTTCAATTACCCATATCCCCAGATC (nucleotides 93-128 of SEQ ID NO:1).

One process for producing the Fas-L protein comprises culturing a host cell transformed with an expression vector comprising a DNA sequence that encodes Fas-L under conditions such that Fas-L is expressed. The Fas-L protein is then recovered from culture medium or cell extracts, depending upon the expression system employed. As the skilled artisan will recognize, procedures for purifying the recombinant Fas-L will vary according to such factors as the type of host cells employed and whether or not the Fas-L is secreted into the culture medium.

For example, when expression systems that secrete the recombinant protein are employed, the culture medium first may be concentrated using a commercially available

protein concentration filter, for example, an Amicon or Millipore Pellicon ultrafiltration unit. Following the concentration step, the concentrate can be applied to a purification matrix such as a gel filtration medium. Alternatively, an anion exchange resin can be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. The matrices can be acrylamide, agarose, dextran, cellulose or other types commonly employed in protein purification. Alternatively, a cation exchange step can be employed. Suitable cation exchangers include various insoluble matrices comprising sulfopropyl or carboxymethyl groups. Sulfopropyl groups are preferred. Finally, one or more reversed-phase high performance liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, (e.g., silica gel having pendant methyl or other aliphatic groups) can be employed to further purify Fas-L. Some or all of the foregoing purification steps, in various combinations, can be employed to provide a substantially homogeneous recombinant protein.

It is also possible to utilize an affinity column comprising the ligand binding domain of Fas to affinity-purify expressed Fas-L polypeptides. Fas-L polypeptides can be removed from an affinity column in a high salt elution buffer and then dialyzed into a lower salt buffer for use. Alternatively, the affinity column may comprise an antibody that binds Fas-L. Example 3 describes a procedure for employing the Fas-L protein of the present invention to generate monoclonal antibodies directed against Fas-L.

Recombinant protein produced in bacterial culture may be isolated by initial disruption of the host cells, centrifugation, extraction from cell pellets if an insoluble polypeptide, or from the supernatant fluid if a soluble polypeptide, followed by one or more concentration, salting-out, ion exchange, affinity purification or size exclusion chromatography steps. Finally, RP-HPLC can be employed for final purification steps. Microbial cells can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

Transformed yeast host cells are preferably employed to express Fas-L as a secreted polypeptide. This simplifies purification. Secreted recombinant polypeptide from a yeast host cell fermentation can be purified by methods analogous to those disclosed by Urdal et al. (*J. Chromatog. 296*:171, 1984). Urdal et al. describe two sequential, reversed-phase HPLC steps for purification of recombinant human IL-2 on a preparative HPLC column.

Antibodies

Antibodies that are immunoreactive with the Fas-L polypeptides disclosed herein are provided. Polyclonal and monoclonal antibodies may be prepared by conventional techniques. See, for example, *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses,* Kennet et al. (eds.), Plenum Press, New York (1980); and

Antibodies: A Laboratory Manual, Harlow and Land (eds.), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1988). Production of monoclonal antibodies that are immunoreactive with Fas-L is further illustrated in example 3 below.

The Fas-L proteins of the present invention or antigenic fragments thereof may be employed as immunogens to generate polyclonal or monoclonal antibodies specific to the Fas-L immunogens. In one embodiment, a soluble Fas-L polypeptide is the immunogen. Cells expressing recombinant Fas-L on the cell surface also may be employed as immunogens. As a further alternative, a fusion protein comprising a Fas-L polypeptide (e.g., a fusion protein comprising the extracellular domain of Fas-L and an antibody Fc region polypeptide) is the immunogen.

Antigen-binding fragments of such antibodies, which may be produced by conventional techniques, are also encompassed by the present invention. Examples of such fragments include, but are not limited to, Fab, F(ab'), and $F(ab')_2$ fragments. Antibody fragments and derivatives produced by genetic engineering techniques are also provided.

The monoclonal antibodies of the present invention include chimeric antibodies, e.g., humanized versions of murine monoclonal antibodies. Such humanized antibodies may be prepared by known techniques, and offer the advantage of reduced immunogenicity when the antibodies are administered to humans. In one embodiment, a humanized monoclonal antibody comprises the variable region of a murine antibody (or just the antigen binding site thereof) and a constant region derived from a human antibody. Alternatively, a humanized antibody fragment may comprise the antigen biding site of a murine monoclonal antibody and a variable region fragment (lacking the antigen-binding site) derived from a human antibody. Procedures for the production of chimeric and further engineered monoclonal antibodies include those described in Riechmann et al. (*Nature 332*:323, 1988), Liu et al. (PNAS 84:3439, 1987), Larrick et al. (*Bio/Technology 7*:934, 1989), and Winter and Harris (*TIPS 14*:139, May, 1993).

One embodiment of the present invention is directed to monoclonal antibodies that block the binding of Fas-L to cell surface Fas antigen. Monoclonal antibodies can be screened in conventional assays for the ability to block binding of Fas-L to cells bearing Fas antigen.

Properties and Uses of Fas-L and Antibodies that Bind Fas-L

The Fas antigen has been reported to mediate apoptosis and is believed to play a role in clonal deletion of self-reactive T-cells. (Itoh et al., *Cell* 66:233, 1991; Watanabe-Fukunage et al., *Nature* 356:314, 1992). Mice carrying the *lpr* mutation do not produce functional Fas protein capable of transducing the apoptotic signal (Watanabe-Fukunage et al., *Nature, supra*). These mice develop lymphoproliferative disorders characterized by the accumulation of $CD4^-CD8^-$ thymocytes in lymph nodes and the spleen,

hypergammaglobulinaemia, autoantibody production, rheumatoid factor, arthritis, and glomerulonephritis. (Watanabe-Fukunage et al., *Nature*, *supra*). Alderson et al. (J. Exp. Med., 178: 2231, 1993) report that, in addition to a role in induction of apoptosis in certain transformed cell lines, the Fas protein may play an important role in the activation and proliferation of normal T cells.

Studies employing the Fas-L and anti-Fas-L antibodies of the present invention thus may provide further insight into the development of self-tolerance by the immune system and the etiology of autoimmune diseases. Interaction of Fas with Fas-L and the mechanism of signal transduction may be investigated. The antibodies may be used to block the interaction of Fas with Fas-L. Fas-L and antibodies reactive therewith may be used to promote or inhibit, respectively, biological effects that are mediated by the binding of Fas-L to Fas. The Fas-L and antibodies thus may be used to regulate such biological effects, *in vitro* or *in vivo*.

The Fas-L DNA and protein of the present invention are useful as research tools in elucidating the etiology of autoimmune disorders. Such disorders include, but are not limited to, systemic lupus erythematosus (SLE), immunoblastive lymphadenopathy (IBL), angioimmunoblastic lymphadenopathy (AIL), lymphogranulomatosis X (LgX), rheumatoid arthritis, diabetes, multiple schlerosis, and allergies. The use of Fas-L in treating graft versus host disease also may be explored.

Fas-L may be employed to induce apoptosis in cells bearing the Fas antigen. The Fas-L thus is a research reagent useful in studies of the mechanisms by which apoptosis occurs.

The Fas-L of the present invention may be used in developing treatments for disorders mediated (directly or indirectly) by Fas-L. Alternatively, a therapeutically effective amount of purified Fas-L protein may be administered to a patient afflicted with a disorder caused by defective or insufficient amounts of Fas-L. As a further alternative, the Fas-L DNA sequences may be employed in developing a gene therapy approach to treating such disorders.

The present invention provides pharmaceutical compositions comprising purified Fas-L and a physiologically acceptable carrier, diluent, or excipient. Such carriers, excipients and diluents will be nontoxic to recipients at the dosages and concentrations employed. Such compositions may comprise buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids, carbohydrates including glucose, sucrose or dextrins, chelating agents such as EDTA, glutathione and other stabilizers and excipients commonly employed in pharmaceutical compositions. Neutral buffered saline or saline mixed with conspecific serum albumin are exemplary appropriate diluents. The composition may be formulated as a lyophilizate using appropriate excipient solutions (e.g. sucrose) as diluents. Additional examples of suitable

carriers and their formulations are described in Remington's Pharmaceutical Sciences, 16th ed., 1980, Mack Publishing Company. Appropriate dosages and the frequency of administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the size and condition of the patient and so forth.

For therapeutic use, purified proteins of the present invention are administered to a patient, preferably a human, for treatment in a manner appropriate to the indication. Thus, for example, the pharmaceutical compositions can be administered by bolus injection, continuous infusion, sustained release from implants, or other suitable technique.

The Fas-L protein employed in the pharmaceutical compositions preferably is purified such that the Fas-L protein is substantially free of other proteins of natural or endogenous origin, desirably containing less than about 1% by mass of protein contaminants residual of production processes. Such compositions, however, can contain other proteins added as stabilizers, carriers, excipients or co-therapeutics. The Fas-L protein preferably is purified to substantial homogeneity, i.e., is detectable as a single protein band when analyzed by SDS-polyacrylamide gel electrophoresis.

The Fas-L polypeptides of the present invention also may be employed in *in vitro* assays for detection of Fas or Fas-L or the interactions thereof. The Fas-L of the present invention can be used in a binding assay to detect cells expressing Fas. For example, Fas-L or an extracellular domain or a fragment thereof can be conjugated to a detectable moiety such as ^{125}I. Radiolabeling with ^{125}I can be performed by any of several standard methodologies that yield a functional ^{125}I-Fas-L molecule labeled to high specific activity. Alternatively, another detectable moiety such as an enzyme that can catalyze a colorometric or fluorometric reaction, biotin or avidin may be used. Cells to be tested for Fas expression can be contacted with labeled Fas-L. After incubation, unbound labeled Fas-L is removed and binding is measured using the detectable moiety.

The Fas ligand proteins disclosed herein also may be employed to measure the biological activity of Fas protein in terms of binding affinity for Fas-L. To illustrate, Fas-L may be employed in a binding affinity study to measure the biological activity of a Fas protein that has been stored at different temperatures, or produced in different cell types. The biological activity of a Fas protein thus can be ascertained before it is used in a research study, for example.

Fas-L proteins find use as reagents that may be employed by those conducting "quality assurance" studies, e.g., to monitor shelf life and stability of Fas protein under different conditions. Fas ligands may be used in determining whether biological activity is retained after modification of a Fas protein (e.g., chemical modification, truncation, mutation, etc.). The binding affinity of the modified Fas protein for a Fas-L is compared to

that of an unmodified Fas protein to detect any adverse impact of the modification on biological activity of Fas.

A different use of a Fas ligand is as a reagent in protein purification procedures. Fas-L or Fas-L/Fc fusion proteins may be attached to a solid support material by conventional techniques and used to purify Fas by affinity chromatography. Antibodies of the present invention may be used to study the effects of inhibiting the biological activity of Fas-L, to detect the presence of Fas-L *in vitro* or *in vivo* , and in affinity chromatography for purifying Fas-L, for example. Antibodies of the present invention also find use when inhibition of Fas-L-mediated apoptosis of Fas antigen-bearing cells is desired. Antibodies (preferably monoclonal antibodies) that block binding of endogenous Fas-L to the Fas antigen on the cells are employed for this purpose. In one embodiment, the antibodies bind to cell surface Fas-L and inhibit binding of the cell surface Fas-L to Fas antigen on target cells.

One embodiment of the invention is directed to a method of treating disorders mediated by the interaction of Fas-L and Fas, comprising administering a composition comprising a therapeutically effective amount of an antibody that inhibits binding of a Fas-L to Fas, and a suitable diluent or carrier, to a patient afflicted with such a disease. The antibody preferably is a monoclonal antibody. Diseases for which therapeutic treatment with Fas-L specific antibodies of the present invention may be beneficial include, but are not limited to, SLE, rheumatoid arthritis, lyme disease, idiopathic CD4+ T lymphocytopenia, and the effects of human immunodeficiency virus (HIV) infection.

Activated human T cells are induced to undergo programmed cell death (apoptosis) upon triggering through the CD3/T cell receptor complex, a process termed activated-induced cell death (AICD). AICD has been observed in T cells freshly isolated from HIV-infected, but not from uninfected, individuals (Groux et al., *J Exp. Med.*, *175*:331, 1992; Meyaard et al., *Science*, *257*:217, 1992). Thus, apoptosis may play a role in the depletion of CD4+ T cells and the progression to AIDS in HIV infected individuals.

For Fas-L to play a role in T cell depletion in HIV infection, two criteria must be fulfilled. First, Fas-L must be expressed, and therefore T cells must be activated by exposure to antigen. Second, T cells must be "primed" to become susceptible to Fas mediated apoptosis. In HIV+ patients, such priming of T cells may come from crosslinking of CD4 by GP-120/anti-GP-120 complexes. Such complexes have been shown to prime normal CD4+ T cells to undergo AICD *in vitro* and to cause T cells to undergo apoptosis in mice that express a human CD4 transgene (Wang et al, *Eur. J. Immunol.*, *24*:1553, 1994). In addition, CD4+ T cells undergo apoptotic cell death in mice treated with antibody to CD4, but this phenomenon does not occur in Fas deficient LPR mice (Wang et al., *Eur. J. Immunol.*, *24*:1549, 1994). The AICD seen in activated normal human T cells (such as PL-1 cells) and in freshly isolated T cells from HIV+ individuals is

qualitatively identical. Therapeutic intervention for HIV-infected individuals with an antibody that inhibits the interaction of Fas-L with Fas thus may be possible.

For therapeutic use, purified antibodies of the present invention are administered to a patient, such as a human, for treatment in a manner appropriate to the indication. Compositions comprising an antibody that binds Fas-L and a suitable diluent or carrier are provided herein. Diluents, carriers, and other components of suitable compositions are as described above. In one embodiment, a pharmaceutical composition comprises a therapeutically effective amount of a monoclonal antibody that inhibits binding of Fas-L to cell surface Fas antigen, and a suitable diluent or carrier.

Nucleic Acid Fragments

The present invention further provides fragments of the Fas-L nucleotide sequences presented herein. In one embodiment, such fragments comprise at least about 14 consecutive nucleotides of the human Fas-L cDNA isolated in example 1 (and deposited as ATCC 69527). Such fragments may comprise at least 14 nucleotides of the coding region of the DNA sequence of SEQ ID NO:1. DNA and RNA complements of said fragments are provided herein, along with both single-stranded and double-stranded forms of the Fas-L DNA.

Among the uses of such Fas-L nucleic acid fragments is use as a probe. As one example, a probe corresponding to the extracellular domain of Fas-L may be employed. The probes find use in detecting the presence of Fas-L nucleic acids in *in vitro* assays and in such procedures as Northern and Southern blots. Cell types expressing Fas-L can be identified as well. Such procedures are well known, and the skilled artisan can choose a probe of suitable length, depending on the particular intended application.

Other useful fragments of the Fas-L nucleic acids are antisense or sense oligonucleotides comprising a single-stranded nucleic acid sequence (either RNA or DNA) capable of binding to target Fas-L mRNA (sense) or Fas-L DNA (antisense) sequences. Antisense or sense oligonucleotides, according to the present invention, comprise a fragment of the coding region of Fas-L cDNA. Such a fragment generally comprises at least about 14 nucleotides, preferably from about 14 to about 30 nucleotides. The ability to create an antisense or a sense oligonucleotide, based upon a cDNA sequence for a given protein is described in, for example, Stein and Cohen, *Cancer Res.* 48:2659, 1988 and van der Krol et al., *BioTechniques* 6:958, 1988.

Binding of antisense or sense oligonucleotides to target nucleic acid sequences results in the formation of duplexes that block translation (RNA) or transcription (DNA) by one of several means, including enhanced degradation of the duplexes, premature termination of transcription or translation, or by other means. The antisense oligonucleotides thus may be used to block expression of Fas-L proteins. Antisense or

sense oligonucleotides further comprise oligonucleotides having modified sugar-phosphodiester backbones (or other sugar linkages, such as those described in WO91/06629) and wherein such sugar linkages are resistant to endogenous nucleases. Such oligonucleotides with resistant sugar linkages are stable *in vivo* (i.e., capable of resisting enzymatic degradation) but retain sequence specificity to be able to bind to target nucleotide sequences. Other examples of sense or antisense oligonucleotides include those oligonucleotides which are covalently linked to organic moieties, such as those described in WO 90/10448, and other moieties that increases affinity of the oligonucleotide for a target nucleic acid sequence, such as poly-(L-lysine). Further still, intercalating agents, such as ellipticine, and alkylating agents or metal complexes may be attached to sense or antisense oligonucleotides to modify binding specificities of the antisense or sense oliginucleotide for the target nucleotide sequence. Antisense or sense oligonucleotides may be introduced into a cell containing the target nucleic acid sequence by any gene transfer method, including, for example, $CaPO_4$-mediated DNA transfection, electroporation, or other gene transfer vectors such as Epstein-Barr virus. Antisense or sense oligonucleotides are preferably introduced into a cell containing the target nucleic acid sequence by insertion of the antisense or sense oligonucleotide into a suitable retroviral vector, then contacting the cell with the retrovirus vector containing the inserted sequence, either *in vivo* or *ex vivo*. Suitable retroviral vectors include, but are not limited to, the murine retrovirus M-MuLV, N2 (a retrovirus derived from M-MuLV), or or the double copy vectors designated DCT5A, DCT5B and DCT5C (see PCT Application WO 90/13641). Alternatively, other promotor sequences may be used to express the oligonucleotide.

Sense or antisense oligonucleotides may also be introduced into a cell containing the target nucleotide sequence by formation of a conjugate with a ligand binding molecule, as described in WO 91/04753. Suitable ligand binding molecules include, but are not limited to, cell surface receptors, growth factors, other cytokines, or other ligands that bind to cell surface receptors. Preferably, conjugation of the ligand binding molecule does not substantially interfere with the ability of the ligand binding molecule to bind to its corresponding molecule or receptor, or block entry of the sense or antisense oligonucleotide or its conjugated version into the cell.

Alternatively, a sense or an antisense oligonucleotide may be introduced into a cell containing the target nucleic acid sequence by formation of an oligonucleotide-lipid complex, as described in WO 90/10448. The sense or antisense oligonucleotide-lipid complex is preferably dissociated within the cell by an endogenous lipase.

The following examples are provided to illustrate particular embodiments and not to limit the scope of the invention.

EXAMPLE 1: Isolation of Murine Fas-L DNA

A 180bp fragment of murine Fas-L DNA was isolated by polymerase chain reaction (PCR), as follows. The template in the PCR was cDNA prepared from RNA derived from murine peripheral blood lymphocytes (PBL) that had been stimulated for 18 hours with phorbol 12-myristate 13-acetate (PMA, Sigma Chemical Co.). The 5' and 3' primers were oligonucleotides based on the rat Fas-L DNA sequence of Suda et al. (*Cell*, 75:1169, 1993). The primers define a 180 bp fragment near the 3' end of the Fas-L DNA. The PCR was conducted by conventional procedures. See, for example, Saiki et al., *Science* 239:487 (1988); *Recombinant DNA Methodology*, Wu et al., eds., Academic Press, Inc., San Diego (1989), pp. 189-196; and *PCR Protocols: A Guide to Methods and Applications*, Innis et al., eds., Academic Press, Inc. (1990).

The 180bp murine Fas-L DNA fragment isolated and amplified by PCR comprised nucleotides 643-822 of the murine Fas-L DNA sequence presented in SEQ ID NO:4. This DNA fragment was labeled with ^{32}P for use as a probe. A random primed DNA labeling kit (Amersham, Arlington Heights, Illinois) was used to radiolabel the probe.

A full-length murine Fas-L DNA was isolated as follows. A murine hybridoma cell line designated SC9 was generated by fusion of the T-cell hybridoma cell line BW5147 with the ∝B10.5 CTL line described by Lynch, D. and R. Miller (*J. Exp. Med.* 179:31, 1994). The ∝B10.5 CTL cells were stimulated with PMA and ionomycin (cultured in 500 ng/ml PMA and 3 μg/ml ionomycin for two hours at 37°C) prior to fusion. The SC9 hybridoma cell line was selected for Fas-L expression. Briefly, cells resulting from the fusion were assayed for Fas-L expression, including an assay for binding to a soluble Fas/Fc fusion protein. SC9 was isolated by fluorescence activated cell sorting (FACS), based on the cells binding to Fas/Fc.

The SC9 cells were stimulated with PMA and ionomycin as above, and RNA was extracted therefrom. cDNA was prepared from the RNA by conventional techniques and inserted and packaged into phage vector λgt10 (Gigapak® Stratagene Cloning Systems, La Jolla, CA). The resulting cDNA library was screened using the 180 bp murine Fas-L DNA fragment prepared above as a probe.

A positive clone was isolated and the nucleotide sequence of the cDNA insert was determined. This murine Fas-L nucleotide sequence is presented in SEQ ID NO:4, and the amino acid sequence encoded thereby is presented in SEQ ID NO:5. The protein comprises a cytoplasmic domain (amino acids 1-78), a transmembrane region (amino acids 79-103), and an extracellular domain (amino acids 104-279).

EXAMPLE 2: Isolation of Human Fas-L DNA

Human Fas-L cDNA was isolated from a cDNA library derived from stimulated human peripheral blood lymphocytes. The procedure was as follows.

Peripheral blood lymphocytes (PBL) were obtained from normal human volunteers and treated with 10 ng/ml of OKT3 (an anti-CD3 antibody), and 10 ng/ml of human IL-2 for six days. The PBL cells were washed and stimulated with 500 ng/ml ionomycin (Calbiochem) and 10 ng/ml PMA for four hours. Messenger RNA was isolated from the stimulated PBL cells. cDNA synthesized on the mRNA template was packaged into λgt10 phage vectors (Gigapak® Stratagene Cloning Systems, La Jolla, CA) according to manufacturer's instructions. Recombinant phage were then plated on *E. coli* strain KW251 and screened using standard plaque hybridization techniques.

The ^{32}P-labeled 180bp murine Fas-L probe prepared in example 1 was hybridized to the recombinant DNA phage at 37°C overnight. Hybridization was followed by washing twice with 6X SSC at room temperature, then washing twice with 2X SSC/0.1% SDS at 55°C. Positive (hybridizing) plaques were visualized by autoradiography.

Two of the positive plaques were purified and the inserts were amplified by PCR. Oligonucleotides that flank the cloning site (the EcoRI site of λgt10 into which the cDNA had been inserted) were employed as primers for the PCR. The cDNA inserts of the recombinant phage from both positive plaques were about 2.0kb in length. The PCR products from one clone were digested with EcoRI, and the desired fragment (containing the cDNA insert) was ligated into an EcoRI-digested cloning vector pBLUESCRIPT®SK (Stratagene Cloning Systems, La Jolla, CA). *E. coli* strain DH5α cells containing the resulting recombinant vector (designated human Fas-L/pBS) were deposited with the American Type Culture Collection, Rockville, Maryland, U.S.A., on January 5, 1994, and assigned accession no. ATCC 69527. The deposit was made under the terms of the Budapest Treaty.

The DNA sequence of this human Fas-L DNA is presented in SEQ ID NO:1. The amino acid sequence encoded by the isolated DNA is presented in SEQ ID NO:2. This human Fas-L protein comprises an N-terminal cytoplasmic domain (amino acids 1-80), a transmembrane region (amino acids 81-105), and an extracellular domain (amino acids 106-281). As will be understood by the skilled artisan, the transmembrane region is identified in accordance with conventional criteria for identifying that type of hydrophobic domain. The exact boundaries of the transmembrane region may vary slightly (most likely by no more than five amino acids on either end) from those presented above. Computer programs useful for identifying such hydrophobic regions in proteins are available.

The human Fas-L coding region DNA sequence (nucleotides 93-938 of SEQ ID NO: 1) is 82.89% identical to the rat Fas-L coding region DNA sequence presented in Suda et al. (*Cell*, 75: 1169, 1993). The human Fas-L amino acid sequence of SEQ ID NO: 2 is 77.98% identical to that of rat Fas-L. The degree of homology between human and rat Fas-L was neither known nor predictable prior to isolation of the human Fas-L cDNA disclosed herein.

The human Fas-L cDNA was excised from pBLUESCRIPT®SK by digestion with SalI and Not I. The desired fragment was isolated and ligated into a mammalian expression vector pDC409 which had been digested with SalI and Not I.

pDC409 is derived from an expression vector designated pDC406, and differs from pDC406 in that a Bgl II site outside the mcs has been deleted so that the mcs Bgl II site is unique. Two Pme 1 sites and one Srf 1 site have been added to the mcs, and three stop codons (TAG) have been positioned downstream of the mcs to function in all three reading frames. A T7 primer/promoter has been added to aid in the DNA sequencing process.

Plasmid pDC406, which has been described by McMahan et al. (*EMBO J.* 10:2821, 1991) and in PCT application WO 91/18982 (hereby incorporated by reference), is an expression vector for use in mammalian cells, but is also replicable in *E. coli* cells.

pDC406 contains origins of replication derived from SV40, Epstein-Barr virus and pBR322 and is a derivative of HAV-EO described by Dower et al., *J. Immunol.* 142:4314 (1989). pDC406 differs from HAV-EO by the deletion of an intron present in the adenovirus 2 tripartite leader sequence in HAV-EO. DNA inserted into a multiple cloning site (containing a number of restriction endonuclease cleavage sites) is transcribed and translated using regulatory elements derived from HIV and adenovirus. The vector contains a gene that confers ampicillin resistance.

The recombinant vector (pDC409 containing human Fas-L DNA) was transfected into the monkey kidney cell line CV-1/EBNA-1 (ATCC CRL 10478). The CV-1/EBNA cell line was derived by transfection of the CV-1 cell line (ATCC CCL 70) with a gene encoding Epstein-Barr virus nuclear antigen-1 (EBNA-1) that constitutively expresses EBNA-1 driven from the human CMV intermediate-early enhancer/promoter as described by McMahan et al., *supra.* The EBNA-1 gene allows for episomal replication of expression vectors, such as pDC409, that contain the EBV origin of replication.

CV1-EBNA-1 cells transformed with the recombinant vector are cultivated in roller bottles to permit express of the Fas-L protein. Other suitable mammalian expression vectors may be substituted for pDC409.

EXAMPLE 3

This example illustrates the preparation of monoclonal antibodies to Fas-L. Fas-L is expressed in mammalian host cells such as COS-7 or CVI-EBNA cells and purified using Fas/Fc affinity chromatography as described herein. The Fas-L may be the human or murine Fas-L described herein, or an immunogenic fragment thereof, e.g., the extracellular domain thereof.

Purified Fas-L can be used to generate monoclonal antibodies against Fas-L using conventional techniques, for example, those techniques described in U.S. Patent 4,411,993. Briefly, mice are immunized with Fas-L as an immunogen emulsified in complete Freund's adjuvant, and injected in amounts ranging from 10-100 μg subcutaneously or intraperitoneally. Ten to twelve days later, the immunized animals are boosted with additional Fas-L emulsified in incomplete Freund's adjuvant. Mice are periodically boosted thereafter on a weekly to bi-weekly immunization schedule. Serum samples are periodically taken by retro-orbital bleeding or tail-tip excision for testing by dot blot assay or ELISA (Enzyme-Linked Immunosorbent Assay), for Fas-L antibodies.

Following detection of an appropriate antibody titer, positive animals are provided one last intravenous injection of Fas-L in saline. Three to four days later, the animals are sacrificed, spleen cells harvested, and spleen cells are fused to a murine myeloma cell line (e.g., NS1 or Ag 8.653). Fusions generate hybridoma cells, which are plated in multiple microtiter plates in a HAT (hypoxanthine, aminopterin and thymidine) selective medium to inhibit proliferation of non-fused cells, myeloma hybrids, and spleen cell hybrids.

The hybridoma cells are screened by ELISA for reactivity against purified Fas-L by adaptations of the techniques disclosed in Engvall et al., *Immunochem.* 8:871, 1971 and in U.S. Patent 4,703,004. Positive hybridoma cells can be injected intraperitoneally into syngeneic BALB/c mice to produce ascites containing high concentrations of anti-Fas-L monoclonal antibodies. Alternatively, hybridoma cells can be grown *in vitro* in flasks or roller bottles by various techniques. Monoclonal antibodies produced in mouse ascites can be purified by ammonium sulfate precipitation, followed by gel exclusion chromatography. Alternatively, affinity chromatography based upon binding of antibody to protein A or protein G can also be used, as can affinity chromatography based upon binding to Fas-L.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: GOODWIN, Raymond G.

(ii) TITLE OF INVENTION: Ligand That Binds Fas Antigen

(iii) NUMBER OF SEQUENCES: 5

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Immunex Corporation
(B) STREET: 51 University Street
(C) CITY: Seattle
(D) STATE: WA
(E) COUNTRY: US
(F) ZIP: 98101

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: Apple Macintosh
(C) OPERATING SYSTEM: Apple 7.1
(D) SOFTWARE: Microsoft Word, Version 5.1a

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 06-JAN-1995
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 08/179,138
(B) FILING DATE: 07-JAN-1994

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Anderson, Kathryn A.
(B) REGISTRATION NUMBER: 32,172
(C) REFERENCE/DOCKET NUMBER: 2805-WO

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1841 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 93..938

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

GAATTCCGGG TCCCGTCCTT GACACCTCAG CCTCTACAGG ACTGAGAAGA AGTAAAACCG 60

```
TTTGCTGGGG CTGGCCTGAC TCACCAGCTG CC ATG CAG CAG CCC TTC AAT TAC      113
                                    Met Gln Gln Pro Phe Asn Tyr
                                      1               5

CCA TAT CCC CAG ATC TAC TGG GTG GAC AGC AGT GCC AGC TCT CCC TGG      161
Pro Tyr Pro Gln Ile Tyr Trp Val Asp Ser Ser Ala Ser Ser Pro Trp
        10                  15                  20

GCC CCT CCA GGC ACA GTT CTT CCC TGT CCA ACC TCT GTG CCC AGA AGG      209
Ala Pro Pro Gly Thr Val Leu Pro Cys Pro Thr Ser Val Pro Arg Arg
    25                  30                  35

CCT GGT CAA AGG AGG CCA CCA CCA CCA CCG CCA CCG CCA CCA CTA CCA      257
Pro Gly Gln Arg Arg Pro Pro Pro Pro Pro Pro Pro Pro Pro Leu Pro
 40                  45                  50                  55

CCT CCG CCG CCG CCG CCA CCA CTG CCT CCA CTA CCG CTG CCA CCC CTG      305
Pro Pro Pro Pro Pro Pro Pro Leu Pro Pro Leu Pro Leu Pro Pro Leu
                60                  65                  70

AAG AAG AGA GGG AAC CAC AGC ACA GGC CTG TGT CTC CTT GTG ATG TTT      353
Lys Lys Arg Gly Asn His Ser Thr Gly Leu Cys Leu Leu Val Met Phe
            75                  80                  85

TTC ATG GTT CTG GTT GCC TTG GTA GGA TTG GGC CTG GGG ATG TTT CAG      401
Phe Met Val Leu Val Ala Leu Val Gly Leu Gly Leu Gly Met Phe Gln
        90                  95                  100

CTC TTC CAC CTA CAG AAG GAG CTG GCA GAA CTC CGA GAG TCT ACC AGC      449
Leu Phe His Leu Gln Lys Glu Leu Ala Glu Leu Arg Glu Ser Thr Ser
    105                 110                 115

CAG ATG CAC ACA GCA TCA TCT TTG GAG AAG CAA ATA GGC CAC CCC AGT      497
Gln Met His Thr Ala Ser Ser Leu Glu Lys Gln Ile Gly His Pro Ser
120                 125                 130                 135

CCA CCC CCT GAA AAA AAG GAG CTG AGG AAA GTG GCC CAT TTA ACA GGC      545
Pro Pro Pro Glu Lys Lys Glu Leu Arg Lys Val Ala His Leu Thr Gly
                140                 145                 150

AAG TCC AAC TCA AGG TCC ATG CCT CTG GAA TGG GAA GAC ACC TAT GGA      593
Lys Ser Asn Ser Arg Ser Met Pro Leu Glu Trp Glu Asp Thr Tyr Gly
            155                 160                 165

ATT GTC CTG CTT TCT GGA GTG AAG TAT AAG AAG GGT GGC CTT GTG ATC      641
Ile Val Leu Leu Ser Gly Val Lys Tyr Lys Lys Gly Gly Leu Val Ile
        170                 175                 180

AAT GAA ACT GGG CTG TAC TTT GTA TAT TCC AAA GTA TAC TTC CGG GGT      689
Asn Glu Thr Gly Leu Tyr Phe Val Tyr Ser Lys Val Tyr Phe Arg Gly
    185                 190                 195

CAA TCT TGC AAC AAC CTG CCC CTG AGC CAC AAG GTC TAC ATG AGG AAC      737
Gln Ser Cys Asn Asn Leu Pro Leu Ser His Lys Val Tyr Met Arg Asn
200                 205                 210                 215

TCT AAG TAT CCC CAG GAT CTG GTG ATG ATG GAG GGG AAG ATG ATG AGC      785
Ser Lys Tyr Pro Gln Asp Leu Val Met Met Glu Gly Lys Met Met Ser
                220                 225                 230
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TAC TGC ACT ACT GGG CAG ATG TGG GCC CGC AGC AGC TAC CTG GGG GCA      833
Tyr Cys Thr Thr Gly Gln Met Trp Ala Arg Ser Ser Tyr Leu Gly Ala
            235                 240                 245

GTG TTC AAT CTT ACC AGT GCT GAT CAT TTA TAT GTC AAC GTA TCT GAG      881
Val Phe Asn Leu Thr Ser Ala Asp His Leu Tyr Val Asn Val Ser Glu
        250                 255                 260

CTC TCT CTG GTC AAT TTT GAG GAA TCT CAG ACG TTT TTC GGC TTA TAT      929
Leu Ser Leu Val Asn Phe Glu Glu Ser Gln Thr Phe Phe Gly Leu Tyr
    265                 270                 275

AAG CTC TAAGAGAAGC ACTTTGGGAT TCTTTCCATT ATGATTCTTT GTTACAGGCA      985
Lys Leu
280

CCGAGAATGT TGTATTCAGT GAGGGTCTTC TTACATGCAT TTGAGGTCAA GTAAGAAGAC   1045

ATGAACCAAG TGGACCTTGA GACCACAGGG TTCAAAATGT CTGTAGCTCC TCAACTCACC   1105

TAATGTTTAT GAGCCAGACA AATGGAGGAA TATGACGGAA GAACATAGAA CTCTGGGCTG   1165

CCATGTGAAG AGGGAGAAGC ATGAAAAAGC AGCTACCAGG TGTTCTACAC TCATCTTAGT   1225

GCCTGAGAGT ATTTAGGCAG ATTGAAAAGG ACACCTTTTA ACTCACCTCT CAAGGTGGGC   1285

CTTGCTACCT CAAGGGGGAC TGTCTTTCAG ATACATGGTT GTGACCTGAG GATTTAAGGG   1345

ATGGAAAAGG AAGACTAGAG GCTTGCATAA TAAGCTAAAG AGGCTGAAAG AGGCCAATGC   1405

CCCACTGGCA GCATCTTCAC TTCTAAATGC ATATCCTGAG CCATCGGTGA AACTAACAGA   1465

TAAGCAAGAG AGATGTTTTG GGGACTCATT TCATTCCTAA CACAGCATGT GTATTTCCAG   1525

TGCAATTGTA GGGGTGTGTG TGTGTGTGTG TGTGTGTGTG TGTGTATGAC TAAAGAGAGA   1585

ATGTAGATAT TGTGAAGTAC ATATTAGGAA AATATGGGTT GCATTTGGTC AAGATTTTGA   1645

ATGCTTCCTG ACAATCAACT CTAATAGTGC TTAAAAATCA TTGATTGTCA GCTACTAATG   1705

ATGTTTTCCT ATAATATAAT AAATATTTAT GTAGATGTGC ATTTTTGTGA AATGAAAACA   1765

TGTAATAAAA AGTATATGTT AGGATACAAA TAAAAAAAAA AAAAAAAAAA AAAAAAAAAA   1825

AAAAAAACCG GAATTC                                                   1841
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 281 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

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

```
Met Gln Gln Pro Phe Asn Tyr Pro Tyr Pro Gln Ile Tyr Trp Val Asp
 1               5                  10                  15
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Ser Ser Ala Ser Ser Pro Trp Ala Pro Pro Gly Thr Val Leu Pro Cys
            20                  25                  30

Pro Thr Ser Val Pro Arg Arg Pro Gly Gln Arg Arg Pro Pro Pro Pro
        35                  40                  45

Pro Pro Pro Pro Pro Leu Pro Pro Pro Pro Pro Pro Pro Pro Leu Pro
    50                  55                  60

Pro Leu Pro Leu Pro Pro Leu Lys Lys Arg Gly Asn His Ser Thr Gly
65                  70                  75                  80

Leu Cys Leu Leu Val Met Phe Phe Met Val Leu Val Ala Leu Val Gly
                85                  90                  95

Leu Gly Leu Gly Met Phe Gln Leu Phe His Leu Gln Lys Glu Leu Ala
            100                 105                 110

Glu Leu Arg Glu Ser Thr Ser Gln Met His Thr Ala Ser Ser Leu Glu
        115                 120                 125

Lys Gln Ile Gly His Pro Ser Pro Pro Pro Glu Lys Lys Glu Leu Arg
    130                 135                 140

Lys Val Ala His Leu Thr Gly Lys Ser Asn Ser Arg Ser Met Pro Leu
145                 150                 155                 160

Glu Trp Glu Asp Thr Tyr Gly Ile Val Leu Leu Ser Gly Val Lys Tyr
                165                 170                 175

Lys Lys Gly Gly Leu Val Ile Asn Glu Thr Gly Leu Tyr Phe Val Tyr
            180                 185                 190

Ser Lys Val Tyr Phe Arg Gly Gln Ser Cys Asn Asn Leu Pro Leu Ser
        195                 200                 205

His Lys Val Tyr Met Arg Asn Ser Lys Tyr Pro Gln Asp Leu Val Met
    210                 215                 220

Met Glu Gly Lys Met Met Ser Tyr Cys Thr Thr Gly Gln Met Trp Ala
225                 230                 235                 240

Arg Ser Ser Tyr Leu Gly Ala Val Phe Asn Leu Thr Ser Ala Asp His
                245                 250                 255

Leu Tyr Val Asn Val Ser Glu Leu Ser Leu Val Asn Phe Glu Glu Ser
            260                 265                 270

Gln Thr Phe Phe Gly Leu Tyr Lys Leu
        275                 280
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Asp Tyr Lys Asp Asp Asp Asp Lys
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1171 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION: 31..870

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GAAGGAAACC CTTTCCTGGG GCTGGGTGCC ATG CAG CAG CCC ATG AAT TAC CCA      54
                                 Met Gln Gln Pro Met Asn Tyr Pro
                                   1               5

TGT CCC CAG ATC TTC TGG GTA GAC AGC AGT GCC ACT TCA TCT TGG GCT      102
Cys Pro Gln Ile Phe Trp Val Asp Ser Ser Ala Thr Ser Ser Trp Ala
     10                  15                  20

CCT CCA GGG TCA GTT TTT CCC TGT CCA TCT TGT GGG CCT AGA GGG CCG      150
Pro Pro Gly Ser Val Phe Pro Cys Pro Ser Cys Gly Pro Arg Gly Pro
 25                  30                  35                  40

GAC CAA AGG AGA CCG CCA CCT CCA CCA CCA CCT GTG TCA CCA CTA CCA      198
Asp Gln Arg Arg Pro Pro Pro Pro Pro Pro Pro Val Ser Pro Leu Pro
                 45                  50                  55

CCG CCA TCA CAA CCA CTC CCA CTG TCG CCA CTG ACC CCT CTA AAG AAG      246
Pro Pro Ser Gln Pro Leu Pro Leu Ser Pro Leu Thr Pro Leu Lys Lys
             60                  65                  70

AAG GAC CAC AAC ACA AAT CTG TGG CTA CCG GTG GTA TTT TTC ATG GTT      294
Lys Asp His Asn Thr Asn Leu Trp Leu Pro Val Val Phe Phe Met Val
         75                  80                  85

CTG GTG GGT CTG GTT GGA ATG GGA TTA GGA ATG TAT CAG CTC TTC CAC      342
Leu Val Gly Leu Val Gly Met Gly Leu Gly Met Tyr Gln Leu Phe His
     90                  95                  100

CTG CAG AAG GAA CTG GCA GAA CTC CGT GAG TTC ACC AAC CAA AGC CTT      390
Leu Gln Lys Glu Leu Ala Glu Leu Arg Glu Phe Thr Asn Gln Ser Leu
105                 110                 115                 120
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AAA GTA TCA TCT TTT GAA AAG CAA ATA GCC AAC CCC AGT ACA CCC TCT      438
Lys Val Ser Ser Phe Glu Lys Gln Ile Ala Asn Pro Ser Thr Pro Ser
                125                 130                 135

GAA AAA AAA GAG CCG AGG AGT GTG GCC CAT TTA ACA GGG AAC CCC CAC      486
Glu Lys Lys Glu Pro Arg Ser Val Ala His Leu Thr Gly Asn Pro His
            140                 145                 150

TCA AGG TCC ATC CCT CTG GAA TGG GAA GAC ACA TAT GGA ACC GCT CTG      534
Ser Arg Ser Ile Pro Leu Glu Trp Glu Asp Thr Tyr Gly Thr Ala Leu
        155                 160                 165

ATC TCT GGA GTG AAG TAT AAG AAA GGT GGC CTT GTG ATC AAC GAA ACT      582
Ile Ser Gly Val Lys Tyr Lys Lys Gly Gly Leu Val Ile Asn Glu Thr
    170                 175                 180

GGG TTG TAC TTC GTG TAT TCC AAA GTA TAC TTC CGG GGT CAG TCT TGC      630
Gly Leu Tyr Phe Val Tyr Ser Lys Val Tyr Phe Arg Gly Gln Ser Cys
185                 190                 195                 200

AAC AAC CAG CCC CTA AAC CAC AAG GTC TAT ATG AGG AAC TCT AAG TAT      678
Asn Asn Gln Pro Leu Asn His Lys Val Tyr Met Arg Asn Ser Lys Tyr
                205                 210                 215

CCT GAG GAT CTG GTG CTA ATG GAG GAG AAG AGG TTG AAC TAC TTC ACT      726
Pro Glu Asp Leu Val Leu Met Glu Glu Lys Arg Leu Asn Tyr Phe Thr
            220                 225                 230

ACT GGA CAG ATA TGG GCC CAC AGC AGC TAC CTG GGG GCA GTA TTC AAT      774
Thr Gly Gln Ile Trp Ala His Ser Ser Tyr Leu Gly Ala Val Phe Asn
        235                 240                 245

CTT ACC AGT GCT GAC CAT TTA TAT GTC AAC ATA TCT CAA CTC TCT CTG      822
Leu Thr Ser Ala Asp His Leu Tyr Val Asn Ile Ser Gln Leu Ser Leu
    250                 255                 260

ATC AAT TTT GAG GAA TCT AAG ACC TTT TTC GGC TTG TAT AAG CTT TAAAA    872
Ile Asn Phe Glu Glu Ser Lys Thr Phe Phe Gly Leu Tyr Lys Leu
265                 270                 275                 280

GAAAAAGCAT TTTAAAATGA TCTACTATTC TTTATCATGG GCACCAGGAA TATTGTCTTG    932

AATGAGAGTC TTCTTAAGAC CTATTGAGAT TAATTAAGAC TACATGAGCC ACAAAGACCT    992

CATGACCGCA AGGTCCAACA GGTCAGCTAT TTCATTTTCT CGAGGTCCAT GGAGTGGTCC   1052

TTAATGCCTG CATCATGAGC CAGATGGAAG GAGGTCTGTG ACTGAGGGGA CATAAAGCTT   1112

GGGGCTGCTG TGTGACAATG CAGAGGCACA GAGGAAAGAA CTGTCTGATG TTAAATGGC    1171
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 279 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Met Gln Gln Pro Met Asn Tyr Pro Cys Pro Gln Ile Phe Trp Val Asp
1 5 10 15

Ser Ser Ala Thr Ser Ser Trp Ala Pro Pro Gly Ser Val Phe Pro Cys
20 25 30

Pro Ser Cys Gly Pro Arg Gly Pro Asp Gln Arg Arg Pro Pro Pro Pro
35 40 45

Pro Pro Pro Val Ser Pro Leu Pro Pro Pro Ser Gln Pro Leu Pro Leu
50 55 60

Ser Pro Leu Thr Pro Leu Lys Lys Lys Asp His Asn Thr Asn Leu Trp
65 70 75 80

Leu Pro Val Val Phe Phe Met Val Leu Val Gly Leu Val Gly Met Gly
85 90 95

Leu Gly Met Tyr Gln Leu Phe His Leu Gln Lys Glu Leu Ala Glu Leu
100 105 110

Arg Glu Phe Thr Asn Gln Ser Leu Lys Val Ser Ser Phe Glu Lys Gln
115 120 125

Ile Ala Asn Pro Ser Thr Pro Ser Glu Lys Lys Glu Pro Arg Ser Val
130 135 140

Ala His Leu Thr Gly Asn Pro His Ser Arg Ser Ile Pro Leu Glu Trp
145 150 155 160

Glu Asp Thr Tyr Gly Thr Ala Leu Ile Ser Gly Val Lys Tyr Lys Lys
165 170 175

Gly Gly Leu Val Ile Asn Glu Thr Gly Leu Tyr Phe Val Tyr Ser Lys
180 185 190

Val Tyr Phe Arg Gly Gln Ser Cys Asn Asn Gln Pro Leu Asn His Lys
195 200 205

Val Tyr Met Arg Asn Ser Lys Tyr Pro Glu Asp Leu Val Leu Met Glu
210 215 220

Glu Lys Arg Leu Asn Tyr Phe Thr Thr Gly Gln Ile Trp Ala His Ser
225 230 235 240

Ser Tyr Leu Gly Ala Val Phe Asn Leu Thr Ser Ala Asp His Leu Tyr
245 250 255

Val Asn Ile Ser Gln Leu Ser Leu Ile Asn Phe Glu Glu Ser Lys Thr
260 265 270

Phe Phe Gly Leu Tyr Lys Leu
275

CLAIMS

What is claimed is:

1. An isolated DNA encoding a human Fas-L polypeptide, wherein said Fas-L polypeptide is characterized by an N-terminal amino acid sequence MQQPFNYPYPQI.

2. An isolated DNA according to claim 1, wherein said DNA is characterized by a 5' sequence comprising nucleotides 93-128 of SEQ ID NO:1.

3. An isolated DNA according to claim 1 wherein said Fas-L polypeptide comprises the amino acid sequence of residues 1-281 of SEQ ID NO:2.

4. An isolated DNA according to claim 3, wherein said DNA comprises the nucleotide sequence of nucleotides 93-938 of SEQ ID NO:1.

5. An isolated DNA according to claim 1, wherein said DNA comprises the cDNA insert of the recombinant vector deposited in strain ATCC 69527.

6. An isolated DNA according to claim 1, wherein said DNA comprises the coding region of the cDNA insert of the recombinant vector deposited in strain ATCC 69527.

7. An isolated DNA encoding a soluble human Fas-L polypeptide, wherein said Fas-L polypeptide comprises the amino acid sequence of residues 106-281 of SEQ ID NO:2.

8. An isolated DNA according to claim 7, wherein said DNA comprises the nucleotide sequence of nucleotides 408-938 of SEQ ID NO:1.

9. An expression vector comprising a DNA according to claim 1.

10. An expression vector comprising a DNA according to claim 2.

11. An expression vector comprising a DNA according to claim 3.

12. An expression vector comprising a DNA according to claim 6.

13. An expression vector comprising a DNA according to claim 7.

14. A process for preparing a Fas-L polypeptide, comprising culturing a host cell transformed with a vector according to claim 9 under conditions promoting expression of Fas-L, and recovering the Fas-L polypeptide from the culture.

15. A process for preparing a Fas-L polypeptide, comprising culturing a host cell transformed with a vector according to claim 10 under conditions promoting expression of Fas-L and recovering the Fas-L polypeptide from the culture.

16. A process for preparing a Fas-L polypeptide, comprising culturing a host cell transformed with a vector according to claim 11 under conditions promoting expression of Fas-L, and recovering the Fas-L polypeptide from the culture.

17. A process for preparing a Fas-L polypeptide, comprising culturing a host cell comprising a vector according to claim 12 under conditions promoting expression of Fas-L, and recovering the Fas-L polypeptide from the culture.

18. A process for preparing a Fas-L polypeptide, comprising culturing a host cell comprising a vector according to claim 13 under conditions promoting expression of Fas-L, and recovering the Fas-L polypeptide from the culture.

19. A purified human Fas-L protein characterized by an N-terminal amino acid sequence MQQPFNYPYPQI.

20. A human Fas-L protein according to claim 19, wherein said protein comprises the amino acid sequence of residues 1-281 of SEQ ID NO:2.

21. A soluble human Fas-L protein comprising the amino acid sequence of residues 106-281 of SEQ ID NO:2.

22. A human Fas-L protein encoded by the cDNA insert of the recombinant vector deposited in strain ATCC 69527.

23. An antibody immunoreactive with a Fas-L protein according to claim 20.

24. An antibody immunoreactive with a Fas-L protein according to claim 21.

25. An antibody according to claim 23, wherein said antibody is a monoclonal antibody.

26. An antibody according to claim 24, wherein said antibody is a monoclonal antibody.

27. An isolated nucleic acid molecule comprising at least about 14 nucleotides of the coding region of SEQ ID NO:1, or the DNA or RNA complement thereof.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00362

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C07H 21/04; C12N 15/19, 15/63; C07K 14/52, 16/24

US CL :536/23.25, 24.3, 530/350, 387.9; 435/69.1, 69.7, 320.1

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. : 536/23.25, 24.3, 530/350, 387.9; 435/69.1, 69.7, 320.1

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
search terms: Fas antigen, apo-1, cd95

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Arthritis and Rheumatism, volume 36(9), issued 1993, Y. Jian et al., "The Fas-ligand and apoptosis in LPR and GLD mice", page s52 (Abstract No. 80), entire document.	1-27
Y	Arthritis and Rheumatism, Volume 36, No. 9, issued 1993, T. Zhou et al., "FAS+ COS cells and anti-FAS antibody for in vivo localization of FAS/FAS ligand binding and apoptosis sites in autoimmune LPR and GLD mice", page s52 (Abstract No. 81), entire document.	1-27
X Y	Cell, Volume 75, issued 17 December 1993, T. Suda et al., "Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family", pages 1169-1178, entire document.	1-22,27 23-26

[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
07 APRIL 1995

Date of mailing of the international search report
18 APR 1995

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/00362

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nature, Volume 356, issued 26 March 1992, R. Watanabe-Fukunaga et al., "Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis", pages 314-317, entire document.	1-27
Y	J. Biol. Chem., Volume 267, No. 15, issued 25 May 1992, A. Oehm et al., "Purification and molecular cloning of the APO-1 cell surface antigen, a member of the tumor necrosis factor/nerve growth factor receptor superfamily", pages 10709-10715, entire document.	1-27
Y	J. Immunol., Volume 148, No. 4, issued 15 February 1992, R. Watanabe-Fukunaga et al., "The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen", pages 1274-1279, entire document.	1-27
Y	J. Exp. Med., Volume 172, issued November 1990, R.D. Allen et al., "Differences defined by bone marrow transplantation suggest that lpr and gld are mutations of genes encoding an interacting pair of molecules", pages 1367-1375, entire document. 1-27	
Y	Current Protocols in Immunology, Supplement 4, Issued 1992, D. Hollenbaugh et al., "Construction of Immunoglobulin Fusion Proteins", pages 10.19.1-10.19.11, entire document.	1-27
Y	Proc. Nat. Acad. Sci. USA, Volume 84, issued December 1987, A. Aruffo et al., "Molecular cloning of a CD28 cDNA by a high-efficiency COS cell expression system", pages 8573-8577, entire document.	1-27
Y	EP,A,0,510,691 (NAGATA ET AL), 28 October 1992, entire document.	1-27
Y	WO,A, 91/10448 (KRAMMER ET AL), 25 July 1991, entire document.	1-27