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(54) **Titre : PROTEINES DE FUSION COMPORTANT DU TNF ET DOMAINE DE TRIMERISATION DE LA FIBRITINE**
(54) **Title: FUSION PROTEINS COMPRISING TNF AND FIBRITIN TRIMERIZATION DOMAIN**

(57) **Abrégé/Abstract:**
The present invention refers to fusion proteins comprising a TNF superfamily (TNFSF) cytokine or a receptor binding domain thereof fused to a trimerization domain and a nucleic acid molecule encoding the fusion protein. The fusion protein is present as a trimeric complex or as an oligomer thereof and is suitable for therapeutic, diagnostic and/or research applications.

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(54) Title: TNF SUPERFAMILY FUSION PROTEINS

(57) Abstract: The present invention refers to fusion proteins comprising a TNF superfamily (TNFSF) cytokine or a receptor binding domain thereof fused to a trimerization domain and a nucleic acid molecule encoding the fusion protein. The fusion protein is present as a trimeric complex or as an oligomer thereof and is suitable for therapeutic, diagnostic and/or research applications.

FUSION PROTEINS COMPRISING TNF AND FIBRITIN TRIMERIZATION DOMAIN

Description

The present invention refers to fusion proteins comprising a TNF superfamily (TNFSF) cytokine or a receptor binding domain thereof fused to a trimerization domain and a nucleic acid molecule encoding the fusion protein. The fusion protein is present as a trimeric complex or as an oligomer thereof and is suitable for therapeutic, diagnostic and/or research applications.

State of the Art

It is known that trimerization of TNSF cytokines, e.g., the CD95 ligand (CD95L), is required for efficient receptor binding and activation. Trimeric complexes of TNF superfamily cytokines, however, are difficult to prepare from recombinant monomeric units.

WO 01/49866 and WO 02/09055 disclose recombinant fusion proteins comprising a TNF cytokine and a multimerization component, particularly a protein from the C1q protein family or a collectin. A disadvantage of these fusion proteins is, however, that the trimerization domain usually has a large molecular weight and/or that the trimerization is rather inefficient.

Schneider et al. (J Exp Med 187 (1989), 1205-1213) describe that trimers of TNF cytokines are stabilized by N-terminally positioned stabilization motifs. In CD95L, the stabilization of the CD95L-receptor binding domain trimer is presumably caused by N-terminal amino acid domains which are located near the cytoplasmic membrane.

Shiraishi et al. (Biochem Biophys Res Commun 322 (2004), 197-202)

describe that the receptor binding domain of CD95L may be stabilized by N-terminally positioned artificial α-helical coiled-coil (leucine zipper) motifs. It was found, however, that the orientation of the polypeptide chains to each other, e.g. parallel or antiparallel orientation, can hardly be predicted. Further, the optimal number of hepta-d-repeats in the coiled-coil zipper motif are difficult to determine. In addition, coiled-coil structures have the tendency to form macromolecular aggregates after alteration of pH and/or ionic strength.

It was an object of the present invention to provide fusion proteins comprising a TNF cytokine or a receptor binding domain thereof, which allow efficient recombinant manufacture combined with good trimerization properties.

Summary of the Invention

The present invention relates to a fusion protein comprising
(i) a TNF-superfamily cytokine or a receptor binding domain thereof
(ii) a flexible linker element between components (i) and (iii), and
(iii) a fibritin trimerization domain.

The invention further relates to a nucleic acid molecule encoding a fusion protein as described herein and to a cell or a non-human organism transformed or transfected with a nucleic acid molecule as described herein.

The invention also relates to a pharmaceutical or diagnostic composition comprising as an active agent a fusion protein, a nucleic acid molecule, or a cell as described herein.

The invention also relates to a fusion protein, a nucleic acid molecule, or a cell as described herein for use in therapy, e.g., the use of a fusion protein, a nucleic acid molecule, or a cell as described herein for the preparation of a pharmaceutical composition in the prophylaxis and/or treatment of disorders

caused by, associated with and/or accompanied by dysfunction of TNF cytokines, particularly proliferative disorders, such as tumors, e.g. solid or lymphatic tumors; infectious diseases; inflammatory diseases; metabolic diseases; autoimmune disorders, e.g. rheumatoid and/or arthritic diseases; degenerative diseases, e.g. neurodegenerative diseases such as multiple sclerosis; apoptosis-associated diseases or transplant rejections.

Description of the Figures

Figure 1: A) SEC analysis of Streptactin affinity purified hs95L-AT4: Affinity purified protein eluted by desthiobiotin from immobilized Streptactin was loaded onto a Superdex200™ column. The protein elution profile of the SEC run was measured at OD280. The retention volume of the respective hs95L-AT4 peak and the fraction numbers are indicated.
B) Analysis of hs95L-AT4 SEC fractions by SDS-PAGE silver stain: SEC fractions shown in **A** were separated by SDS-PAGE and subsequently analysed by silver staining. The fraction number and the molecular wheight (in kDa) of standard proteins is indicated.

Figure 2: Determination of the native apparent molecular weight for hs95L-AT4
The apparent molecular weight of purified hs95L-AT4 was determined based on calibration of the Superdex 200 column with gel filtration standard proteins (Bio-Rad GmbH, München, Germany). The elution volume of the calibration standards were plotted against the logarithm of the respective molecular weights to create a calibration curve. The apparent Mw of hs95L-AT4 was calculated based on the respective elution volume of 13.85ml. The table summarizes the results of the SEC analysis.

Figure 3: Analysis of hs95L-AT4 SEC fractions (shown in Figure 1) by their potential to induce apoptosis in Jurkat cells. The protein content of the SEC fractions matches their ability to induce Caspase activity.

Figure 4: Inhibition of hs95L-AT4 induced apoptosis by APG101
Hs95L-AT4 was incubated for 30min with different amounts of APG101, added to Jurkat cells and subsequently apoptosis was measured by analysing caspase activity. The graphic shows the dose dependent antagonizing effect of APG101 on hsCD95-AT4 induced apoptosis.

Figure 5: A) SEC analysis of Streptactin affinity purified hs95L-A69: Affinity purified protein eluted by desthiobiotin from immobilized Streptactin was loaded onto a Superdex200 column. The protein elution profile of the SEC run was measured at OD280. The retention volume of the respective hs95L-A69 peak and the fraction numbers are indicated.
B) Analysis of SEC fractions by SDS-PAGE silver stain: SEC fractions shown in **A** were separated by SDS-PAGE and subsequently analysed by silver staining. The fraction number and the molecular weight (in kDa) of standard proteins is indicated.
C) Analysis of hs95L-A69SEC fractions (shown in **A**) by their potential to induce apoptosis in Jurkat cells. The protein content of the SEC fractions matches their ability to induce Caspase activity.

Figure 6: A) SEC analysis of Streptactin affinity purified hsTRAIL-AT4: Affinity purified protein eluted by desthiobiotin from immobilized Streptactin was loaded onto a Superdex200 column. The protein elution profile of hsTRAIL-AT4 peak and the fraction numbers are indicated.
B) Analysis of hsTRAIL-AT4 SEC fractions by SDS-PAGE silver stain: SEC fractions shown in **A** were separated by SDS-PAGE and subsequently analysed by silver staining. The fraction number and the molecular weight (in kDa) of standard proteins is indicated.
C) Analysis of hsTRAIL-AT4 SEC fractions (shown in **A**) by their potential to induce apoptosis in Jurkat cells. The protein content of the SEC fractions matches their ability to induce Caspase activity.

Detailed Description of the Invention

Thus, the present invention relates to a fusion protein comprising
(i) a TNF-superfamily cytokine or a receptor binding domain thereof
(ii) a flexible linker element between components (i) and (iii), and
(iii) a fibritin trimerization domain.

The fusion protein may be a monomeric protein or a multimeric protein. Preferably, the fusion protein is present as a trimeric complex consisting of three monomeric units which may be identical or different. Preferably, a trimeric complex consists of three identical fusion proteins. The trimeric complex as such shows biological activity. It was found, however, that oligomers of the trimeric complex, e.g. defined complexes wherein the basic trimeric structure is present 2, 3 or 4 times, also have biological activity.

Component (i) of the fusion protein is a cytokine of the TNF superfamily or a receptor binding domain thereof. Preferably, component (i) is a mammalian, particularly human cytokine or a receptor binding domain thereof including allelic variants and/or derivatives thereof. Further, it is preferred that the TNF cytokine is a receptor binding domain thereof capable of binding to the corresponding cytokine receptor and preferably capable of receptor activation, whereby apoptotic or proliferative activity may be caused. The cytokine may e.g. be selected from TNF superfamily members, e.g. human TNFSF-1 to -18 as indicated in Table 1, preferably from LTA (SEQ ID NO:25), TNFα (SEQ ID NO:26), LTB (SEQ ID NO:27), OX40L (SEQ ID NO:28), CD40L (SEQ ID NO:29), CD95L (SEQ ID NO:30), CD27L (SEQ ID NO:31), CD30L (SEQ ID NO:32), CD137L (SEQ ID NO:33), TRAIL (SEQ ID NO:34), RANKL (SEQ ID NO:35), TWEAK (SEQ ID NO:36), APRIL 1 (SEQ ID NO:37), APRIL 2 (SEQ ID NO:38), BAFF (SEQ ID NO:39), LIGHT (SEQ ID NO:40), TL1A (SEQ ID NO:41), GITRL (SEQ ID NO:42), EDA-A1 (SEQ ID NO:43), EDA-A2 (SEQ ID NO:44), or a receptor binding domain thereof. Preferred receptor binding domains of the respective proteins are indicated in Table 1 (NH_2-aa to COOH-aa) and, e.g., comprise amino acids 59-205 or

60-205 of LTA (SEQ ID NO:25), 86-233 of TNFα (SEQ ID NO:26), 82-244 or 86-244 of LTB (SEQ ID NO:27), 52-183 or 55-183 of OX40L (SEQ ID NO:28), 112-261 or 117-261 of CD40L (SEQ ID NO:29), 51-193 or 56-193 of CD27L (SEQ ID NO:31), 97-234, 98-234 or 102-234 of CD30L (SEQ ID NO:32), 86-254 of CD137L (SEQ ID NO:33), 161-317 of RANKL (SEQ ID NO:35), 103-249, 104-249 or 105-249 of TWEAK (SEQ ID NO:36), 112-247 of APRIL 1 (SEQ ID NO:37), 112-250 of APRIL 2 (SEQ ID NO:38), 140-285 of BAFF (SEQ ID NO:39), 91-240 of LIGHT (SEQ ID NO:40), 91-251 or 93-251 of TL1A (SEQ ID NO:41), 52-177 of GITRL (SEQ ID NO:42), 245-391 of EDA-A1 (SEQ ID NO:43), 245-389 of EDA-A2 (SEQ ID NO:44).

More preferably, component (i) is selected from CD95L, TRAIL or TNFα or a receptor binding domain thereof. In an especially preferred embodiment, component (i) comprises the extracellular portion of a TNF cytokine including the receptor binding domain without membrane located domains. In an especially preferred embodiment, component (i) of the recombinant fusion protein is selected from human CD95L, particularly amino acids 142-281 or 144-281 of SEQ ID NO:30, or human TRAIL, particularly amino acids 116-281, 118-281 or 120-281 of SEQ ID NO:34.

In a further preferred embodiment of the invention, the cytokine of the TNF superfamily or a receptor binding domain thereof, e.g., TRAIL, of the fusion protein as described herein comprises a mutant of the cytokine of the TNF superfamily or a receptor binding domain thereof which binds and/or activates TRAIL-receptor 1 (TRAILR1) and/or TRAIL-receptor 2 (TRAILR2). The binding and/or activity of the mutant may be, e.g., determined by the assays as disclosed in van der Sloot et al. (PNAS, 2006, 103:8634-8639), Kelley et al. (J. Biol. Chem., 2005, 280:2205-2215), or MacFarlane et al. (Cancer Res., 2005, 65: 11265-11270).

The mutant may be generated by any technique and is known by the skilled person, e.g., the techniques disclosed in an der Sloot et al. (PNAS, 2006, 103:8634-8639), Kelley et al. (J. Biol. Chem., 2005, 280:2205-2215), or

MacFarlane et al. (Cancer Res., 2005, 65: 11265-11270) any may comprise any type of structural mutations, e.g., substitution, deletion, duplication and/or insertion of an amino acid. A preferred embodiment is the generation of substitutions. The substitution may affect at least one amino acid of the cytokine of the TNF superfamily or a receptor binding domain thereof as described herein. In a preferred embodiment, the substitution may affect at least one of the amino acids of TRAIL, e.g., human TRAIL (e.g., SEQ ID NO:34). Preferred substitutions in this regard affect at least one of the following amino acids of human TRAIL of SEQ ID NO:34: R130, G160, Y189, R191, Q193, E195, N199, K201, Y213, T214, S215, H264, I266, D267, D269. Preferred amino acid substitutions of human TRAIL of SEQ ID NO:34 are at least one of the following substitutions: R130E, G160M, Y189A, Y189Q, R191K, Q193S, Q193R, E195R, N199V, N199R, K201R, Y213W, T214R, S215D, H264R, I266L, D267Q, D269H, D269R, or D269K.

The amino acid substitution(s) may affect the binding and/or activity of TRAIL, e.g., human TRAIL, to or on either the TRAILR1 or the TRAILR2. Alternatively, the amino acid substitution(s) may affect the binding and/or activity of TRAIL, e.g., human TRAIL, to or on both, the TRAILR1 and the TRAILR2. The binding and/or activity of the TRAILR1 and/or TRAILR2 may be affected positively, i.e., stronger, more selective or specific binding and/or more activation of the receptor. Alternatively, the binding and/or activity of the TRAILR1 and/or TRAILR2 may be affected negatively, i.e., weaker, less selective or specific binding and/or less or no activation of the receptor.

Examples of mutants of TRAIL with amino acid substitution(s) of the invention that affect binding and/or activity of both TRAILR1 and TRAILR2 may be found, e.g., in Table 1 of MacFarlane et al. (cf. above) and may comprise a human TRAIL mutant with the following two amino acid substitutions of SEQ ID NO:34 Y213W and S215D or with the following single amino acid substitution Y189A.

Examples of mutants of TRAIL with amino acid substitution(s) of the

invention that affect binding and/or activity of TRAILR1 may be found, e.g., in Table 1 of MacFarlane et al. (cf. above) and may comprise a human TRAIL mutant with the following four amino acid substitutions of SEQ ID NO:34 N199V, K201R, Y213W and S215D or with the following five amino acid substitutions Q193S, N199V, K201R, Y213W and S215D or may be found in Table 2 of Kelley et al. (cf. above) and may comprise a human TRAIL mutant with the following six amino acid substitutions Y213W, S215D, Y189A, Q193S, N199V, and K201R or with Y213W, S215D, Y189A, Q193S, N199R, and K201R.

Examples of mutants of TRAIL with amino acid substitution(s) of the invention that affect binding and/or activity of TRAILR2 may be found, e.g., in Table 1 of MacFarlane et al. (cf. above) or in Table 2 of Kelley et al. (cf. above) and may comprise a human TRAIL mutant with the following six amino acid substitutions of SEQ ID NO:34 Y189Q, R191K, Q193R, H264R, I266L, and D267Q or may be found in Table 2 of van der Sloot et al. (cf. above) and may comprise a human TRAIL mutant with the following single amino acid substitution D269H, with the following two amino acid substitutions D269H and E195R or with D269H and T214R.

Thus one preferred embodiment is a fusion protein as described herein wherein component (i) comprises a mutant of TRAIL or of a receptor binding domain thereof which binds and/or activates TRAILR1 and/or TRAILR2.

One preferred embodiment of a fusion protein comprising a mutant of TRAIL or of a receptor binding domain as described herein is a fusion protein wherein component (i) comprises at least one amino acid substitution.

Such an amino acid substitution affects at least one of the following amino acid positions of human TRAIL (SEQ ID NO:34): R130, G160, Y189, R191, Q193, E195, N199, K201, Y213, T214, S215, H264, I266, D267, D269.

Such an amino acid substitution is at least one of the following: R130E,

G160M, Y189A, Y189Q, R191K, Q193S, Q193R, E195R, N199V, N199R, K201R, Y213W, T214R, S215D, H264R, I266L, D267Q, D269H, D269R, or D269K.

Component (ii) is a flexible linker element located between components (i) and (iii). The flexible linker element preferably has a length of 5-20 amino acids, particularly a length of 6, 9, 12, 15 or 18 amino acids. The linker element is preferably a glycine/serine linker, i.e. a peptide linker substantially consisting of the amino acids glycine and serine. In an especially preferred embodiment, the linker has the amino acid sequence $(GSS)_a(SSG)_b(GS)_c(S)_d$, wherein a, b, c, d is each 0, 1, 2, 3, 4, or 5. Examples of specific linker sequences are GSS GSS GSS GS (a=3 b=0, b=0, c=1; d=0) (see also amino acids 161-171 of SEQ ID NO:19 or amino acids 182-192 of SEQ ID NO:20), or SSG SSG SSG S (a=0; b=3, c=0; d=1). It is clear to the skilled person that in cases in which the cytokine of the TNF superfamily or a receptor binding domain thereof already terminates with a G, e.g. human TRAIL (SEQ ID NO:34) such a G may form the first G of the linker in the linker sequence $(GSS)_a(SSG)_b(GS)_c(S)_d$ (see amino acid 182 of SEQ ID NO:20).

Component (iii) is a fibritin trimerization domain, particularly a bacteriophage fibritin trimerization domain, more particularly a fibritin trimerization domain from bacteriophage T4 or related bacteriophages such as T - even bacteriophages or phage RB69 or phage AR1 as shown in Table 2. The T4 fibritin trimerization domain is e.g. described in US 6,911,205 or WO 01/19958, and has the sequence of SEQ ID NO:23. The RB69 fibritin trimerization domain has the sequence of SEQ ID NO:24.

More preferably, component (iii) comprises the amino acid sequence (G) YIPEAPRDGQ AYVRKDGEWV LLSTFL (SEQ ID NO:8 or amino acids 458-484 or 459-484 of SEQ ID NO:23) or a sequence variant having an identity of at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99%

thereto. Examples of preferred sequence variants are shown in Table 3.

More preferably, component (iii) comprises the amino acid sequence (G) YIEDAPSDGKFYVRKDGAWVELPTA (SEQ ID NO:9 or amino acids 455-480 or 456-480 of SEQ ID NO:24) or a sequence variant having an identity of at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% thereto.

Further, it is preferred that component (iii) has a length of from 20 up to 30 amino acids.

In the fusion protein of the invention, it is preferred that component (i) is located N-terminally of component (iii). The invention, however, also refers to embodiments, wherein component (iii) is located N-terminally of component (i). The components (i) and (iii) may directly flank each other or be separated by, e.g., a linker sequence as described herein (see, e.g., SEQ ID NOs:19 and 20).

The fusion protein may additionally comprise an N-terminal signal peptide domain, which allows processing, e.g. extracellular secretion, in a suitable host cell. Preferably, the N-terminal signal peptide domain comprises a protease, e.g. a signal peptidase cleavage site and thus may be removed after or during expression to obtain the mature protein. Further, the fusion protein may additionally comprise a C-terminal flexible element, having a length of e.g. 1-50, preferably 10-30 amino acids which may include or connect to a recognition/purification domain, e.g. a FLAG domain, a Strep-tag domain and/or a poly-His domain.

Examples of specific fusion proteins of the invention are SEQ ID NOs:1, 19, and 20.

A further aspect of the present invention relates to a nucleic acid molecule encoding a fusion protein as described herein. The nucleic acid molecule may be a DNA molecule, e.g. a double-stranded or single-stranded DNA

molecule, or an RNA molecule. The nucleic acid molecule may encode the fusion protein or a precursor thereof, e.g. a pro- or pre-proform of the fusion protein which may comprise a signal sequence or other heterologous amino acid portions for secretion or purification which are preferably located at the N- and/or C-terminus of the fusion protein. The heterologous amino acid portions may be linked to the first and/or second domain via a protease cleavage site, e.g. a Factor X_a, thrombin or IgA protease cleavage site.

Examples of specific nucleic acid sequences of the invention are SEQ ID Nos:2, 21, and 22.

The nucleic acid molecule may be operatively linked to an expression control sequence, e.g. an expression control sequence which allows expression of the nucleic acid molecule in a desired host cell. The nucleic acid molecule may be located on a vector, e.g. a plasmid, a bacteriophage, a viral vector, a chromosal integration vector, etc. Examples of suitable expression control sequences and vectors are described for example by Sambrook et al. (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Press, and Ausubel et al. (1989), *Current Protocols in Molecular Biology*, John Wiley & Sons or more recent editions thereof.

Various expression vector/host cell systems may be used to express the nucleic acid sequences encoding the fusion proteins of the present invention. Suitable host cells include, but are not limited to, prokaryotic cells such as bacteria, e.g. *E.coli*, eukaryotic host cells such as yeast cells, insect cells, plant cells or animal cells, preferably mammalian cells and, more preferably, human cells.

Further, the invention relates to a non-human organism transformed or transfected with a nucleic acid molecule as described above. Such transgenic organisms may be generated by known methods of genetic transfer including homologous recombination.

The fusion protein, the respective nucleic acid encoding therefor, the transformed or transfected cell as well as the trimeric complexes or oligomers of the trimeric complexes, all as described herein may be used for pharmaceutical, diagnostic and/or research applications.

A further aspect of the present invention relates to a pharmaceutical or diagnostic composition comprising as an active agent at least one fusion protein, one respective nucleic acid encoding therefor, one transformed or transfected cell as well as one trimeric complexe or oligomer of the trimeric complexes, all as described herein.

At least one fusion protein, one respective nucleic acid encoding therefor, one transformed or transfected cell as well as one trimeric complexe or oligomer of the trimeric complexes, all as described herein may be used in therapy, e.g., in the prophylaxis and/or treatment of disorders caused by, associated with and/or accompanied by dysfunction of TNF cytokines, particularly proliferative disorders, such as tumors, e.g. solid or lymphatic tumors; infectious diseases; inflammatory diseases; metabolic diseases; autoimmune disorders, e.g. rheumatoid and/or arthritic diseases; degenerative diseases, e.g. neurodegenerative diseases such as multiple sclerosis; apoptosis-associated diseases or transplant rejections.

The term “dysfunction of TNF cytokines” as used herein is to be understood as any function or expression of a TNF cytokine that deviates from the normal function or expression of a TNF cytokine, e.g., overexpression of the TNF gene or protein, reduced or abolished expression of the TNF cytokine gene or protein compared to the normal physiological expression level of said TNF cytokine, increased activity of the TNF cytokine, reduced or abolished activity of the TNF cytokine, increased binding of the TNF cytokine to any binding partners, e.g., to a receptor, particularly a TRAIL receptor or another cytokine molecule, reduced or abolished binding to any binding partner, e.g. to a receptor, particularly a TRAIL receptor or another cytokine molecule, compared to the normal physiological activity or binding of said

TNF cytokine.

The composition may be administered as monotherapy or as combination therapy with further medicaments, e.g. cytostatic or chemotherapeutic agents, corticosteroids and/or antibiotics.

The fusion protein is administered to a subject in need thereof, particularly a human patient, in a sufficient dose for the treatment of the specific conditions by suitable means. For example, the fusion protein may be formulated as a pharmaceutical composition together with pharmaceutically acceptable carriers, diluents and/or adjuvants. Therapeutic efficacy and toxicity may be determined according to standard protocols. The pharmaceutical composition may be administered systemically, e.g. intraperitoneally, intramuscularly or intravenously or locally, e.g. intranasally, subcutaneously or intrathecally. Preferred is intravenous administration.

The dose of the fusion protein administered will of course be dependent on the subject to be treated, on the subject's weight, the type and severity of the disease, the manner of administration and the judgement of the prescribing physician. For the administration of fusion proteins, a daily dose of 0.001 to 100 mg/kg is suitable.

Example

1. Manufacture of a fusion protein

In the following, the basic structure of the recombinant proteins of the invention is shown exemplified for the receptor binding domain of the human CD95 ligand.

1.1 <u>Polypeptide structure</u>

A) Amino acids Met1-Gly20

IgKappa-signal peptide, assumed signal peptidase cleavage site between the amino acids Gly20 and Glu21

B) Amino acids Glu21-Leu160
Receptor binding domain of the human CD95 ligand (CD95L; amino acids 142-281 of SEQ ID NO:30))

C) Amino acids Gly161-Ser171
Flexible linker element providing a distance of up to 30 Å between CD95L and the trimerization domain.

D) Amino acids Gly172-Leu198
Trimerization domain of the bacteriophage T4-fibritin (amino acids 458-484 of SEQ ID NO:23)

E) Amino acids Ser199-Lys222
Flexible element with a 6xHis-Streptag II motif

The resulting protein was designated hs95L-AT4.

1 METDTLLLWV LLLWVPGSTG ELRKVAHLTG KSNSRSMPLE WEDTYGIVLL SGVKYKKGGL
61 VINETGLYFV YSKVYFRGQS CNNLPLSHKV YMRNSKYPQD LVMMEGKMMS YCTTGQMWAR
121 SSYLGAVFNL TSADHLYVNV SELSLVNFEE SQTFFGLYKL GSSGSSGSSG SGYIPEAPRD
181 GQAYVRKDGE WVLLSTFLSG PSSSSSHHHH HHSAWSHPQF EK (SEQ ID NO:1)

1.2 Gene cassette encoding the polypeptide

The synthetic gene may be optimized in view of its *codon-usage* for the expression in suitable host cells, e.g. insect cells or mammalian cells.

```
                Cpo-I      Nco-I
1               CGGTCCGAAACCATGGAGACCGATACACTGCTCTTGTGGGTACTCTTGCTGTGGGTTCCG
1                             M  E  T  D  T  L  L  L  W  V  L  L  L  W  V  P
                     BshT-I
61              GGATCTACCGGTGAACTCCGTAAAGTCGCCCATCTGACAGGAAAGTCCAACTCCCGATCA
17               G  S  T  G  E  L  R  K  V  A  H  L  T  G  K  S  N  S  R  S

121             ATGCCTCTTGAGTGGGAAGACACCTACGGAATCGTCCTGTTGAGCGGAGTGAAGTACAAG
```

```
     37          M  P  L  E  W  E  D  T  Y  G  I  V  L  L  S  G  V  K  Y  K

    181          AAGGGTGGTCTGGTCATCAACGAGACAGGCTTGTACTTCGTGTACTCCAAGGTGTACTTC
     57           K  G  G  L  V  I  N  E  T  G  L  Y  F  V  Y  S  K  V  Y  F

    241          CGTGGTCAATCGTGCAACAACCTTCCACTCTCACACAAGGTCTACATGCGTAACTCGAAG
     77           R  G  Q  S  C  N  N  L  P  L  S  H  K  V  Y  M  R  N  S  K

    301          TATCCGCAGGATCTGGTGATGATGGAGGGCAAGATGATGAGCTACTGCACGACCGGACAG
     97           Y  P  Q  D  L  V  M  M  E  G  K  M  M  S  Y  C  T  T  G  Q

    361          ATGTGGGCACGTAGCTCATACCTGGGTGCTGTCTTCAACCTGACCAGTGCAGACCACCTG
    117           M  W  A  R  S  S  Y  L  G  A  V  F  N  L  T  S  A  D  H  L

    421          TACGTGAACGTGTCCGAACTGTCGCTCGTGAACTTCGAGGAGAGCCAGACGTTCTTCGGT
    137           Y  V  N  V  S  E  L  S  L  V  N  F  E  E  S  Q  T  F  F  G

                                BamH-I                    Xho-I
    481          CTCTACAAGCTGGGATCCTCAGGATCGAGTGGCTCGAGTGGTTCTGGATACATCCCAGAA
    157           L  Y  K  L  G  S  S  G  S  S  G  S  S  G  S  G  Y  I  P  E

    541          GCACCCAGAGACGGTCAGGCTTATGTCCGCAAAGACGGAGAATGGGTTCTGCTCTCGACC
    177           A  P  R  D  G  Q  A  Y  V  R  K  D  G  E  W  V  L  L  S  T
                          Sac-I                                      Eco47-III
    601          TTCTTGTCGGGTCCGAGCTCAAGCTCATCTCATCATCATCATCATCATAGCGCTTGGTCT
    197           F  L  S  G  P  S  S  S  S  S  H  H  H  H  H  H  S  A  W  S

                                   Oli-I              Not-I       Hind-III
    661          CACCCGCAGTTCGAGAAATGACACCATAGTGATAAGTAGCGGCCGCAGTAAGCTT
    217           H  P  Q  F  E  K STOP
```

(SEQ ID NO:2 and SEQ ID NO:3)

1.3 Cloning strategy of hs95L-AT4

The synthetic gene is excised from the transfer plasmid by means of Cpo-I/Hind-III hydrolysis and cloned into a suitable vector.

The sequence coding for the C-terminal Streptag-II may be deleted, e.g. by simultaneous hydrolysis with the *blunt-end cutters Eco47-III* and *Oli-I* and religation of the vector. A stop codon is therefor introduced by the fusion of the restriction enzyme *half-sites* downstream of the 6xHistag:

A) 3' terminus of the cassette prior to hydrolysis with Eco47-III and Oli-I

```
Eco47-III                    Oli-I              Not-I       Hind-III
AGCGCTTGGTCTCACCCGCAGTTCGAGAAATGACACCATAGTGATAAGTAGCGGCCGCAGTAAGCTT
 S  A  W  S  H  P  Q  F  E  K STOP
```

(SEQ ID NO:4 and SEQ ID NO:5)

B) 3' terminus of the cassette after hydrolysis and religation

Not-I ***Hind-III***

AGCTAGTGATAAGTA***GCGGCCGC***AGT***AAGCTT***

S STOP

(SEQ ID NO:6)

Sequence of the synthetic gene:

CGG TCC GAA ACC ATG GAG ACC GAT ACA CTG CTC TTG TGG GTA CTC TTG
CTG TGG GTT CCG GGA TCT ACC GGT GAA CTC CGT AAA GTC GCC CAT CTG
ACA GGA AAG TCC AAC TCC CGA TCA ATG CCT CTT GAG TGG GAA GAC ACC
TAC GGA ATC GTC CTG TTG AGC GGA GTG AAG TAC AAG AAG GGT GGT CTG
GTC ATC AAC GAG ACA GGC TTG TAC TTC GTG TAC TCC AAG GTG TAC TTC
CGT GGT CAA TCG TGC AAC AAC CTT CCA CTC TCA CAC AAG GTC TAC ATG
CGT AAC TCG AAG TAT CCG CAG GAT CTG GTG ATG ATG GAG GGC AAG ATG
ATG AGC TAC TGC ACG ACC GGA CAG ATG TGG GCA CGT AGC TCA TAC CTG
GGT GCT GTC TTC AAC CTG ACC AGT GCA GAC CAC CTG TAC GTG AAC GTG
TCC GAA CTG TCG CTC GTG AAC TTC GAG GAG AGC CAG ACG TTC TTC GGT
CTC TAC AAG CTG GGA TCC TCA GGA TCG AGT GGC TCG AGT GGT TCT GGA
TAC ATC CCA GAA GCA CCC AGA GAC GGT CAG GCT TAT GTC CGC AAA GAC
GGA GAA TGG GTT CTG CTC TCG ACC TTC TTG TCG GGT CCG AGC TCA AGC
TCA TCT CAT CAT CAT CAT CAT CAT AGC GCT TGG TCT CAC CCG CAG TTC
GAG AAA TGA CAC CAT AGT GAT AAG TAG CGG CCG CAG TAA GCT T

(SEQ ID NO:7)

2. Expression and Purification

a) Cloning, expression and purification of hs95L-AT4

Hek 293T cells grown in DMEM + GlutaMAX (GibCo) supplemented with 10% FBS, 100 units/ml Penicillin and 100 µg/ml Streptomycin were transiently transfected with a plasmid containing an expression cassette for hs95L-AT4. Cell culture supernatant containing recombinant hs95L-AT4 was

harvested three days post transfection and clarified by centrifugation at 300 g followed by filtration through a 0.22 µm sterile filter. For affinity purification Streptactin Sepharose was packed to a column (gel bed 1 ml), equilibrated with 15 ml buffer W (100 mM Tris-HCl, 150 mM NaCl pH 8.0) and the cell culture supernatant was applied to the column with a flow rate of 4 ml/min. Subsequently, the column was washed with 15 ml buffer W and bound hs95L-AT4 was eluted stepwise by addition of 7 x 1 ml buffer E (100 mM Tris HCl, 150 mM NaCl, 2.5 mM Desthiobiotin pH 8.0). The protein amount of the eluate fractions was quantified and peak fractions were concentrated by ultrafiltration and further purified by size exclusion chromatography (SEC).

SEC was performed on a Superdex 200 column using an Äkta chromatography system (GE-Healthcare). The column was equilibrated with phosphate buffered saline and the concentrated, Streptactin purified hs95L-AT4 was loaded onto the SEC column at a flow rate of 0.5 ml/min. The elution profile of hs95L-AT4 monitored by absorbance at 280 nm showed a prominent protein peak at 13.85 ml (Figure 1A). Peak fractions were subsequently analysed under denaturing conditions by SDS-PAGE and silver staining (Figure 1B). Based on calibration with standard proteins hs95L-AT4 runs at about 30 KDa. The calculated theoretical molecular weight of hs95L-AT4 monomer is 22.4 KDa. The higher apparent molecular weight of about 30 KDa after SDS-PAGE is probably due to carbohydrate modifications of hs95L-AT4.

For determination of the apparent molecular weight of purified hs95L-AT4 under native conditions a Superdex 200 column was loaded with standard proteins of known molecular weight. Based on the elution volume of the standard proteins a calibration curve was calculated and the apparent molecular weight of purified hs95L-AT4 was determined to be 90.3 KDa indicating a stable trimeric structure of hs95L-AT4 (Figure 2; Table 4).

b) Cloning, expression and purification of human CD95L-A69 (hs95L-A69) and human TRAIL-AT4 (hsTRAIL-AT4)

The amino acid sequence of the hs95L-A69- and hsTRAIL-AT4 -constructs (SEQ ID NO:19 and SEQ ID NO:20) were backtranslated and their codon usage optimised for mammalian cell-based expression. Gene synthesis was done by ENTELECHON GmbH (Regensburg, Germany).

Finally, the hs95L-A69 and hsTRAIL-AT4 - expression-cassettes (SEQ ID NO:21 and SEQ ID NO:22) were subcloned into pCDNA4-HisMax-backbone (INVITROGEN), using unique Hind-III- and Not-I-sites of the plasmid.

The hs95L-A69 and hsTRAIL-AT4 proteins were purified from tissue culture supernatants of Hek293T cells transiently transfected with plasmids encoding the respective cDNA-constructs, as described for hsCD95L-AT4 (see 2a). Briefly, the recombinant expressed proteins were first purified via Streptactin affinity chromatography. In a second step the affinity peak fractions were further purified and analysed via SEC (Fig. 5A and 6A). To check the purity of the purified proteins, SEC fractions were subsequently analysed by SDS-PAGE and Silver staining (Fig. 5B and 6B). Data from SEC were in addition used to determine the native apparent molecular weight of the respective proteins.

3. Apoptosis Assay

A cellular assay with a Jurkat A3 permanent T-cell line was used to determine the apoptosis inducing activity of different CD95-ligand (CD95L) constructs. Jurkat cells were grown in flasks with RPMI 1640-medium + GlutaMAX (GibCo) supplemented with 10% FBS, 100 units/ml Penicillin and 100 µg/ml Streptomycin. Prior to the assay, 100,000 cells were seeded per well into a 96-well microtiterplate. The addition of different concentrations of CD95L to the wells was followed by a 3 hour incubation at 37°C. Cells were lysed by adding lysis buffer (250 mM HEPES, 50 mM $MgCl_2$, 10 mM EGTA, 5% Triton-x-100™, 100 mM DTT, 10 mM AEBSF, pH 7.5) and plates were put on ice for 30 minutes. Apoptosis is paralleled by an increased activity of

Caspase 3 and Caspase 7. Hence, cleavage of the specific Caspase 3/7 substrate Ac-DEVD-AFC (Biomol) was used to determine the extent of apoptosis. In fact, Caspase activity correlates with the percentage of apoptotic cells determined morphologically after staining the cells with propidium iodide and Hoechst-33342. For the Caspase activity assay, 20 µl cell lysate was transferred to a black 96-well microtiterplate. After the addition of 80 µl buffer containing 50 mM HEPES, 1% Sucrose, 0.1% CHAPS, 50 µM Ac-DEVD-AFC, and 25 mM DTT, pH 7.5, the plate was transferred to a Tecan GeniosPro microtiterplate reader and the increase in fluorescence intensity was monitored (excitation wavelength 400 nm, emission wavelength 505 nm). Exemplarily, Figure 3 demonstrates the induction of caspase activity of SEC fractions of the CD95 ligand hs95L-AT4 in this cellular apoptosis assay. The extent of caspase activity is well in line with the hs95L-AT4 content of SEC fractions as shown in Figures 1A and 1B.

A cellular assay with a Jurkat A3 permanent T-cell line was also used to determine the apoptosis inducing activity of hs95L-A69 and hsTRAIL-AT4. Jurkat cells (100,000 cells per well) were incubated with the ligands for 3 hours at 37°C. Cells were lysed and apoptosis induction was monitored by determination of cleavage of the specific Caspase 3/7 substrate Ac-DEVD-AFC.

Based on their apparent molecular weights both purified proteins, hs95L-A69, and hsTRAIL-T4, were expressed and purified as stable homotrimeric proteins that induced apoptosis on Jurkat cells. A summary comparing the apparent molecular weights determined by SDS-PAGE and SEC with the theoretical molecular weights calculated on basis of the primary amino acid sequence is shown in Table 4.

This apoptosis assay was also used for the determination of biological activity of APG101. APG101 is a fusion protein comprising the extracellular domain of the human CD95-receptor (the in vivo binding partner of CD95

ligand) with human Fc. APG101 antagonizes the apoptosis inducing effect of CD95L by binding to the ligand. Prior to the addition of CD95L to the Jurkat cells, CD95L at a constant concentration was incubated for 30 minutes at 37°C with different concentrations of APG101. An example of the effect of APG101 is shown in Figure 4. The CD95 ligand hs95L-AT4 induces caspase activity in a dose dependent manner, an effect which is abolished by APG101.

Table 1

Approved Gene symbol	TNFSF-number	Synonyms	Accession	NH2-aa	COOH-aa	Length
LTA	TNFSF-1	LTA	gi|6806893|ref|NP_000586.2|	Ser59 Thr60	Leu205 Leu205	147aa 146aa
TNF	TNFSF-2	TNF-alpha	gi|25952111|ref|NP_000585.2|	Asp86	Leu233	148aa
LTB	TNFSF-3	LTB	gi|4505035|ref|NP_002332.1|	Asp82 Gly86	Gly244 Gly244	163aa 159aa
TNFSF4	TNFSF-4	OX40L/GP34	gi|4507603|ref|NP_003317.1|	Val52 Arg55	Leu183 Leu183	132aa 129aa
CD40LG	TNFSF-5	CD40L	gi|4557433|ref|NP_000065.1|	Asp117 Glu112	Leu261 Leu261	150aa 145aa
FASLG	TNFSF-6	CD95L/APO-L/FAS-L	gi|4557329|ref|NP_000630.1|	Glu142 Arg144	Leu281 Leu281	140aa 138aa
TNFSF7	TNFSF-7	CD27L	gi|4507605|ref|NP_001243.1|	Glu51 Asp56	Pro193 Pro193	143aa 138aa
TNFSF8	TNFSF-8	CD30L	gi|4507607|ref|NP_001235.1|	Lys97 Ser98 Leu102	Asp234 Asp234 Asp234	138aa 137aa 133aa
TNFSF9	TNFSF-9	4-1BB/CD137L	gi|4507609|ref|NP_003802.1|	Asp86	Glu254	169aa
TNFSF10	TNFSF-10	TRAIL	gi|4507593|ref|NP_003801.1|	Glu116 Gly118	Gly281 Gly281	166aa 164aa
TNFSF11	TNFSF-11	TRANCE/RANK L	gi|4507595|ref|NP_003692.1|	Glu161	Asp317	157aa
TNFSF12	TNFSF-12	TWEAK/Apo-3	gi|4507597|ref|NP_003800.1|	Ala103 Arg104 Arg105	His249 His249 His249	147aa 146aa 145aa
TNFSF13	TNFSF-13	APRIL/TALL-2/TRDL-1	gi|26051248|ref|NP_742085.1|	Lys112	Leu247	136aa
TNFSF13	TNFSF-13	APRIL/TALL-2/TRDL-1	gi|4507599|ref|NP_003799.1|	Lys112	Leu250	139aa
TNFSF13B	TNFSF-13B	BAFF/Blys	gi|5730097|ref|NP_006564.1|	Glu140	Leu285	146aa
TNFSF14	TNFSF-14	LIGHT	gi|25952144|ref|NP_003798.2|	Glu91	Val240	150aa
TNFSF15	TNFSF-15	TL1A/VEGI	gi|23510445|ref|NP_005109.2|	Asp91 Asp93	Leu251 Leu251	161aa 159aa
TNFSF18	TNFSF-18	GITRL	gi|4827034|ref|NP_005083.1|	Glu52	Ser177	126aa
EDA		EDA-A1	gi|4503449|ref|NP_001390.1|	Glu245	Ser391	147aa
EDA		EDA-A2	gi|54112101|ref|NP_001005609.1|	Glu245	Ser389	145aa

Table 2

gi|2104653|emb|CAA31379.1| whisker antigen control protein (AA 1-487) [Enterobacteria phage T4].

gyipeaprdgqayvrkdgewvllstfl

(Gly458-Leu485)

Natural variants:

gi|32453655|ref|NP_861864.1| Wac fibritin neck whiskers [Enterobacteria phage RB69]

gi|22652096|gb|AAN03610.1|fibritin protein gpwac [phage AR1]

GYIPEAPRDGQAYVRKDGEWVLLSTFL T4-foldon (SEQ ID NO:8)

GYIEDAPSDGKFYVRKDGAWVELPTA Enterobacteria phage RB69 (SEQ ID NO:9)

GYIPEAPKDGQAYVRKDGEWVLLSTFL phage AR1 (SEQ ID NO:10)

Table 3

T4 foldon

GYIPEAPRDGQAYVRKDGEWVLLSTFL

T4 foldon muteins

GYIPEAPRDGQAYVRKDGEWVLLSTFL

GYIPEAPRDGQAYVRRDGDWVLLSTFL (SEQ ID NO:11)

GYIPEAPKDGQAYVRKDGEWVLLSTFL (SEQ ID NO:12)

GYIPDAPRDGQAYVRKDGEWVLLSTFL (SEQ ID NO:13)

GYIPEAPREGQAYVRKDGEWVLLSTFL (SEQ ID NO:14)

GYIPEAPRDGQAYVRKDGEWVFLSTFL (SEQ ID NO:15)

GYIPEAPRDGQAYVRKDGEWVLLSTFV (SEQ ID NO:16)

GYIPEAPRDGQAYVRKDGEWVLLSTFI (SEQ ID NO:17)

GYIPDAPREGQAYVRKDGEWVFLSTFV (SEQ ID NO:18)

Table 4

Comparison of theoretical and experimental determined molecular weights

Construct:	Theoretical MW of monomer (kDa)	Apparent MW based on SDS-PAGE (kDa)	Apparent MW: based on SEC (kDa)	ELUTION volume (SEC in ml)
hsCD95L-AT4	22.4	30	90,3	13.85
hsCD95L-A69	22.5	31	80	14.07
hsTRAIL-AT4	25.3	25	61	14.56

CLAIMS

1. A fusion protein comprising:

(i) a TNF-superfamily cytokine or a receptor binding domain thereof,

(ii) a flexible linker element between components (i) and (iii), and

(iii) a fibritin trimerization domain, wherein component (i) is located N-terminally of component (iii),

wherein component (i) is selected from the group consisting of CD95L, TNFα1 and a receptor binding domain thereof.

2. The fusion protein of claim 1, wherein component (i) comprises amino acids 142-281 or 144-281 of SEQ ID NO:30.

3. The fusion protein of claim 1 or 2, wherein component (ii) has a length of from 5-20 amino acids.

4. The fusion protein of claim 3, wherein component (ii) has a length of 6, 9, 12, 15 or 18 amino acids.

5. The fusion protein of claims 3 or 4, wherein component (ii) is a glycine/serine linker.

6. The fusion protein of claim 5, wherein component (ii) has the amino acid sequence $(GSS)_a(SSG)_b(GS)_c(S)_d$ wherein a, b, c, d is each 0, 1 , 2, 3, 4, or 5.

7. The fusion protein of any one of claims 1-6, wherein component (iii) is a bacteriophage fibritin trimerization domain.

8. The fusion protein of claim 7, wherein component (iii) is

a) a bacteriophage T4 fibritin trimerization domain, or

b) a bacteriophage RB69 fibritin trimerization domain.

9. The fusion protein of claims 7 or 8, wherein component (iii) comprises

a) the amino acid sequence (G)YIPEAPRDGQ AYVRKDGEWV LLSTFL as depicted as amino acids 458-484 or 459-484 of SEQ ID NO:23;

b) the amino acid sequence GYIEDAPSDGKFYVRKDGAWVELPTA as depicted as amino acids 455-480 or 456-480 of SEQ ID NO:24 ; or

c) an amino acid sequence having an identity of at least 70% to the amino acid sequence of a) or b).

10. The fusion protein of any one of claims 1-9, which additionally comprises at least one of

a) an N-terminal signal peptide domain, which may comprise a protease cleavage site; and

b) a C-terminal flexible element which may comprise or connect to a recognition/purification domain.

11. The fusion protein of any one of claims 1-10, which is present as a trimeric complex or as an oligomer of the trimeric complex.

12. The fusion protein of claim 11, wherein the complex consists of three identical fusion proteins.

13. A nucleic acid molecule encoding the fusion protein of any one of claims 1-12.

14. The nucleic acid molecule of claim 13, in operative linkage to an expression control sequence.

15. The nucleic acid molecule of claim 13 or 14, comprising the sequence of SEQ ID NO:2 or SEQ ID NO:21.

16. The nucleic acid molecule of any one of claims 13-15, which is located on a vector.

17. A cell transformed or transfected with the nucleic acid molecule of any one of claims 13-16, wherein the cell is selected from a prokaryotic cell, and a eukaryotic cell.

18. The cell of claim 17, wherein said cell is a mammalian.

19. The cell of claim 18, wherein said mammalian cell is a human cell.

20. A pharmaceutical composition comprising the fusion protein of any one of claims 1-12, the nucleic acid molecule of any one of claims 13-16, or the cell of any one of claims 17-19, and a carrier.

21. A diagnostic composition comprising the fusion protein of any one of claims 1-12, the nucleic acid molecule of any one of claims 13-16, or the cell of any one of claims 17-19, and a carrier.

22. The fusion protein of any one of claims 1-12, the nucleic acid molecule of any one of claims 13-16, or the cell of any one of claims 17-19 for use in prophylaxis or treatment of a disorder caused by, associated with or accompanied by dysfunction of TNF cytokines.

23. The fusion protein of claim 22, wherein the dysfunction of TNF cytokines is selected from the group consisting of proliferative disorders; infectious diseases; inflammatory diseases; metabolic diseases; autoimmune disorders; degenerative diseases; apoptosis associated diseases and transplant rejections.

24. The fusion protein of claim 23, wherein said proliferative disorders are tumors.

25. The fusion protein of claim 24, wherein said tumors are solid tumors or lymphatic tumors.

26. The fusion protein of claim 23, wherein said autoimmune disorders are rheumatoid diseases or arthritic diseases.

27. The fusion protein of claim 23, wherein said degenerative diseases are neurodegenerative diseases.

28. The fusion protein of claim 27, wherein said neurodegenerative diseases are multiple sclerosis.

Figure 1A

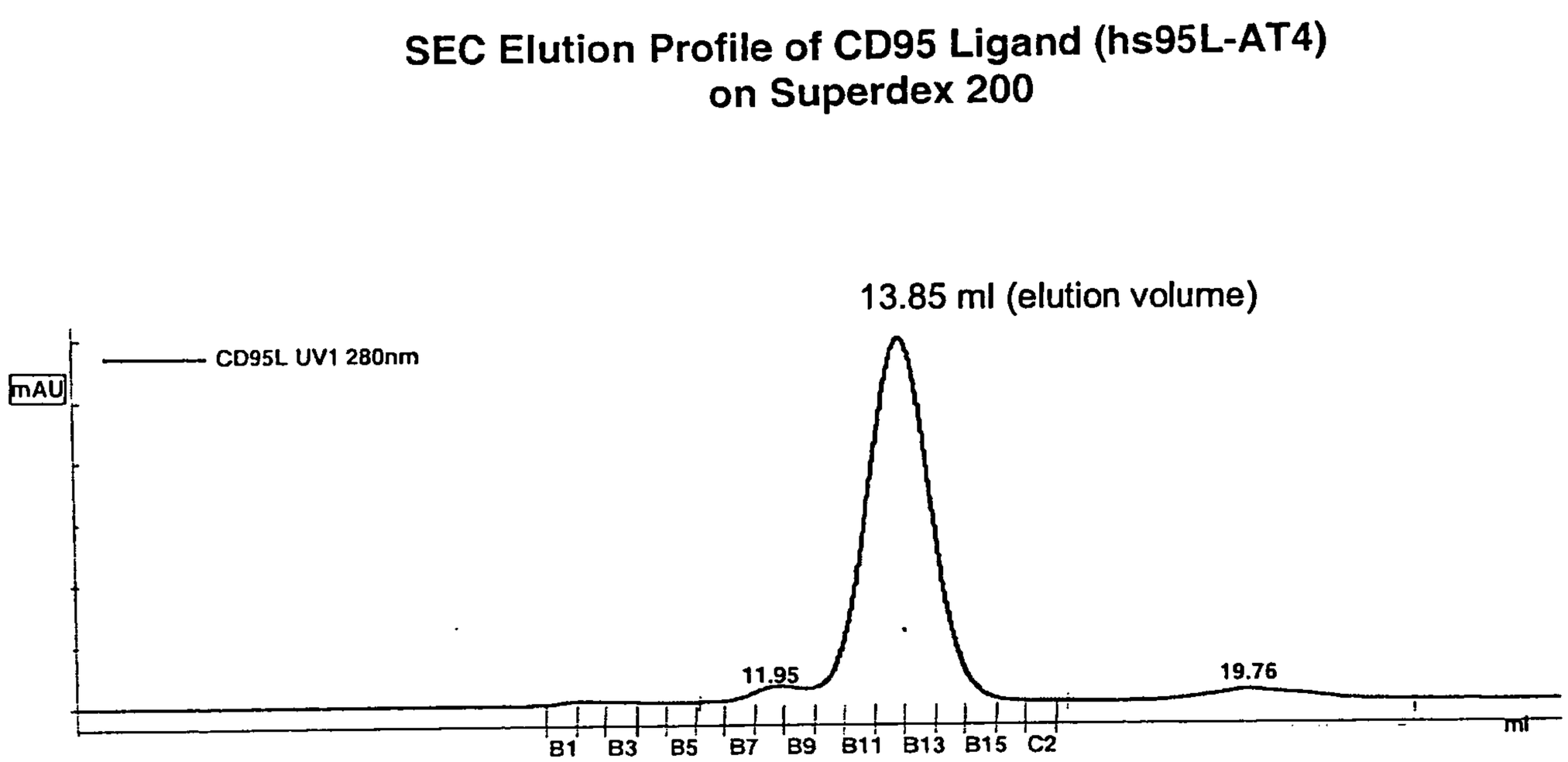


Figure 1B

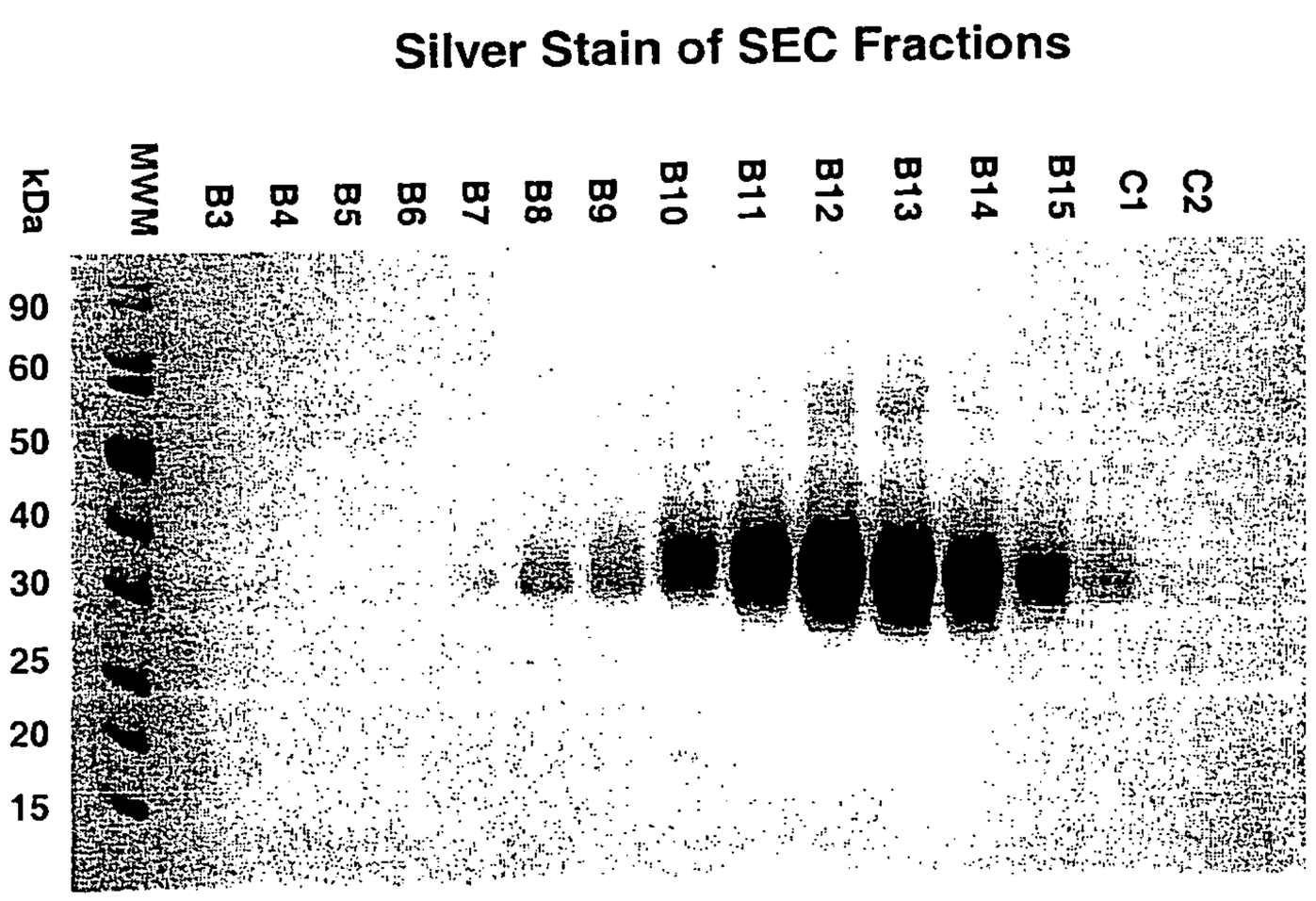
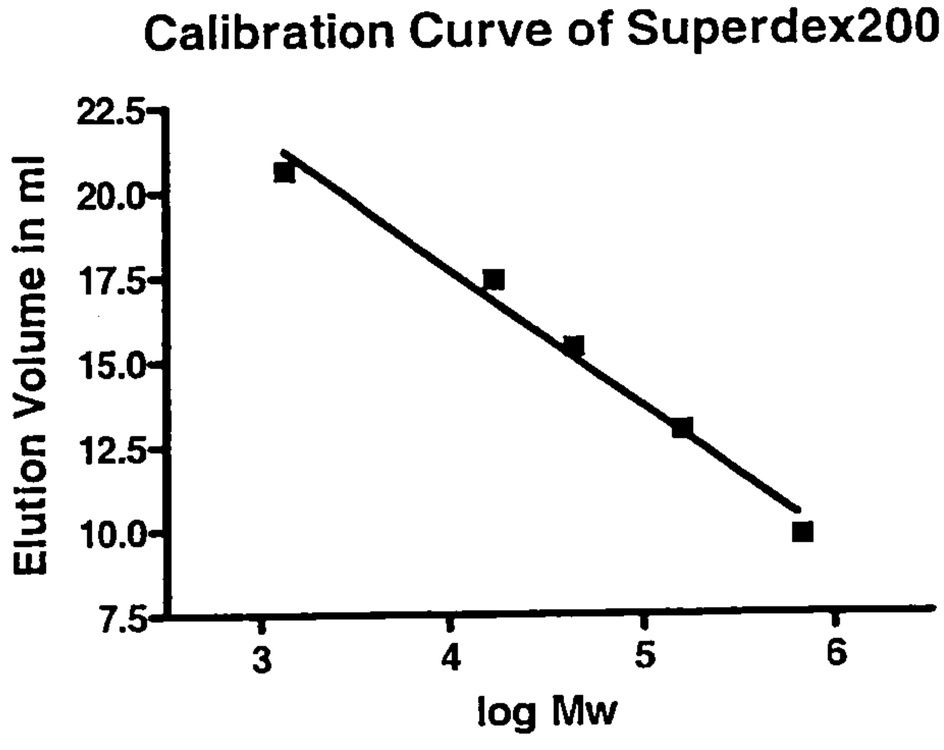


Figure 2



Protein	SEC Elution in ml	Mw in kDa	Log Mw
Thyroglobulin	9.82	670	5.826
y-Globulin	12.97	158	5.199
Ovalbumin	15.44	44	4.643
Myoglobin	17.43	17	4.230
Vitamin B12	20.65	1.4	3.130
CD95 Ligand (hs95L-AT4)	13.85	*90.3*	4.956

Figure 3

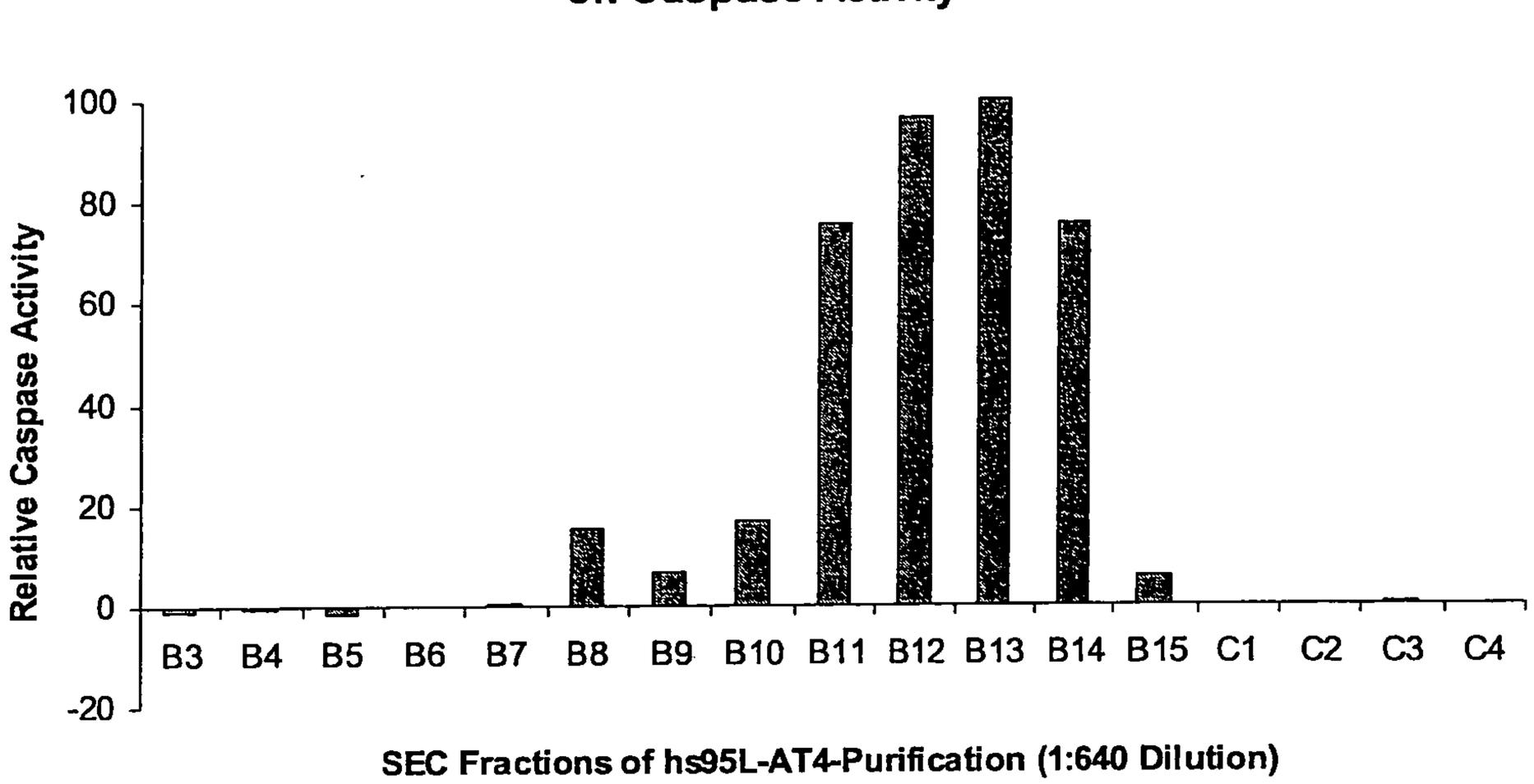

Cellular Apoptosis Assay with Jurkat Cells:
Impact of CD95 Ligand (hs95L-AT4, Different SEC Fractions)
on Caspase Activity
Relative Caspase Activity
100
80
60
40
20
0
-20
B3
B4
B5
B6
B7
B8
B9
B10
B11
B12
B13
B14
B15
C1
C2
C3
C4
SEC Fractions of hs95L-AT4-Purification (1:640 Dilution)

Figure 4

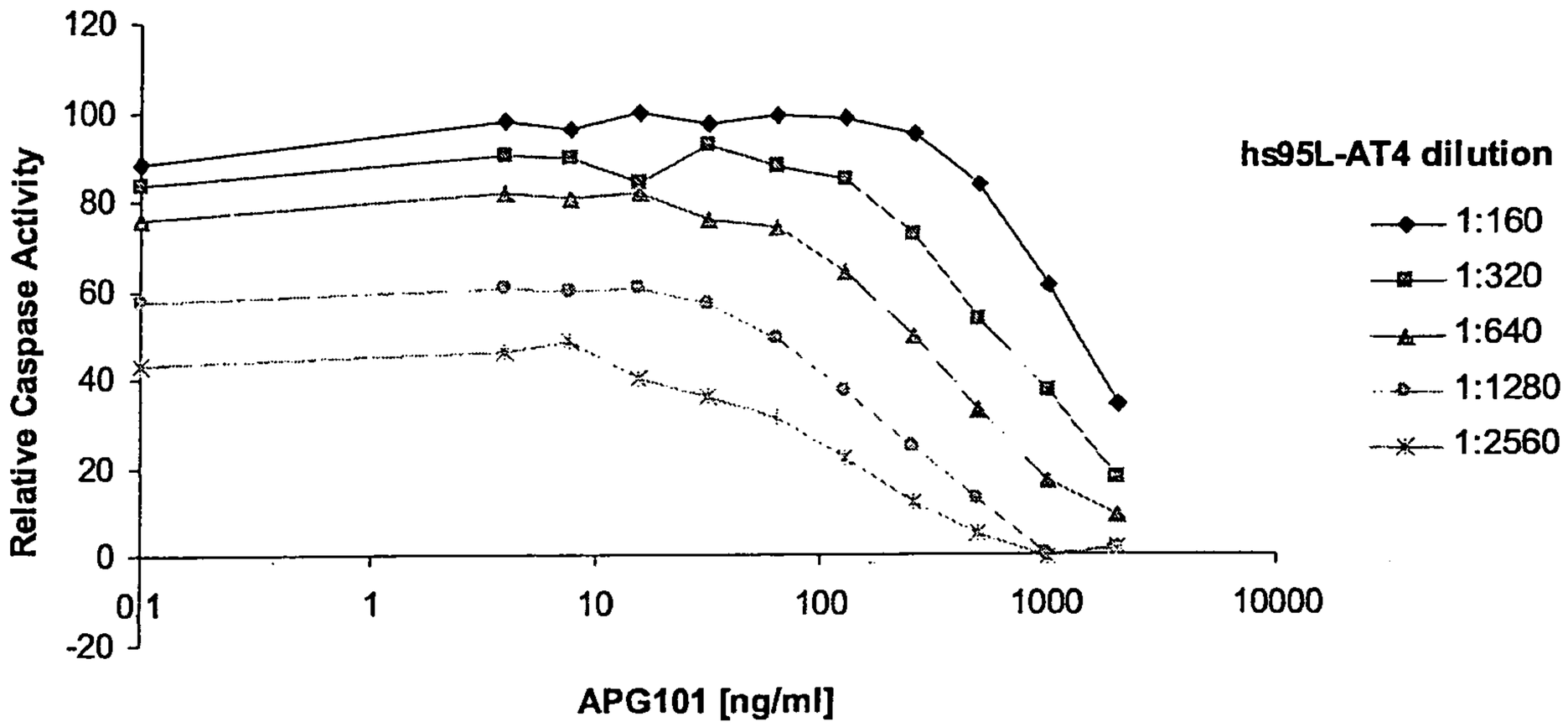
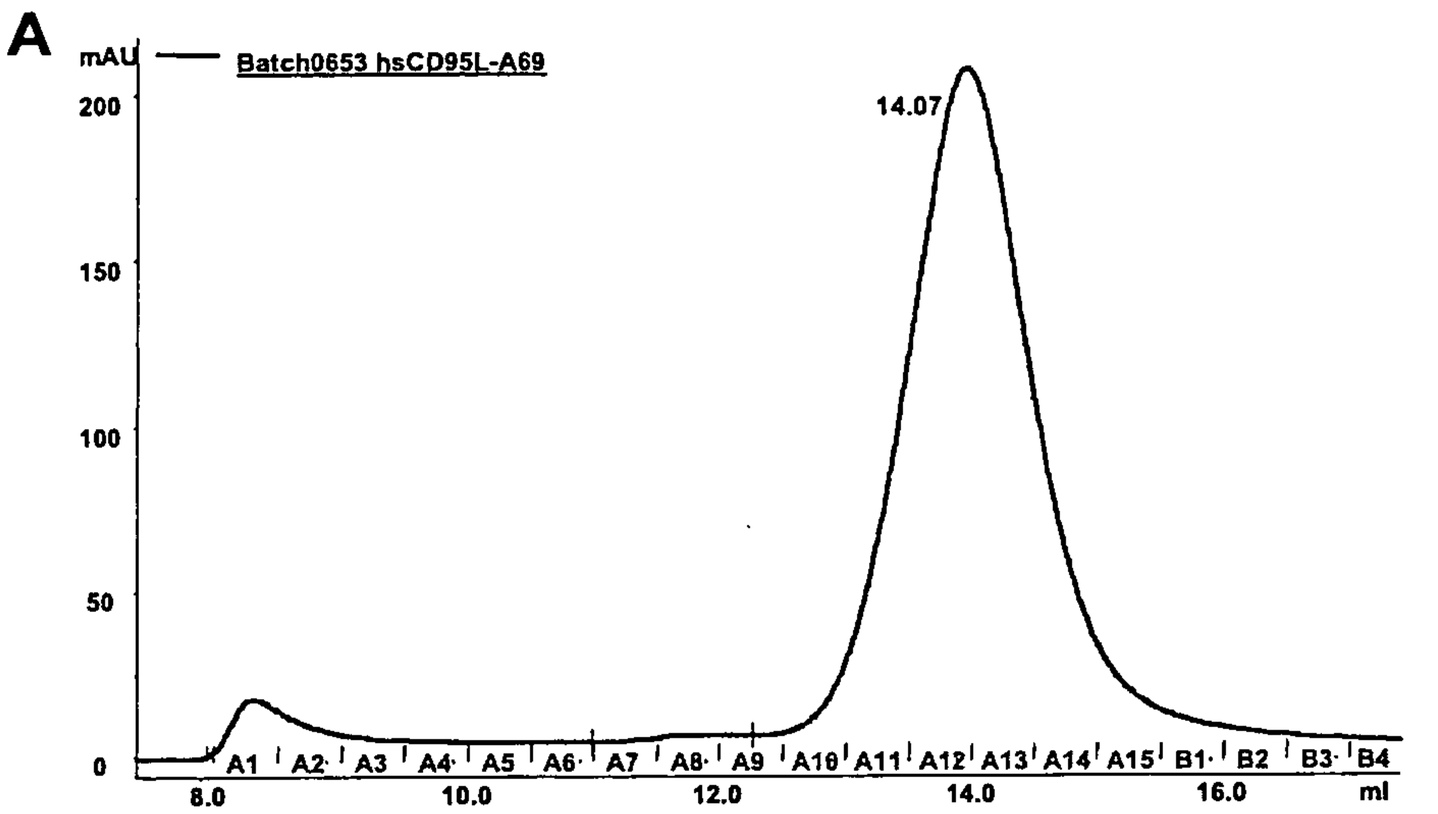
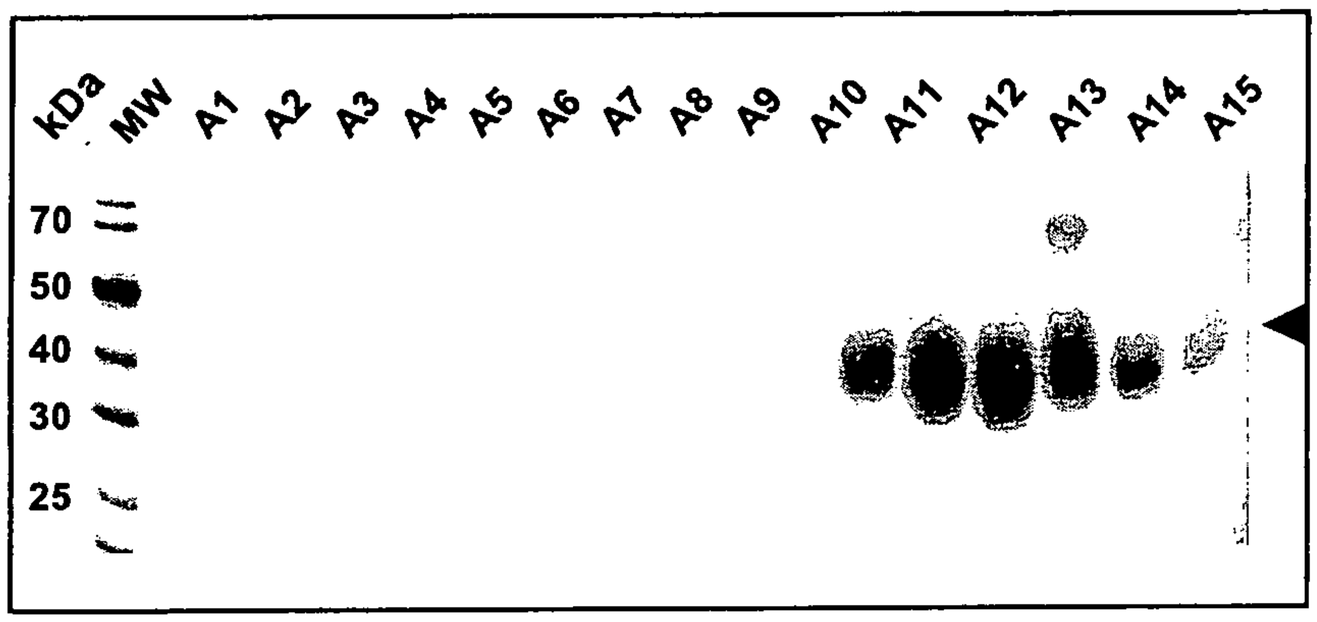

Cellular Apoptosis Assay with Jurkat Cells:
APG101 Antagonizes the Dose-Dependent Induction of
Caspase Activity by the CD95 Ligand hs95L-AT4
Relative Caspase Activity
120
100
80
60
40
20
0
-20
0
1
1
10
100
1000
10000
APG101 [ng/ml]
hs95L-AT4 dilution
1:160
1:320
1:640
1:1280
1:2560

Figure 5

Purification of hsCD95-A69





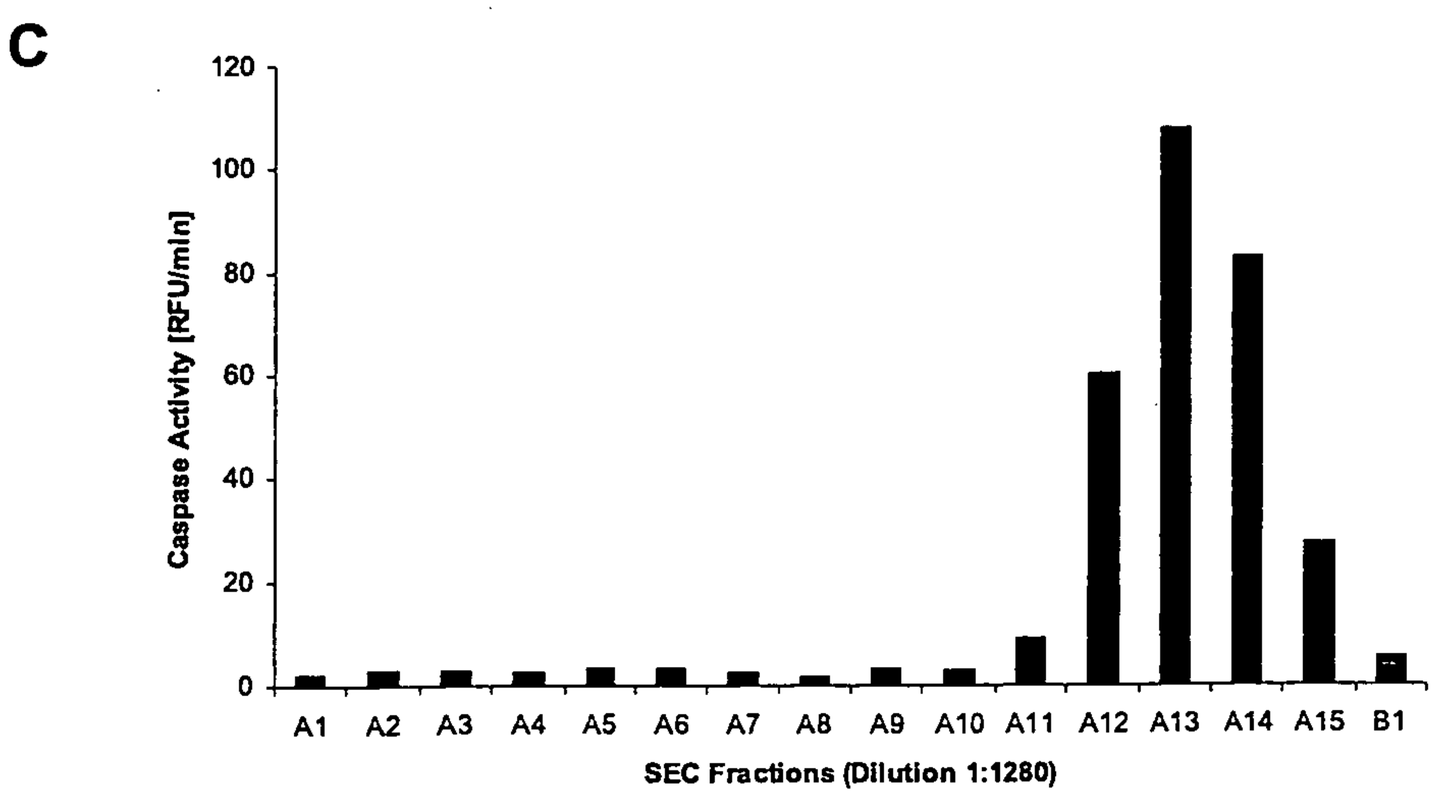


Figure 6

Purification of hsTRAIL-AT4: