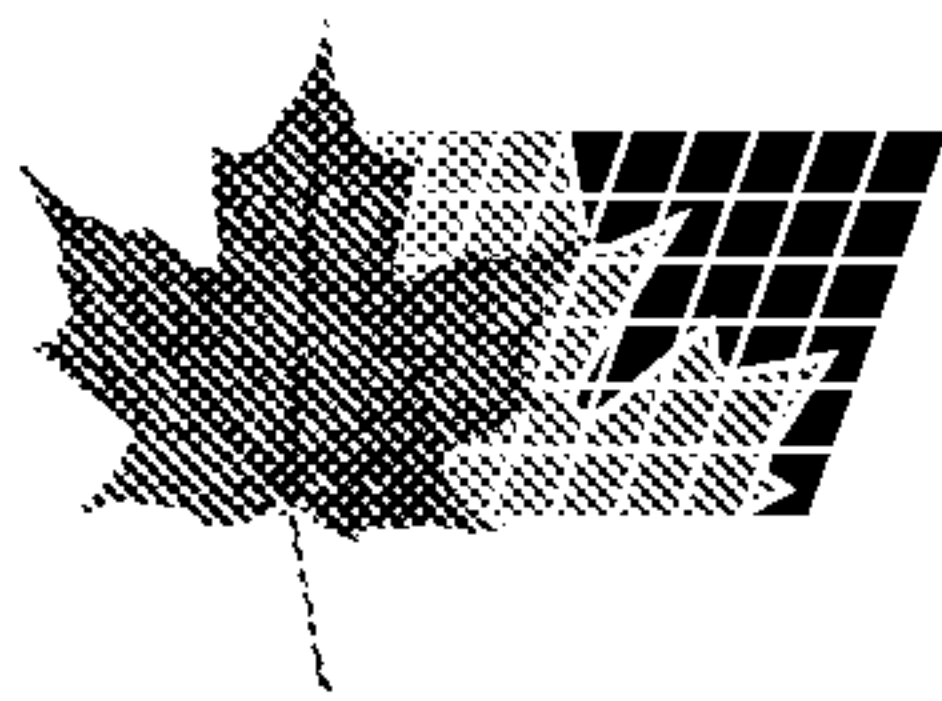




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(54) **FLIP-GENE ET FLIP-PROTEINE**
(54) **FLIP GENE AND FLIP PROTEIN**

(57) L'invention concerne des séquences ADN ayant au moins un domaine effecteur de la mort cellulaire, des vecteurs d'expression à séquences ADN ayant au moins un domaine effecteur de la mort cellulaire, des cellules hôtes transformées par ces vecteurs d'expression, un procédé pour l'expression ou l'isolement de produits géniques ayant au moins un domaine effecteur de la mort cellulaire et des produits géniques purifiés ou des fragments de ces produits géniques ayant au moins un domaine effecteur de la mort cellulaire.

(57) The invention relates to DNA sequences with at least one death effector domain, expression vectors with DNA sequences with at least one death effector domain, host cells transformed with these expression vectors, a method for expressing or isolating gene products with at least one death effector domain, and purified gene products or segments of these gene products with at least one death effector domain.



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(54) Title: FLIP GENE AND FLIP PROTEIN (54) Bezeichnung: FLIP-GEN UND FLIP-PROTEIN (57) Abstract The invention relates to DNA sequences with at least one death effector domain, expression vectors with DNA sequences with at least one death effector domain, host cells transformed with these expression vectors, a method for expressing or isolating gene products with at least one death effector domain, and purified gene products or segments of these gene products with at least one death effector domain. (57) Zusammenfassung Offenbart werden DNA-Sequenzen mit mindestens einer Todeseffektordomäne, Expressionsvektoren mit DNA-Sequenzen mit mindestens einer Todeseffektordomäne, mit diesen Expressions vektoren transformierten Wirtszellen, Verfahren zur Expression oder Isolation von Genprodukten mit mindestens einer Todeseffektordomäne und gereinigte Genprodukte oder Abschnitte dieser Genprodukte mit mindestens einer Todeseffektordomäne.				

FLIP GENE AND FLIP PROTEIN

DNA sequences with at least one death effector domain, expression vectors with DNA sequences with at least one death effector domain, host cells transformed with these expression vectors, a method for expressing or isolating gene products with at least one death effector domain, and purified gene products or segments of these gene products with at least one death effector domain.

The invention relates to DNA sequences coding for proteins or protein segments with at least one death effector domain and inhibiting cell apoptosis, and further, expression vectors with the above DNA sequences with at least one death effector domain, host cells that have been transformed by these expression vectors, procedures for the expression or isolation of gene products with at least one death effector domain, and purified gene products or segments of these gene products with at least one death effector domain, whereby the invention, by means of the various subjects of the invention, intervenes in the regulation of programmed cell death.

Programmed cell death is known as apoptosis and occurs in embryogenesis, metamorphosis, tissue regeneration, elimination of diseased cells, and necrotization of immunologic thymocytes and T-cells. During T-cell maturation in the thymus, T-cells learn how to distinguish heterologous from autologous antigens. T-cells recognizing autologous antigens are killed off during the maturation process. The loss of these cells represents an apoptotic process.

Within the immune system apoptosis also serves as a defense strategy for eliminating potentially harmful agents. Apoptosis occurs in particular with parasites having their reproductive cycle within the cell (such as viruses, certain intracellular bacterial parasites, as *Mycobacterium tuberculosis*, or *Mycobacterium leprae*, or *Bordetella pertussis* or types of *Lysteria* and also protozoa, such as trypanosomas or toxoplasmas. The apoptotic processes of virus-infected cells are regulated by T-lymphocytes. This may occur in several ways. Thus a T-lymphocyte may initiate apoptosis by releasing proapoptotic proteins (for instance perforin or granzymes), or by expressing CD95-(APO-1/Fas)-ligand (Tschopp & Hofmann, Trends in Microbiology 4, 91-94). In addition apoptosis of infected cells may in such a case also be regulated by an involuntary mechanism. The cells react to the presence of parasitizing intracellular viruses and respond to this danger by means of a cell-suicide.

In apoptosis, Fas receptors play a central role. The Fas receptor conveys an extracellular signal to the cell and, after it goes through a signal cascade, this leads to the apoptosis of the cell. Thus, for example, the Fas protein (CD95) is expressed on activated T-cells, B-cells and neutrophilic leucocytes. It is a 45 kD-protein (Itoh et al., Cell 66:232, 1991; Watanabe-Fukunaga et al., Nature 356:314, 1992). A vigorous expression of Fas-mRNA may also be observed in the thymus, the liver, the heart muscle, the lung and/or in the ovaries of mice (Watanabe-Fukunaga et al., Journal of Immunology, 148:1274, 1992). Crosslinking of specific monoclonal antibodies against the Fas receptor leads to the induction of cell death (apoptosis)

with numerous cell types (Yonehara et al., Journal of Experimental Medicine, 169:1747, 1989; Traut et al., Science, 245: 301, 1989).

There is also further evidence that in addition to the Fas receptor other receptors, after binding of an extracellular ligand, may initiate the signal cascade leading to cell death. Thus the membranous receptors TNFR-1 and TRAMP (wsl/DR-3/Apo-3) are involved in initiating apoptosis through binding of an extracellular ligand. (Nagata, Cell 88, 355-365, 1997; Bodmer et al., Immunity 6, 79-88, 1997; Kitson et al., Nature 384, 372-375, 1996; Yu et al., Science 274, 990-992, 1996). The above receptors are also called "death receptors." All of them belong to the tumor necrosis factor receptor family (TNF-R).

These receptors convey the death signal (the apoptotic signal) by means of a cytoplasmic sequence motif also called death domain (DD). This death domain interacts with the adaptor molecules FADD and/or TRADD (Nagata, Cell 88, 355-365, 1997), i.e. the death domain causes an attachment of adaptor molecules. The adaptor molecule FADD is known to associate with the so-called ICE-like protease FLICE I (also called caspase-8, Mch5, or MACH) (Muzio et al., Cell 85, 817-827, 1996; Boldin, Goncharov, Goltsev, Y.V. & Wallach, Cell 85, 803-815, 1996). This association is produced via the so-called death effector domains (DEDs), which are present at the C-terminal of the adaptor molecule, for instance FADD, as well as at the N-terminal of the protease FLICE. The complex made up of receptor, adaptor molecule and protease is also called DISC ("Death Inducing Signaling Complex") (Kischkel et al., EMBO J 14, 5579-5588, 1995). The FLICE protein linked

within the DISC-complex finally regulates the remaining proteolytical activities of other proteins of the ICE protease group. This proteolytical cascade finally leads to the apoptotic reaction of the cell (Muzio et al., Cell 85, 817-827, 1996; Boldin, Goncharov, Goltsev, Y.V. & Wallach, Cell 85, 803-815, 1996).

As already described, apoptosis is a defensive strategy of the immune system for killing off virus-infected cells. In their turn viruses have developed strategies in order to avoid the apoptosis which would inhibit their reproductive cycle. Certain viruses are therefore equipped with genes whose genetic products block the apoptotic signal transduction mechanism (Shen, Y. & Schenk, Curr. Biology 5, 105-111, 1995). Examples are the gene products CrmA of the cowpox virus or protein p35 of the baculovirus (Shen, Y. & Schenk, Curr. Biology 5, 105-111, 1995). As expected, as inhibitor proteins these gene products above all block the effector proteases of the apoptotic signal transduction mechanism. Their inhibitory effect on the cysteine proteases, which belong to the ICE-like (or Caspase-) protein group, deserves particular mention (Henkart, Immunity 4, 195-201, 1996). But other viral genes with anti-apoptotic properties have been identified also. These are similar to the mammalian bcl-2-gene (Shen, Y. & Schenk, Curr. Biology 5, 105-111, 1995).

Through the publications of Boldin M.P. et al. (J. Biol. Chem. 270, 7795-7798 (1995) and Chinnaiyan et al. (Chinnaiyan, A.M., O'Rourke, K., Tewari, M. & Dixit, V.M., Cell 81, 505-512 (1995)) it is known that deletion mutants of the adaptor molecule FADD, then only including one death domain, or of the protease FLICE, then only including two death effector domains, exercise a dominant negative inhibitory effect on the early events of the apoptotic signal cascade.

The present invention seeks to identify genes or their gene products that likewise exhibit a blocking effect on cell apoptosis and thus exercise a regulatory effect on apoptotic events.

Within the scope of the present invention, genes, i.e. segments of DNA sequences, and gene products coded with these genes, i.e. proteins having inhibitory characteristics toward the signal cascade, are disclosed. The invention above all discloses viral, human and murine genes, various gene transcripts and their expressed proteins that suppress proteolytical signal transduction and thus block the apoptosis.

The subject of the present invention therefore is a DNA sequence coding for a gene product or a gene product segment that inhibits cell apoptosis and includes at least one death effector domain. Gene product or gene product segment in particular is defined as a coded protein or coded protein segment. A primary transcript, for instance mRNA, may however also be called a gene product.

Within the scope of the present invention, all derivatives or alleles of the DNA sequence according to the invention are included in the subject of Claim 1, provided they are functionally homologous to the natural sequence. All DNA sequences currently existing in organic nature exhibiting the same functions, but showing characteristic mutations of the natural DNA sequence due to evolutionary development, qualify as alleles.

Furthermore, all derivatives of the natural DNA sequence, be they natural or artificial, are claimed in Claim 1. Artificial alterations of the natural DNA sequence may be introduced by all known methods. The mutations may be introduced at a certain DNA

sequence site by synthesizing the appropriate oligonucleotides including a certain mutation sequence.

In that case they are flanked by restriction sites, which promote binding of the synthesized oligonucleotide with the natural sequence. After ligation an altered sequence with a certain amino acid, or a certain amino acid insertion, substitution or deletion is obtained.

Alternatively oligonucleotides may be used to alter genes by means of "site-specific mutagenesis." This results in changing certain codons, whereby the desired substitution, deletion or insertion is made possible. Exemplary methods for introducing alterations into a DNA sequence have been published by Walder *et al.* (Gene 42:133, 1986), Bauer *et al.* (Gene 37:73, 1985), Craik (Bio Techniques, January 1985, 12-19), and Smith *et al.* (Genetic Engineering: Principles and Methods, Plenum Press, 1981).

Variants defined as derivatives may have any desired substitutions, insertions or deletions of a natural DNA sequence, as long as the signal cascade-inhibitory function according to the invention is present. They may in particular exhibit conservative substitutions in that one amino acid has been exchanged for another amino acid with similar physicochemical properties. Examples of conservative substitution are the substitution of an aliphatic amino acid for another aliphatic amino acid, such as isoleucin, valin, leucin or alanin for one another, or also a substitution of polar amino acids for other polar amino acids, for instance the substitution of lysin for arginin.

Within the scope of this invention, DNA sequences in which a DNA

sequence according to the invention has been recombined with other DNA sequences are likewise included in the claim. This may occur through fusion at the C- or N-terminals of the DNA sequence according to the invention. Thus one might conceive of certain "reporter genes" being linked to a DNA sequence according to the invention by means of their C or N-terminals. Reporter genes could, within certain limits, reveal information regarding the quantity of the expression. On the other hand, "leader sequences" may occur at the N- and/or C-terminal of the gene product of the DNA sequence according to the invention, but particularly at the N-terminal. Leader sequences will allow the specific regulation of the import or export of the gene product into certain cell organelles or into extra-cellular space.

Segments of the gene product correlating with an appropriate DNA sequence according to the invention are defined as abbreviated forms of the physiological gene product. These abbreviated forms may be abbreviated either at the N- or the C-terminals of the natural sequence by means of a process known *per se*. However, abbreviated forms lacking an amino acid sequence segment within their internal sequencing range are also conceivable. Likewise, various internal amino acid sequence segments may also have been removed from the natural amino acid sequence. The pertinent DNA sequences, in which degeneration of the genetic code for one amino acid sequence always results in a multitude of DNA sequences in accordance with the invention, in all of the above cases correspond to the inventive idea.

The condition for having a DNA sequence, be it a derivative of the natural DNA sequence or an allele of the natural DNA sequence, or a segment of the natural DNA sequence of any composition, claimed in Claim 1, is that the DNA sequence according to the invention should code for a gene product or the

segment of a gene product that inhibits cell apoptosis.

Within the scope of this invention, DNA sequences inhibiting cell apoptosis under physiological conditions by means of at least one death effector domain are being disclosed for the first time.

Death effector domains have so far been known only as protein-binding domains with the function of transmitting the extracellular signal for initiating the apoptosis. In this case the death effector domain served to bind caspase proteins to adaptor molecules in order to initiate the apoptotic events. Thus the death effector domain has an activation effect on the transmission of the signal which finally leads to the apoptosis of the cell. This has also up to now been the functional definition of the death effector domain. It serves as a signal-transmitting, linking domain within the scope of the signal cascade in order to activate the apoptosis (Nagata, Cell, 88, 355-365, 1997). The DNA sequences according to the invention, on the other hand, code for proteins or protein segments which do not serve to transmit the signal for initiating apoptosis, but instead, block the signal cascade for initiating the apoptotic events. Therefore all DNA sequences having an inhibitory effect on cells that have already been prepared for apoptosis by an external signal, are the subject of this invention, as long as they code for at least one death effector domain. Any derivatives or alleles, by no means limited to those enumerated in these few examples, are included in the subject of the invention if they likewise interrupt the apoptotic signal cascade. This makes them functionally analogous to the gene product of the natural DNA sequence according to the invention. With respect to the regulation of the signal cascade, the DNA sequences according to the invention, together with their gene products, thus perform functions exactly opposite those of the known proteins with death

effector domains, such as FLICE or Mch4.

Therefore, quite surprisingly, the enabling disclosure of the present invention has revealed that there are DNA sequences for coding proteins with death effector domains that may to a great extent prevent cell apoptosis in the most varied life forms, such as viruses and mammals.

Thus the expression of a DNA sequence according to the invention may save 70% and possibly more than 90% of the cells after addition of an apoptosis-stimulating agent, for instance by addition of the CD95 ligand.

In a preferred embodiment of the DNA sequence according to the invention, under Claim 1, a DNA sequence is claimed which exhibits a significance level of $p < 10^{-2}$ in a comparison of the sequence of the death effector domain with a search profile according to Figure 1a or a search profile according to Figure 1b. Such a search profile is established when related sequences with homologous functions are to be identified within the scope of a data base search.

Indeed, a multitude of protein sequences currently are stored in data bases, to name, for instance, the data bases of SWISSPROT, established by EMBO (Eur. Mol. Biol. Org.), or the database "GenBank" (GenBank data base, s. Benson et al. (Nucleic Acids Research 25: 1-6 (1997))), each of them with stored DNA sequences and/or amino acid sequences of proteins. The physiologically closely related function for most of these DNA sequences and/or amino acid sequences is not known. By using sequencing homologues, and based on sequences of known functions, an attempt is being made to identify similar sequences within the data base possibly having the same or at least physiologically closely

related functions. The success of such a data base search on the one hand depends on the success of such a data base search, but on the other hand, the search profile, which is being created in accordance with the sequence or sequences of known functions, is of key importance. In accordance with the invention, two search profiles were designed for proteins exhibiting so-called death effector domains as described above. The amino acid sequences for each of the proteins FADD, FLICE and Mch4 (also called caspase-10), coded by the appropriate DNA sequences, served as a basis for the profile design for the present invention (Fernandes-Alnemri, T., et al., Proc. Natl. Acad. Sci. USA 93, 7464-7469 (1996)) on the one hand. This profile can be seen in Fig. 1A. Fig. 1b, on the other hand, shows a search profile derived from a generalized search profile on the basis of six amino acid sequences coded by DNA sequences of viral provenance according to the invention (see below). These DNA sequences according to the invention represent genes, which will be called FLIP genes in the following, except if derived from viruses, when they will be called vFLIP genes. These genes code for the vFLIP proteins. The profiles are hereby aimed at the amino acid sequences of the known death effector domains. According to the profile, newly identified proteins or DNA sequences shall include at least one death effector domain.

The data base search was carried out with the algorithm by Bucher et al. (Bucher, P. Karplus, K., Moeri, N. & Hofmann, K.A., Computer Chem. 20, 3-24 (1996)). The present amino acid sequences and the DNA sequences on which they are based were identified in the database "GenBank." However all commercial or non-commercial data bases with amino acid sequence entries may come under consideration for homology searches. A search in DNA data bases with a DNA search profile for death effector domains is another possibility. The invention is not limited by the current status

of the entries, that is, by the number of protein sequences entered by the filing date for the patent. All future data base entries with their pertinent DNA sequences according to the invention and their corresponding protein sequences are included in this preferred embodiment of the invention, provided they meet the design criteria of the profiles in Figures 1A or 1B.

In accordance with the invention under the preferred embodiment of Claim 2, a significance level of $p < 10^{-2}$ must result when the sequence of the death effector domain is compared with a search profile according to Figure 1A or a search profile according to Figure 1B. In order to calculate the statistical significance of the hits identified in the data base according to the search profile, the pertinent p-values reveal statistical significance values resulting from a conversion of normalized profile scores (Nscores). The programs *pfscan* and *pfsearch* (both included in the program package *pftools*) in their output file yield so-called "normalized scores" (Nscores) as a comparison standard for the significance of a profile/sequence. *pftools* is a freely available program package for generalized profile applications. It has been described in the publication by Bairoch et al. (Nucleic acids Research 25: 217-221 (1997)). It is available on the Internet under <ftp://ulrec3.unil.ch/pub/pftools> or by inquiring with Dr. Philippe Buchner, Swiss Institute of Exp. Cancer Research, H-1066 Epalinges, Switzerland. The program *pfsearch* is used for searching a sequence data base using a profile, while the program *pfscan* is a tool for searching a profile data base by means of a sequence. The normalized scores are calculated from raw scores by applying scaling parameters. Some of the scaling parameters are derived through profile construction based on a search in a randomized data base, whereby the parameters become part of the profile. The randomized data base, which may, for instance, be

based on the SWISSPROT data base, is then created by means of so-called "sectional shuffling" with a window latitude [?] of 20 residuals (Pearson and Lipman, Proceedings of National Academy of Science, USA, 85, 244-2448, 1988). Further information about the algorithm can also be found in the publications by Bucher et al. (Computer Chemistry 20, 3-24, 1996) and Hoffmann et al. (TIBS 20, 347-349, 1995).

Nscores may be interpreted as a base 10 logarithm of the number of amino acid residues in a randomized data base, in which one would accidentally find exactly one protein of the desired quality. One may, for instance, state that an Nscore of 9.0 may be expected to occur exactly one time by accident in a randomized data base of 10^9 residues. Since the protein data base currently contains around 58,000,000 residues, which corresponds to a magnitude of $10^{7.76}$, a protein with an Nscore of 7.76 could according to the statistical average be expected to occur once. Higher Nscores signify better protein/profile hits than could be expected in a data base of the current size by pure accident. In practice an Nscore of 8.5 to 8.7 has established itself as a significance limit. The probability p of finding such a hit in the protein data base by accident is about 0.1. This is based on the following calculation:

$$p = 10^{-(\text{Nscore} - \log[\text{data base size}])}.$$

This equation holds for Nscores > 8.5.

In sum it may be said that the probability of error has hereby been defined as the probability that a hit of the desired quality will be accidental and does not have any evolutionary relationship to a cause. Further information about the search method in a data base by means of a search profile may be found at the following web sites :

<http://ul-rec3.unil.ch/profile/profile/html>, and

<http://ulrec3.unil.ch/profile/scoredoc.html>

A special embodiment is represented by the coding of the DNA sequences for proteins according to the invention, that have two death effector domains. The presence of two death effector domains permits a strong link with the protein to be bound, for instance to the adaptor molecule FADD. While the two death effector domains of the DNA sequence according to the invention show distinct similarities, they preferably are not identical. Variations in the amino acid sequence of the two death effector domains result in a specificity of each domain that is functionally significant. Therefore the linking behavior of the proteins coded by the DNA sequences according to the invention with proteins of the apoptosis signal transduction path, may be optimally regulated. Given the cellular concentration of the coded protein according to the invention, the intensity of the apoptotic signal may be regulated through the binding constant. The scope of this invention, however, in addition lays claim to artificially recombinant DNA sequences, which code for proteins with two identical death effector domains. The recombination of certain DNA sequences according to the invention (for instance, of DED1 from HHV-8 and DED2 from MCV (159L) or duplication of DED1 from HHV-8 or similar combinations) allows certain differentiated inhibitory effects on the apoptotic reaction to be achieved.

In a particular embodiment of the present invention the proteins (or gene products) coded by the DNA sequence according to the invention bind to a protein of the apoptosis signal transduction path. This provides a way for the gene products coded by the DNA sequence according to the invention to intervene in the apoptosis signal transduction path and regulate it. Proteins including

death effector domains make particularly good target proteins for the linkage. Within the signal transduction path these target proteins can either function as adaptor proteins, as does FADD, or as proteases, especially as caspase-like proteases. In this manner signal transmission from one protein of the apoptosis signal transduction mechanism to the following elements in the signal cascade may be blocked. In that case the apoptosis signal triggered outside the cell that has been transmitted via the receptor is dead-ended. Further signal transmission to the proteolytic members of the signal cascade therefore is no longer possible. The apoptosis of the cell is being prevented.

The specific binding of gene products of the DNA sequences according to the invention by means of one or several death effector domains of proteins in the apoptosis signal transduction path enjoys particular preference.

Particular preference is given to those DNA sequences whose gene product matches the search profiles shown and who at the same time, in their earliest intracellular stage of apoptosis signal transmission, already produce a blockage of the extracellular apoptosis signal. This occurs through gene products which bind directly or indirectly to the cytoplasmic segment of a membranous, cellular receptor of the apoptosis signal transduction path.

This is the case above all with the receptors for the tumor necrosis factor type. Worth mentioning are the receptors TRAMP, CD95 and TNFR-1, and also the receptor for the death-inducing ligand TRAIL (Wiley *et al.*, Immunity 3, 673-682 (1995)).

In addition, those DNA sequences are particularly preferred which code for gene products binding to soluble intracellular proteins

of the apoptosis signal transduction path. An interaction between the gene product of the DNA sequence according to the invention with the protein FADD may be mentioned as an example. If this interaction takes place via the death effector domains involved, then an interaction between the adapter molecules, such as FADD or TRADD, and the cytoplasmic segment of the receptor still can occur. However, signal transmission from the adapter molecule to the proteins of the signal cascade functioning proteolytically will be blocked. This means that interactions between the death domains of the cytoplasmic segment of the apoptosis receptor and the adapter molecule still are possible, but that the subsequent signal transmission step, for instance from FADD to FLICE, has been blocked.

To sum up it may be said that during a particularly preferred embodiment of the DNA sequence according to the invention its gene product attaches to an adaptor molecule in such a manner that a complex made up of the receptor in the form of its cytoplasmic segment, of the adaptor molecule, and the gene product of the DNA sequence according to the invention is created, and thus inhibition of the apoptotic signal cascade is assured.

As the signal cascade of the apoptosis progresses, the DISC-complex described above is completed. The DISC-complex is composed of several elements which are deposited on the CD95 protein. The elements are named CAP1 to CAP6, whereby CAP1 represents a FADD protein, CAP2 a pro-FLICE protein, CAP3 an until recently unknown protein with the N-terminus of the FLICE terminus, CAP4 a pro-FLICE protein, and CAP5 or CAP6, FLICE domains already split off, which may serve as indicators during an activation of FLICE protein within the DISC complex. In case of an agonistic stimulus to the CD95 receptor, these components

are deposited together with the CD95 protein. In the presence of a gene product of the DNA sequence according to the invention, the DISC complex is not fully completed, although CAP1, as expected, deposits on the CD95 protein, because at the same time the presence of CAP4 and CAP3 (each of which is a FLICE or FLICE-like component) has been severely reduced. Beyond that, the components CAP5 and CAP6 cannot be detected during the appropriate experiments.

Within a special embodiment therefore, the deposit and activation of the FLICE protein is nearly blocked by the gene product of a DNA sequence according to the invention. Therefore no components occurring later in the signal cascade and functioning proteolytically can be activated. The apoptosis signal is therefore being interrupted. Just as with the CD95 receptor, so too with the TRAMP receptor, the FLICE protein is activated by association with the adaptor proteins TRADD and FADD. Here too inhibition of the exogenously stimulated cell death by means of a co-expression of gene products of the DNA sequences according to the invention will occur. These gene products of the DNA sequences according to the invention may also block apoptosis signal transmission if the protein TRAIL, likewise a member of the TNF-ligand class, is added exogenously to the cells as an apoptosis stimulant. In all of the cases mentioned there is, however, a dose-response correlation between blockage of the apoptosis signal and the concentration of the gene product of the DNA sequences in accordance with the invention.

In a particularly preferred embodiment the gene product of the DNA sequences according to the invention completely inhibits cell apoptosis by preventing signal transmission to the proteolytical proteins in the cascade.

In another embodiment the DNA sequences according to the invention occur in viral, prokaryotic, or eukaryotic DNA-hereditary informational molecules.

The DNA sequences according to the invention may therefore occur in all forms of life.

DNA sequences leading to the expression of gene products, with at least one death effector domain, and inhibiting cell apoptosis, mainly occur with viruses. Here the use of the search profile according to Figure 1a or Figure 1b has resulted in identification of several proteins, whose sequence is homologous to the known cellular apoptosis signal proteins FLICE and Mch4. DNA sequences according to the invention may for example, occur with the *molluscum contagiosum* virus (MCV). Here in particular human MCV deserves mention as a carrier of the inventive sequences. With this virus a DNA sequence according to the invention occurs in ORF 159L or in ORF 160L. These ORFs code for proteins that may inhibit cell apoptosis. Amino acid sequences of DNA sequences according to the invention have been entered in the data base GenBank with an access code of U60315 (for the ORF 159L or for the ORF 160L of the MCV). In addition, N-terminal amino acid sequences of the two ORFs are shown in Figure 2. This site has two death effector domains. Both amino acid sequences have a C-terminal extension indicated by arrows in Figure 2. The extensions are either 66 amino acids long (ORF 259L), or 102 amino acids long (ORF 160L).

In a particularly preferred embodiment the DNA sequences according to the invention occur with herpes viruses, particularly with γ herpes viruses. These herpes viruses are ones with double-stranded DNA, whose morphogenesis occurs in the nucleus of the host cell.

Claim 15 in particular claims a DNA sequence whose gene product includes an amino acid sequence according to GenBank"-access code U20824 (E8), where E8 indicates the ORF. This amino acid sequence is derived from the virus EHV-2. With this sequence it is remarkable that in four areas of the domain DED I no agreement exists with the pertinent sequences otherwise listed among those in Figure 2. In these positions the EHV-2 sequence alone shows deviations.

Claim 16 in particular claims a DNA sequence whose gene product includes an amino acid sequence in accordance with figure 16. It represents an amino acid sequence of the virus HHV-8. This sequence is in ORF 71. A DNA sequence according to the invention on which it is based has been listed in the GenBank under access code (U90534 (71)).

The claim particularly concerns a DNA sequence whose gene product includes an amino acid sequence with the GenBank access code X64346 (ORF 71). This sequence is present with the HSV virus.

Finally the claim also particularly includes a DNA sequence whose gene product includes an amino acid sequence according to Figure 17. This viral sequence (BHV-4) has a GenBank data entry for a DNA sequence in accordance with the invention under Z46385.

As defined by this invention these genes or gene products are called vFLIP genes or vFLIP proteins (abbreviation for viral FLICE Inhibition Proteins).

Here are found two sequence motifs with a highly significant homology for the death effector domains. The significance level p for the search profile according to Figures 1a or 1b according to the invention is smaller than 10^{-2} . All of them have two death

effector domains each (DED 1 or DED II). These were determined via the search profile shown in Figure 1a using the algorithm by Buchner et al. (see above). They thus exhibit the characteristic sequence motifs of a death effector domain.

In order for viral FLIP proteins to function, at least one death effector domain, but preferably two domains of this type, are as necessary as under physiological conditions. With these five viruses (EHV-2, HHV-8, HVS, BHV-4, and MCV) the two death effector domains are linked by a sequence of at least 16 amino acids. Within this linkage sequence, only a weak homology between the five viruses mentioned exists. The linkage sequence preferably has a length of 16 to 19 amino acids. The DNA sequences according to the invention may code for gene products, in which at least one or both of these death effector domain amino acid sequence segments is included.

In an embodiment of the present invention, the DNA sequence according to the invention is present within the hereditary information of mammalian cells. In this connection all imaginable mammalian cells are included. The invention relates particularly to human DNA sequences of the type defined by the invention.

A DNA sequence is included in the subject of the invention particularly if its gene product has or includes an amino acid sequence, as is shown in one of Figures 4a or 4b or 4c. Figures 4a and 4b show the amino acid sequences and the DNA sequences of human proteins pertaining to them that are homologous with the viral vFLIP proteins. The sequences were determined by screening cDNA libraries of activated human peripheral hemolymphocytes (PBL). During this procedure several clones coding for a protein closely related to the viral FLIP protein were identified. This protein has two death effector domains (DED), which allow only a

short C-terminal sequence to connect. In the following this human protein will be known as FLIP_S. A human FLIP_S DNA sequence in accordance with the invention is shown in Figure 4a. It includes 1190 nucleotides.

A longer version of a human FLIP-DNA sequence is illustrated in Figure 1b. This kind of DNA sequence according to the invention, for coding a protein, called FLIP_L in the following, and also isolated from activated human peripheral hemolymphocytes, includes 2143 nucleotides. FLIP_L and FLIP_S are alternative SPLICE variants of the DNA sequence according to the invention. According to the invention, all SPLICE variants exhibiting the characteristics of the invention, specifically that of coding for a gene product with at least one death effector domain and at the same time, inhibiting cell apoptosis, are included in the present inventive idea. The invention demonstrates that three different RNA species exist in the form of transcripts of the DNA sequence according to the invention, that is, a DNA sequence of at least one and preferably two death effector domains with apoptosis-inhibiting activity. The shortest RNA transcript (1.1 - 1.3 kB) is most vigorously expressed in muscle tissue and in the peripheral hemolymphocytes. This shortest transcript does not include a caspase-homologous domain, as is the case with the longer version. In addition two longer transcripts were detected (2.2 kB and 3.8 kB respectively) both of which exhibit a caspase-homologous domain. Compared to the number of the shorter FLIP_S transcripts, the number of the longer FLIP_L transcripts synthesized in the cell is small. They are mainly limited to muscle and lymphatic tissue, particularly to the spleen and small intestine, or to peripheral hemolymphocytes. Small numbers of transcripts may however also be detected in other tissues.

Figure 4c additionally shows a murine DNA sequence, a long

version with a caspase-homologous domain. Its amino acid sequence is also revealed in Figure 4c. The DNA sequence of this cellular FLIO protein has 2452 nucleotides. The murine gene was identified within a cDNA library of murine cardiac cells using human cDNA samples.

The present invention includes DNA sequences made up of cellular DNA of eukaryotic FLIP_L sequences that, besides at least one death effector domain, also exhibit a caspase-homologous domain. FLIP_S sequences, on the other hand, only have at least one death effector domain.

An embodiment of the present invention claims a DNA sequence whose gene product has two death effector domains and one caspase-homologous domain, whereby the caspase-homologous domain is not functional. A Caspase-homologous domain is defined as a domain of the proteases of the caspase type (i.e. the cysteine-protease type), whereby this homologous domain equips the caspases for their proteolytical activity. A non-functional caspase-homologous domain is characterized by no longer exercising any cysteine-proteolytical activity. This type of functional loss may be based on a sequence-specific mutation. With the particularly preferred DNA sequences for gene products of the long cellular FLIP proteins, the cysteine residue within the active center critical to proteolytical activity, which for instance equips FLICE protein for its proteolytical activity, mutates.

Figure 3 shows that with the present sequences the cysteine residue has been substituted for a tyrosin residue. In addition, for instance as compared to the functional protease domains of FLICE, characteristic mutations occur at different sites of the caspase-homologous domain of long FLIP proteins. One might

mention mutations in position -1, as related to the critical cysteine residue, and in positions +1 and +2, likewise related to the critical cysteine residue. The critical cysteine residue indicated by a star in Figure 3, has also mutated in the mouse FLIP_L. In this case also, the caspase-homologous domain can therefore not fulfill any proteolytic function dependent on cysteine residue. According to this invention, non-functionality of a caspase-homologous domain is merely defined as a loss of proteolytic activity which depends on the critical cysteine residue. Even so the caspase-homologous domain may have another functionality. Such caspase-homologous domains, according to this invention, are also non-functional.

The two death effector domains of the human or murine FLIP proteins likewise interact with adapter proteins, for instance, FADD. The long protein version (55 kD) and also the short form (34 kD) associate vigorously with the FADD protein in case of a co-expression. However the caspase-homologous sequence section of the long FLIP proteins does not show any FADD-binding affinity. Binding of a FLIP protein identified in a mammalian cell with a DNA sequence according to the invention to another protein, independent of any caspase-homologous domain, takes place via at least one death effector domain. Preferably this binding is promoted by two death effector domains. The appropriate experiments also prove that the proteins of the DNA sequences according to the invention made up of eukaryotic cells, in particular of mammalian cells including human cells, may also form a stable tri-complex with Fas/FADD. The gene products of these cellular DNA sequences according to the invention, as a result of activation of the apoptotic signal cascade, attach to the membranous death receptor domains. As soon as FADD has linked with FLIP and FAS, large aggregates insoluble even in SDS can be observed.

Without being confined to any one scientific theory, it can be determined that the caspases during their signal transmission as dimers are first activated and then processed through autocatalysis, finally forming the stable complex $(p10/p20)_2$. On the other hand the long FLIP proteins made up of eukaryotic cells coded by the DNA sequences according to the invention, in particular mammalian or human cells, do not show any dimer or oligomer formation. The FLIP proteins rather interact with the FLICE proteins in such a way that hetero-dimers or hetero-oligomers form between the active FLICE and the FLIP_L protein without cysteine proteolysis activity. Thus the use of FLIP proteins with non-functional, caspase-homologous domains and with at least one death effector domain for binding to proteases, in particular to cysteine proteases functioning as elements of the apoptotic signal cascade by means of their death effector domain, is another aspect of the present invention. This binding may then block apoptotic signal transmission.

Signal cascade inhibition is based on following detailed mechanism: The FLICE protein is activated by the signal for apoptosis initiated at the death receptor, possibly by homo-dimer or homo-oligomer formation. As it is activated, either an autocatalytic fission at the p10/p20 intersection with the sequential segment of the caspase domain may occur, or the FLIP protein may bind first. In every case after the FLIP protein binds with the non-functional caspase-homologous domain, proteolysis of the FLIP protein through proteolytic activity of a FLICE protein within the FLICE/FLIP heterodimer or hetero-oligomer follows. After certain conditions which initiate apoptosis, for instance superexpression of the CD95 receptor, further proteolytic fission (processing) of the FLICE protein may occur. The FLICE protein takes on a form corresponding to a large extent to the short cellular FLIP protein (FLIP_S).

Proteolytic fission therefore takes place at about the connection between the C-terminal death effector domain and the caspase domain of the FLICE protein.

As with the viral FLIP protein, the examples of the embodiment demonstrate that the cellular forms of the FLIP protein (human or murine, long or short FLIP protein) coded by DNA sequences according to the invention have an inhibiting effect on numerous apoptosis signals triggered by or via death receptors.

An important aspect of all DNA sequences according to the invention therefore is their utilization for immortalizing cells. This immortalization may occur *in vitro* or *in vivo*; it may involve all cell types imaginable that are capable of undergoing an apoptotic reaction. *In vivo* processes include all gene-therapeutic methods known to one skilled in the art.

In an embodiment of the present invention a DNA sequence according to the invention is effectively linked with a promoter. In this embodiment one promoter, preferably arranged upstream, thus binds with a DNA sequence according to the invention. This promoter may, depending on the experimental goal, be either a prokaryotic or eukaryotic promoter.

Promoters used in prokaryotic host cells might be the β -lactamase promoter or the lactose promoter, or the tryptophane promoter system or hybrid promoters, as for instance the tac promoter. The appropriate promoters are chosen by one skilled in the art, depending on the bacterial host cell. Their nucleotide sequences have been published. Through information in the literature one skilled in the art is in a position of being able to bind the promoters to the DNA sequences according to the invention

(Siebenlist *et al.*, Cell, 20: 269, 1980). Promoters in bacterial systems generally also include a Shine-Dalgarno (SD) sequence.

Suitable promoter sequences in host cells may for example include the 3-phosphoglycerate-kinase promoter or promoters of other glycolytic enzymes (examples include: enolase, glycerialdehyde-3-phosphate-D-hydrokinase, hexokinase, pyruvate decarboxylase, phosphofructokinase and others).

The transcription of a DNA sequence according to the invention in cells of higher eukaryotes, in particular mammalian cells, is regulated by promoters that may be derived from differing natural systems. Thus promoters from viral genomes may be used. Examples would be polyoma viruses, SV40, adenoviruses, retroviruses, hepatitis B viruses, cytomegaloviruses and the like. With mammalian cells a possibility would be the β -actin promoter. In the current invention the S α promoter is particularly preferred.

In particular the early promoter of the human cytomegalovirus, which contains a HindIII-E restriction site (Greenway *et al.*, Gene, 18: 355-360 (1982)) lends itself to use as a promoter of cytomegaloviruses. Of course promoters of the mammalian or human host cells in each case also come into consideration.

In a particularly preferred embodiment of the invention, additional regulating elements for transcription and/or translation have been added to the DNA sequence according to the invention. Thus the transcription of a DNA sequence according to the invention apparently is considerably increased when an enhancer element is present in the expression vector. Enhancers are cis-acting elements of DNA which usually include 10 to 300

base pairs and act upon the promoter in order to raise the transcription rate. They may be arranged in the 3' or 5' position of the DNA sequence according to the invention, but also in the coding sequence itself or within an intron which is first cut out by SPLICE procedures. Typically an enhancer is selected from a virus of eukaryote cells. Examples are the SV40 enhancer or the enhancer of the early promoter of the cytomegalovirus. Enhancers of adenoviruses are also possible. Of course numerous enhancers are also known to derive from mammalian genes (for instance globin, elastase, or albumin). The enhancers typically are integrated with the 3' or 5' position in the expression vector of a DNA sequence according to the invention. Preferably the enhancer is positioned at 5' in relation to the promoter. Further regulating elements may serve to regulate transcription termination, so that the expression of mRNA is involved.

Another aspect of the present invention consists of expression vectors including a DNA sequence according to the invention, typically with a promoter and, if appropriate, with another of the above regulating elements of the transcription and/or translation. They serve to express and multiply the nucleotide sequence according to the invention in specific host cells. In general expression vectors are used that can autonomously replicate independently of the host chromosome. They have their own "origin of replication." Such sequences are present in bacteria, yeasts or viruses. On the other hand these origins are not required in the expression vectors of mammals.

If necessary the expression vectors with the DNA sequence according to the invention are developed as so-called shuttle vectors, that is, they are able to replicate in a host system and can then be transfected into another host system for purposes of expression. For instance a vector can at first be cloned in

E.coli and then be inoculated into a yeast or mammalian cell for expression. In such a case it is no longer absolutely necessary for the replication to occur independently of host cell chromosome replication.

The expression vector pCR-3 or all derivatives of this vector with an EcoR1-intersection are particularly preferred. A DNA sequence according to the invention preferably is transfected into another vector, which allows stable expression in cells such as Jurkat and Raji. Typically such expression and cloning vectors then have a selection gene exercising a marker function. This is a gene coding for a protein allowing host cells to survive or grow after being transformed by the vector. Typical selection genes code for proteins which permit a resistance toward antibiotics or other toxins. This, for instance, includes puromycin or ampicillin or neomycin. Within the scope of this invention, resistances to puromycin are particularly preferred with regard to selecting the transformed host cells.

An additional aspect of the present invention is a process for isolating gene products with at least one death effector domain, whereby the host cells are transformed by means of an expression vector in accordance with the invention and then cultivated under appropriate circumstances favoring the expression, so that the gene product finally may be purified out of the culture. This allows the protein of the DNA sequence according to the invention to be isolated out of a culture medium or out of cell extracts. One skilled in the art will immediately recognize that the isolation method and the purification process of the recombinant protein DNA-coded according to the invention in each case depends on the type of host cell, and also on whether the protein is secreted into the medium. Thus expression systems leading to secretion of the recombinant protein may be used. In that case

the culture medium must be concentrated by means of a commercially available protein concentration filter, such as Amicon or Millipore Pelicon. After the concentration step a purification step may follow, for instance a gel filtration step. Alternatively an ion exchanger with a DEAE matrix may also be used.

All materials known from protein purification, such as acrylamide, agarose, dextran or similar, may be used as matrices. A cation exchanger may also be used, which typically would contain carboxymethyl groups. Further purification of a protein coded by means of a DNA according to the invention could then proceed by means of HPLC steps. Particularly the "reversed phase" method is being used. By means of these steps an essentially homogenous recombinant protein of the DNA sequence according to the invention is obtained.

Besides bacterial cell cultures for isolating the gene product, transformed yeast cells may also be used. In this case the translated protein may be secreted in order to simplify protein purification. Secreted recombinant protein from a yeast host cell may be obtained by methods published by Urdal et al. (J. Chromato. 296:171 (1994)).

Another aspect of the present invention is a process for the expression of gene products with at least one death effector domain, whereby host cells are transformed by means of an expression vector including a DNA sequence according to the invention. The purpose of this process for expressing gene products based on a DNA sequence according to the invention is not to concentrate and purify the gene product, but rather to influence cell metabolism by means of introducing the DNA sequences according to the invention through the expression of

the pertinent gene product. In this connection the use of host cells transformed by means of expression vectors for purposes of immortality must in particular be considered. Through use of a so-called constitutive promoter these cells may express constant protein concentrations based on sequences according to the invention. By this means apoptosis, either initiated by or through the mediation of, the death receptors, will permanently be prevented. The appropriate cell lines thereby become resistant to a large number of apoptotic stimuli. These cells may also be inoculated into mammalian or human organisms as the need arises. In this manner gene-therapeutic use of the DNA sequences according to the invention will be come possible by means of the cells being manipulated in the lab with expression vectors according to the invention, and their subsequent inoculation into the organism. This calls for the transfection of expression vectors with sequences according to the invention into cells which due to disease, are being eliminated as a result of apoptotic conditions in the organism.

Such a process makes possible the steady restoration of these cells endangered by apoptosis.

However, the inventive idea is also accompanied by a gene-therapeutic procedure that may be carried out *in vivo*. In this procedure vectors are used (for example liposomes or adenoviruses or retroviruses or the like), which insert the DNA sequences according to the invention in a specific manner into each of the organism's cells which, due to pathological conditions, exhibit an increased tendency toward cell death.

The DNA sequences according to the invention, their alleles, derivatives or fragments may also be used in the laboratory as samples. Thus, for example, the DNA sequence of the viral FLIP

gene might be used to identify the appropriate viruses with *in vitro* test systems. This might particularly be applied with Northern or Southern blots. The samples would have the appropriate DNA sequence length depending on the requirements of the experiment. A DNA sequence on which a death effector domain of the type according to the invention was based, might for instance be used as a detection probe.

Another useful application of the DNA sequences according to the invention is their employment as single strand DNA sequences. It is possible on the RNA and on the DNA levels. Employing both the DNA and RNA of the coding strand ("sense") and its complementary strand ("antisense") is conceivable. Which one to use would depend on the choice of the target protein. The appropriate oligonucleotides typically include between 20 and 40 nucleotides of the DNA sequence in accordance with the invention. In this connection it should be mentioned that the binding of anti-sense or sense DNA leads to the formation of duplex molecules

either blocking translation (on the RNA level), or transcription (on the DNA level). Thus the expression of FLIP proteins in the cell can be prevented by means of the DNA sequences according to the invention. Another possible application for this technology would result during the conception of anti-viral substances. By inoculation of these single-strand oligonucleotides, in particular the tumor-like qualities of the viruses expressing FLIP, particularly those of herpes viruses, could be combated. The oligonucleotides could be chemically altered by the appropriate modification to protect them against enzymatic decomposition. Inoculation of the oligonucleotide single strand into the cell might be performed via all known methods, for instance through vectors for gene transfer, or establishing electronic fields.

Host cells transformed with an expression vector according to the invention are another subject of the present invention. Appropriate host cells for cloning or expressing the DNA sequences according to the invention are prokaryotic yeasts or higher eukaryotic cells. With prokaryotes gram-negative or gram-positive organisms are expressly included. *E. coli* or bacilli are worth mentioning. The *E. coli* 294, *E. coli* B and *E. coli* X1776 as well as *E. coli* W3110 strains are disclosed as the preferred host cells for cloning the DNA sequences according to the invention, and among the bacilli, *Bacillus subtilis*, *Salmonella typhimurium* and the like. As mentioned, if the expression vectors typically include a signal sequence for transporting the protein into the culture medium, prokaryotic cells will be used. Besides prokaryotes, eukaryotic microbes which have been transfected with the expression vector, are also under consideration. Thus filamentous fungi or yeasts may be used as suitable host cells for DNA sequence-coding vectors according to the invention. Above all worth mentioning is *Saccharomyces cerevisiae* or ordinary baker's yeast (Stinchcomb et al., Nature, 282: 39 (1997)).

In a preferred embodiment, cells for expressing DNA sequences according to the invention are being selected from multicellular organisms. This also takes place before a background of a possibly needed glycolysis of the coded proteins. This function may be carried out in an appropriate manner in higher eukaryote cells - as compared to prokaryote cells. In principle every higher eukaryotic cell culture is available as a host cell, even though cells of mammals, such as monkeys, rats, hamsters and humans, are particularly preferred. One skilled in the art is familiar with a large number of established cell lines. The following cell lines are mentioned as part of a by no means exhaustive list: 293T (embryo kidney cell line) (Graham et al., .

Gen. Viro., 36:59 (1997)), BHK (baby hamster kidney cells), CHO (cells from hamster ovaries), (Urlaub and Chasin, P.N.A.S. (USA) 77:4216, (1980)), HELA (human cervix carcinoma cells) and other cell lines.

In accordance with the present invention, preferably cells of the mammalian immune system, above all the human immune system, have been transfected with expression vectors with DNA sequences in accordance with the invention. Here T-lymphocytes and D-lymphocytes are particularly preferred as host cells.

The case of an HIV infection demonstrates another application of the DNA sequences according to the invention which could immortalize the host cell. With an HIV infection cells perish as a result of apoptotic mechanisms. Even non-infected CD4⁺ cells are subjected to cell death. To counteract an HIV infection, immortalization of the non-infected immune cells would therefore be desirable. This would allow the basic functionality of the immune system to be preserved.

Another aspect of the present invention are the gene products of the DNA sequences according to the invention. Gene products in accordance with this invention include both primary transcripts, that is, RNA, preferably mRNA, and also proteins. These proteins according to the invention have at least one death effector domain and inhibit cell apoptosis. These proteins include all proteins coded according to the invention, including proteins expressed by DNA derivatives, DNA fragments or DNA alleles. In addition the proteins may be chemically modified. Thus a protective group may exist at the N-terminal. Glycosyl groups may be attached to hydroxyl or amino groups, lipids may have covalent bonds with a protein according to the invention, likewise phosphates or acetyl groups, or the like. Any chemical substance,

compound or group may bind to the protein according to the invention by any type of synthesis. Additional amino acids may be fused with the N and/or C terminals, for instance as individual amino acids, as peptides or as protein domains and the like. In particular, so-called signal or "Leader" sequences are present at the N terminal of the amino acid sequence according to the invention, leading the peptide into a certain cell organelle or into extra-cellular space (or into the culture medium), either co-translationally or post-translationally. At the N or at the C-terminal amino acid sequences may also be present, which due to their role as antigens allow the amino acid sequence according to the invention to bind to antibodies. Here Flag-peptide, whose sequence in the amino acid one-letter code reads DYKDDDDK, deserves special mention. This sequence has very antigenous properties and therefore allows fast testing and easy purification of the recombinant protein. Monoclonal antibodies binding the Flag peptide are available from the Eastman Kodak Co., Scientific Imaging Systems Division, New Haven, Connecticut. The DNA sequences according to the invention may also be deposited on the strand of the hereditary informational molecule in the form of numerous exons separated from each other by introns. Thus all conceivable SPLICE variants (on the mRNA-level) are likewise included among the gene products of the subject of the invention. Even the proteins coded by these various SPLICE variants are included in this invention.

Besides the covalent modifications of the protein, also protein aggregates, for instance dimers of the proteins according to the invention or higher level aggregates, are included in the subject of the present invention.

In an embodiment of the present invention the protein as a gene product according to the invention has a significance level of

$p < 10^{-2}$ in a comparison of the sequence with a search profile according to Figure 1a or Figure 1b. As described above, a search profile is established in order to identify possible death effector domains in a data base. Two data bases are available. The data base can either be compared with a search profile, or the search profile compared to the data base. According to the invention, all gene products or segments of these gene products in an embodiment are included in the subject of the invention if a primary transcript (RNA) is coded by one of claims 1 through 24.

In a preferred embodiment, FLIP proteins according to the invention have an amino acid sequence according to one of Figures 4a, 4b, or 4c (for the cellular proteins), an amino acid sequence according to Figures 16 or 17, or GenBank access codes U60315 (MCV 159L), U60315 (MCV 160L), U20824 (E8), or X64346 (ORF 71).

Numerous applications for a protein according to the invention are disclosed. Thus the purified protein according to the invention may, according to current usage, be administered to patients, particularly to human patients, given an appropriate pathological indication. The protein according to the invention may freely be Galenically prepared with other proteins or pharmacological additives or support materials, or co-therapeutic materials. In this connection binding studies with other proteins are feasible. Within the scope of such binding studies the biological activity of the proteins according to the invention may be tested. A study of proteins according to the invention binding to adapter proteins such as FADD is conceivable. Application of proteins according to the invention within the scope of protein purification processes may be addressed. A protein according to the invention may be attached to a support material, such as a column, with this application serving for

binding and isolating possible cell proteins physiologically interacting with the protein according to the invention.

The Invention is Further Explained by Means of the Figures Below

In Figure 1 two search profiles for identifying death effector domains have been described. In accordance with their one-letter code, the amino acids are alphabetically arranged (i.e. Alanin: A, in the first position). A systematic series of amino acids like this follows for each of the positions in the sequence. The positions in the sequence have been marked by "/M:". This is followed by the information as to which amino acid at this sequence position has the greatest probability (SY = 'P' for the first position in the profile sequence according to Figure 1a), of occurring in a homologous sequence. Then, after "M =", a systematically alphabetized series of amino acids with a probability weighting according to the profile for each of the amino acids at the sequence position specified follows. The more negative the numerical value of an amino acid, the less is the probability (with a protein having a homologous function) of finding this amino acid in this position in the sequence. In this fashion a matrix for all amino acids in all sequence positions has been constructed (<http://expasy.hcuge.ch/txt/profile.txt>).

Figure 1a shows the search profile created from the proteins FADD, Mch4, and FLICE. It includes two pages.

Figure 1b shows the search profile created from the proteins FADD, Mch4, FLICE and the viral FLIP proteins. It was used to identify the murine and human FLIP sequences. This search profile also includes two pages.

In Figure 2 the amino acid sequences of proteins known to have

death effector domains (FADD, FLICE and Mch4) are compared with the amino acid sequences of viral proteins of the viruses EHV-2 (equine herpes virus 2), HHV-8 (human herpes virus 8), HVS (herpes virus Saimiri), BHV-4 (bovine herpes virus 4), and MCV (Molluscum contagiosum virus) found by means of a profile search. The sequence comparison shows homology with the first and second death effector domain (DED1 or DED2), while only a minor homology exists in the linking section between the first and second death effector domain. In the sequence comparison at hand a black background for the amino acid sign (one-letter code of amino acids, described in Stryer, Biochemistry (1995)), corresponds to a sequence match of at least 50%, while a gray background for the amino acid sign stands for at least 50% matching through conservative amino acid substitution. The arrows at the C-terminal of the second death effector domain of the MCV 159L and MCV 160L sequences relate to their C-terminal extensions, 66 (ORF 159L) and 202 (ORF 160L) amino acids in length. The amino acid sequences of the viral FLIPs have been deposited in the data base GenBank under the access code numbers U20824 (ORF E8 in EHV-2), X64346 (ORF 71 in HVS), and U60315 (ORF 159L and ORF 160L in MCV). The amino acid sequences of the viral FLIPs for HHV-8 (ORF 71) and for BHV-4 have been shown in Figure 16 (ORF 71 for HHV-8), and in Figure 17 (v-FLIP of BHV-4). A DNA sequence coding for the amino acid sequence of the viral FLIPs has been deposited in the data base GenBank under the access number U20824 (ORF E8 in EHV-2), Z46385 (BHV-4), X64346 (ORF 71 in HVS), U90534 (ORF 71 in HHV-8) and U60315 (ORF 159L and ORF 160L in MCV).

Figure 3 shows the homology of the amino acid sequences of the human (HS) and the murine (MM) form of the long (FLIP_L) and the short (FLIP_S) version of FLIP with the amino acid sequences of FLICE and MCH4. The N-terminal 202 amino acids of the shorter splicing variant of the human FLIP (FLIP_S) are identical with the

sequence of the longer form of human FLIP (FLIP_L) the sequence of the shorter protein (FLIP_S) ends after a C-terminal extension of 19 additional amino acids (this is the only extension that has been entered for the human FLIP_S in this figure), which are not included in the longer form (FLIP_L). As do the viral FLIPs (see for example the amino acid sequence of the FLIPs coded by ORF E8 of EHV-2), the murine (MM) homologue FLIP_L and the human (HS) homologues FLIP_L and FLIP_S both include two death effector domains each (DED1 or DED2). The murine and human FLIP_L form in addition have a C-terminal domain which is homologous with the protease domain of the caspases. The structural organization of FLIP_L (2 N-terminal death effector domains linked with a caspase) thus corresponds to the structural organization of FLICE and Mch4, but compared to the caspases the preserved cystein residue of the active protease domain is missing: in human and in murine FLIP_L the corresponding amino acid position is occupied by a tyrosin residue (in the figure that position is marked by a star).

Figure 4a shows the DNA and the amino acid sequence of the human FLIP_S which was isolated from a cDNA base of activated T-cells by screening with a DNA probe with a ³²P marker (EcoRI/RsaI fragment of the 5'-terminal of the DNA insert of EST clone No. 309776).

Figure 4b (2 pages) shows the DNA and the amino acid sequence of the human c-FLIP_L isolated from a cDNA data base of activated T-cells by screening with the probe described in Figure 4a.

Figure 4c (consisting of 3 pages) shows the cDNA and the amino acid sequence of murine c-FLIP_L, isolated from a murine cardiac muscle cDNA data base by screening with the probe described in Figure 4a.

Figure 5 shows by cotransfection experiments in eukaryotic 239T cells by the example of the viral FLIP_s of EHV-2 (ORF E8, Fig. 5a) and of MCV (ORF 159L, fig. 5b) that the viral FLIP_s bind to the adaptor protein FADD, and that this link does not prevent the attachment of the adaptor protein FADD to the cytoplasmic protein segment that includes the death domain of the CD95 death receptor. This binding of the viral FLIP_s E8 (from EHV-2) and 159L (from MCV) to FADD makes possible an attachment of the viral FLIP_s, by means of the adapter FADD, to the cytoplasmic protein segment of the death receptor CD95.

The lower part of the illustrations 5a and 5b in each case identifies the expression of the appropriate proteins (viral FLIP, FADD, and cytoplasmic protein segment of CD95 by means of the Western blot analysis described in the literature, of cell extracts for the transfected 293T-cells. In the upper part of illustration 5 the above associations (FLIP-FADD, FADD-CD95 and FLIP-FADD-CD95) are demonstrated through immune precipitations (IP). Each column corresponds to a probe, and the plus signs above the figures indicate the combination of expression vectors for the transfection of the 293T cells for each probe. In immune precipitates of Flag-marked E8 or Flag-marked 159L by means of an anti-Flag antibody, FADD can be detected in the anti-Fadd Western blot (in each case, second part of the figure from above) only if the appropriate FLIP (E8 or 159L) was co-transfected with FADD (if this is the case, it is indicated by plus signs in the first and second line of a column). An association of the viral FLIPs with the myc-marked cytoplasmic protein segment of CD95 (detected by anti-myc Western blot in the uppermost part of the figure) is possible only in the presence of FADD, i.e. only if v-FLIP, FADD and the myc-marked cytoplasmic protein segment of CD95 were expressed at the same time (plus sign in all 3 lines of a column).

Figure 6 shows the attachment of the viral FLIP protein E8 of EHV-2 with the agonistically stimulated CD95 receptor complex in human Raji B-cell clones, which have been stably transfected with an expression vector for the E8 protein. Figure 6a shows the expression of the Flag-marked E8 protein in cell extracts of two Raji B-cell clones called RE8/11 and RE8/19, which were transfected with an expression vector for E8, by means of Western blot analysis. On the other hand, no E8 protein was detected in the control clones called RCo/1 and RCo/3, which were transfected with the appropriate expression vector without the insertion of E8. Figure 6b shows the result of the analysis of immune precipitations of the CD95 death receptor stimulated agonistically with the antibody APO-1 (Kischkel et al., EMBO Journal 14, 5579-5588 (1995)) from ³⁵S metabolically marked Raji cell clones Rco/1 (a control clone not expressing E8). A white arrowhead marks the migration position of the radioactively marked E8 protein that is included in the CD95 attachment complex of the RE8/11 clone, but not in the corresponding complex of the RCo/1 control clone.

In Figure 7a the association of ³⁵S-marked proteins with the CD95 death receptor stimulated agonistically (Kischkel et al., EMBO Journal 14, 5579-5588 (1995)) in the non-transfected Raji control cells (clone Rco/3, left part of the illustration) is compared by two-dimensional gel electrophoresis analysis of CD95 (anti-APO-1) immune precipitations to the same association for such cells that were transfected with an expression vector for the viral FLIP E8 of the EHV-2 (clone RE8/19, right part of the illustration). The figure shows that the viral protein E8 associates with the agonistically stimulated CD95 death receptor without interfering with the attachment of the adaptor molecule FADD (CAP 1) to the receptor (the FADD protein called CAP1 is included in the agonistically stimulated CD95-associated signal transduction

complex of the control clone RC0/# that does not express E8, and also in the Raji clone RE8/19 which does express E8; compare right and left part of the figure). The figure further shows that in the cells expressing the viral FLIP, the attachment of FLICE (CAP4) and of FLICE-like molecules (CAP3) to the receptor is prevented, and therefore the conversion of FLICE into its two fission products CAP5 and CAP6 taking place in the receptor attachment complex is blocked (absence of the proteins with radioactive markers 4-6 in clone RE8/19 expressing E8 only in the right, but not in the left part of the figure).

Figure 7b shows that the FLICE fission activity of the agonistically stimulated receptor of the Raji B-cell clone RE8/19 which expresses E8, induced by treatment of cells with the agonistically stimulating anti-CD95 antibody APO-1, is considerably less than the corresponding activity of the control clone Rco/3 (which does not express E8). The presence of FLICE fission activity in the CD95 attachment complex is quantifiable through the formation of the FLICE fission products p43, p26, p17, p12, and p9 from radioactively marked FLICE by means of CD95 (anti-APO-1) immune precipitate of APO-1-treated (+) cells, but practically not at all of untreated (-) cells. In the FLICE fission experiment the bands for the molecular mass described above are 43, 26, 17, 12, and 9kD for the APO-1-stimulated (+), but practically not at all for the unstimulated (-) Raji control clone RC0'3, which does not express E8. On the other hand the fission activity of the CD95 attachment complex of the clone RE8/19 expressing E8 induced by APO-1 treatment is much reduced (the corresponding FLICE fission products are almost undetectable for this clone). Figure 7 therefore shows that the presence of the FLIP E8 from EHV-2 in the DISC of the death receptor CD95 impedes the attachment of FLICE as well as its activation caused by proteolytic fission.

Figure 8 shows that through expression of a viral FLIP (ORF E8 of EHV-2 or ORF 159L of MCV) eukariotic cells become considerably more resistant to apoptosis induction by the CD95 death receptor than do control cells. Figure 8 shows the relationship of the percentage of the apoptotic Raji B-cells (Y-axis) to the concentration (in the culture medium for 24h at 34°C) of the agonistic anti-CD95 (APO-1)-antibody (X-axis) for two Raji control clones not expressing E8 (Rco/1 and RDp/3). The two clones expressing E8 proved distinctly more resistant to the the CD95-apoptosis than the control clone. A APO-1 concentration in the culture medium of 100ng/ml was sufficient under the selected experimental conditions to induce apoptosis in over 60% of the control clones not expressing E8, while under the same conditions only about 20% of the cells of the two Raji clones RE8/11 and RE8/19 expressing E8 were apoptotic. Inducing apoptosis in ca. 50% of the Raji Raji clones RE8/11 and RE8/19 expressing E8 required a more than 10 times higher APO-1 concentration was required than for the of the control clones RCo/1 and Rco/3 not expressing E8.

Figure 8b shows that through expression of the viral FLIP of the E8 ORF of EHV-2, human Jurkat T-cells will also acquire resistance against induction of apoptosis via the CD95 death receptor. The experiments were conducted with E8 expressing Jurkat clones (JE8/1, JE8/10 and JE8/13 and a Jurkat clone JE8/5 not expressing E8, as well as control vector-transfected clones (jCo/2 and JCo/4). The sensitivity of the clones toward CD95 ligand-induced apoptosis was determined by means of incubation of the clones for 3 h at 37° with supernatant of CD95L-producing neuronal cells diluted 1/10 in a culture medium (Rensing-Eh et al., Eur. J. Immunol. 25, 2253-2258 (1995)), and subsequent analysis of the propidiumiodide marked cells in the FACScan flow-through cytometer (Nicoletti et al., J. Immunol. Methods 139,

271-279 (1991)). Figure 8b shows in particular that the amount of the viral FLIPs marked with Flag expressed in the clones (identified by the Western blot described in the literature, of cell extracts with anti-Flag antibodies, upper section of Figure 8b) correlates with a reduction of the apoptosis sensitivity toward the CD95 ligand (lower section of Figure 8b), for the percentage of the apoptotic cells induced by the CD95 ligand (as indicated on the X-axis) is the lower, the more vigorous the E8-expression of the clone detected by the Western blot in the upper section of Figure 8b. In particular, under the experimental conditions chosen, the percentage of the apoptotic cells with control clones not expressing E8 (JCo/2, JCo/3, JCo/4 and JE8/5) was at least three times as high as the percentage of the apoptotic cells for the Jurkat clone with the most vigorous E8 expression (clone JE8/13).

Figure 8c shows the shielding effect of vFLIPs (it shows an experiment with the gene product of ORF E8 from EHV-2) with induced apoptosis by means of over-expression of the death receptor CD95 in 293T-cells (a human embryo kidney cell line). For this experiment 293T cells with expression vectors for CD95 were transfected together with the quantities of expression vectors stated for the above-mentioned V-FLIP in Figure 8c and harvested 30h after transfection. The apoptosis induction in the cells was determined through photometric quantification of apoptotic histone-DNA complexes (the apoptotic index stated on the Y-axis correlates with the optical density (OD) of the samples at 405 nm as determined with the Elisa cell-death detection system by Boehringer). Cells transfected with expression vector without insertion (mock), or transfected with the expression vector for CD95, after transfection were incubated in medium with 25 μ M z-VAD-fmk until harvested, served as negative controls with apoptosis induction. The figure shows that the gene

product of E8 of the EHV-2 can shield 293T cells, from apoptosis induction through CD95 over-expression, whereby the shielding effect rises with the concentration of the v-Flip expression vector (this corresponds to a lowering of the apoptotic index with the rising v-FLIP expression vector concentration in Figure 8c). With the highest doses used (co-expression of 1 μ g each of the expression vectors for E8 with the amount of the CD95 expression vector stated in Figure 8c), the shielding effect of the viral FLIPs is comparable to the protection achieved through the protease inhibitor z-VAD-fmk.

Figure 9 shows the shielding effect of v-FLIPs (shown are experiments with the gene products of the ORF E8 of EHV-2, ORF 159L of MCV and ORF 71 of HVS) on induced apoptosis by over-expression of the death receptor TRAMP in 293T-cells (a human embryo kidney cell line). With these experiments 293 T cells with expression vectors for TRAMP (Bodmer et al., Immunity 6, 79-88 (1997)) were transfected together with the indicated quantities of expression vectors for the above v-FLIPs and harvested 30 hours after transfection. Cells were transfected with an expression vector without insertion (mock), or were transfected with the expression vector for CD95, and after transfection were incubated in medium with 25 μ M z-VAD-fmk until harvested, served as negative controls for the apoptosis induction. The apoptosis induction in the cells was determined as described in Figure 8. For all 3 of the v-FLIPs shown, with increasing concentration of the v-FLIP expression vector there is a reduction of the apoptosis induced by TRAMP over-expression (quantified via the optical density at 405nm as described above). For each of the greatest quantities of the v-FLIP expression vectors used in the experiments (the expression vector quantity has been stated below the figures in μ g) an apoptosis shield of the TRAMP over expressing cells of at least 70% was achieved (as compared to

100% protection through the protease inhibitor z-VAD-fmk).

Figure 10 shows that the human Jurkat T-cells through expression of viral FLIP of the E8 ORF of EHV-2, will acquire resistance against induction of apoptosis via the receptor for death ligand TRAIL. Here the quantity of the viral FLIP expressed in the clones (as determined by Western blot test on cell extracts, upper section of figure 8b) correlates with the amount of cell viability in the presence of the apoptosis-inducing death receptor ligand TRAIL (Figure 10). The sensitivity of the above-mentioned clones toward TRAIL-induced apoptosis was determined by means of incubation with the concentrations of recombinant soluble Flag-marked TRAIL and cross-linked anti-Flag antibody for 20 h at 37° and subsequent determination of the proliferation rate of the cells (Y-axis) by means of a cell profile assay (Cell titer 96 AQ, Promega). With increasing TRAIL concentration the Jurkat clones not expressing E8 (JCo/2, JCo/3, JCo/4 and JE8/5) in this test showed a definite reduction in proliferation (sigmoid curve, reduction of optic density with decrease in proliferation). With Jurkat clone JE8/13, on the other hand, with the most vigorous E8 expression, the observed cell proliferation remained nearly the same (horizontal curve). The two Jurkat clones with an intermediate E8 expression level (J8/1 and J8/10) showed only a slight proliferation decrease due to the effect of being treated with TRAIL.

Figure 11 demonstrates the correlation of the expression of a viral FLIP (OrF 71 of HVS) in the course of the viral infection of the host cell OMK (owl monkey kidney) with the shield of the host cell against CD95 ligand induced apoptosis. Figure 11a shows a Northern blot analysis for detecting transcripts of ORF 71 of HVS in probes of non-virus-infected control cells (OMK), in probes of OMK cells, infected with the HVS strain C488 for 4 days

(C488, OMK d1-d4), or were infected with the HVS strain A11 for 4 days (OMK d4-A1), or of probes of a semi-permissive T-cell line producing small amounts of virus particles (P-1079). In virus-infected OMK cells a specific transcript of ca 5kb can be detected on the fourth day of the infection. This correlates with the inhibition of the CD95 ligand induced apoptosis of virus infected cells shown in figure 11b as compared to non-infected cells at this time. The seemingly slight apoptosis protection on day 5 of the virus infection is due to the onset, at this time, of a massive cell lysis due to infection (ca. 50% lysis on day 5 compared to less than 10% lysis on day 4). Inhibition of the CD95 ligand -induced apoptosis of virus-infected OMK cells as compared to non-infected control cells was determined through incubation of the cells with recombinant CD95 ligand and cross-linked anti-Flag antibody for 20h and subsequent quantification of apoptotic histone-DNA complexes (Cell-death detection ELISA, Boehringer).

Figure 12 shows through co-transfection experiments in eucaryotic 293 T cells that the short and long form of human FLIP, (FLIP_S and FLIP_L), but not the protein segment with caspase homology (FLIP_P) will bind to the adapter protein FADD. The figure further shows that the linkage of FLIP_S and FLIP_L to the adaptor protein FADD, does not prevent the attachment of FADD to the cytoplasmic protein segment of the CD95 death receptor which includes the death domain. By means of the linkage of human FLIP_S and FLIP_L to the FADD adapter protein an attachment of these FLIP forms to the cytoplasmic protein segment of the CD95 death receptor is made possible via the adapter FADD.

In the lower part of Figures 12a and 12b the expression of the appropriate proteins (FLIP_S, FLIP_P, FLIP_L, FLIP_L, FADD and the cytoplasmic protein segment of CD95) in each case is verified through Western blot analysis of cell extracts of the transfected

293T cells, while in the upper part of the figures 12a and 12b the protein associations described above (FLIP_S, or FLIP_L with FADD, FADD with CD95 and FLIP_S or FLIP_L via FADD with CD95) are demonstrated through immune precipitations (IP). Every column here corresponds to a probe, and plus signs above figure sections 12a and 12b characterize the combination of expression vectors for the transfection of the 293T cells. Within immune precipitations of Flag-marked human FLIP by means of an anti-Flag antibody the forms of the human FLIPs (either FLIP_L or FLIP₃), for the two N-terminal death effector domains, and FADD can be detected in the anti-FADD Western blot (uppermost part of the figure) only if a FADD expression vector was used during transfection (plus sign in the appropriate lines of the column). The caspase-homologous protein segment (FLIP_P) of the human FLIPs which does not include the two death effector domains cannot associate with FADD (result of the anti-FADD Western blot of the probes in columns 5 and 9 in Figure 12a). An association of the forms FLIP_S or FLIP_L, but not the protein segment of FLIP_P, with the myc-marked cytoplasmic protein segment of CD95 (detected by anti-myc Western blot, see second part of the figure from the top) is possible only in the presence of FADD, i.e. only if FLIP_S or FLIP_L, FADD and the myc-marked cytoplasmic protein segment of CD95 were expressed together (column 7 of Figure 12a and column 2 of Figure 12b).

Figure 13 shows by cotransfection experiments in eukaryotic 239T cells that the human FLIP_S and FLIP_L bind to the cystein protease FLICE. The figure further shows that both the N-terminal protein segment including the death effector domains of FLIP_L and also the caspase homology endowed C-terminal protein segment of FLIP_L contribute to binding FLIP_L to FLICE. Expression of the HA-marked FLICE protein in cell extracts of the transfected probes was determined in the lower-most part of the figure by means of a

Western blot test with anti-HA-antibodies. The central portion of the illustration by means of an anti-Flag Western blot shows that comparable quantities of three different Flag-marked FLIP proteins or protein segments were precipitated out of the transfected cells by means of anti-Flag immune precipitation. The uppermost figure portion, by means of an anti-HA Western blot analysis of the anti-Flag precipitates, that the HA-marked FLICE binds to FLIP_L and FLIP_S and also to the caspase -homologous C-terminal protein segment of FLIP_L (FLIP_p).

Figure 14 shows that through expression of a human FLIP_S or FLIP_L eukariotic cells become considerably more resistant to apoptosis induction via the CD95 death receptor than do control cells. It also demonstrates that the longer form of the human FLIP (FLIP_L) provides more effective protection against CD95-induced Apoptosis than the shorter form FLIP_S. Figure 14a shows that human Jurkat T-cells through expression of the human FLIP_S or FLIP_L will also acquire resistance against induction of apoptosis through the Fas(CD95) ligand (FasL). Here the quantity of the human VSV-marked FLIP_S or FLIP_L expressed in the clones (as determined by Western blot test of cell extracts, upper section of Figure 14a) correlates with the amount of cell viability in the presence of the apoptosis-induced FasL (lower part of Figure 14a). The sensitivity of the Jurkat clones towards FasL-induced apoptosis was determined by incubation with the concentrations stated for the X-axis of recombinant soluble flag-marked FasL (sFasL) and cross-linked anti-Flag antibodies for 20h at 37degrees C and subsequent determination of the proliferative ability of the cells (Y-axis), by means of a cell proliferation test (cell titer 96 AQ, Promega). The parental Jurkat T-cells not transfected (Jurkat, Co) the non- FLIP_S expressing Jurkat clone JFS1 and the poorly FLIP_S -expressing Jurkat clone JFS5 in this test show an overlapping sigmoid curve (decrease of proliferation with

increase in sFasL concentration). On the other hand, a shift to the right JFS7 was observed with the proliferation curve for the more vigorous FLIP_S-expressing clone. To achieve a decrease in proliferation comparable to that of the parental control clone with clone JFS7, an approximately 5 times higher FasL concentration was needed as compared to the parental clone. An even more vigorous resistance against FasL was observed for the two FLIP_L-transfected Jurkat clones JFL1 and JFL2. Although the protein expression of the VSV-marked FLIP_S was merely weak (JFL2) or did not show up in the Western blot at all (JFL1), (upper part of Figure 14a) the two clones were approximately 10 times (JFL1) or more than 25 times (JFL2) more resistant against treatment with sFASL than the parental Jurkat clone.

Figure 14b shows that also human Raji B-Cells through expression of a human FLIP_S become considerably more resistant to apoptosis induction via sFasL. For the experiments the Raji clones RFS3, RFS7, RFS8, and RFS11, expressing the human FLIP_S, and a Raji clone not expressing the human FLIP_S (RFS1) and the non-transfected parental Raji clone (Raji wild type wt) were used and the sensitivity of the clones to sFasL-induced apoptosis determined as in Figure 14a. Figure 14b shows that Raji clone expressing VSV-marked FLIP_S (see anti-VSV western blot in the upper part of Figure 14b) show a partial resistance against sFasL. Under the selected experimental conditions these clones were at least 5 times more resistant against the obstruction to proliferation than the non FLIPS-expressing control clones Raji (wt) and RFS1.

Figure 14c finally shows by means of the experimental approach described with Figure 14a that human Raji B cells through expression of human FLIP_L may acquire a practically total resistance to treatment with sFasL. In particular the Raji clones

with a vigorous (RFL12) and medium (RFL2, RFL42, and RFL47) expression level of the VSV-marked FLIP_L were almost completely resistant against treatment with sFasL at the concentrations shown for sFasL in the X-axis (this showed in a nearly horizontal curve, indicating unchanged cell proliferation behavior of the clone in question, even during high (above 1 μ g/ml) sFasL concentrations).

Figure 15 shows that human Jurkat T cells acquire resistance to apoptosis induction by the receptor for the death ligand TRAIL through expression of the human FLIP_S or FLIP_L. Here the quantity of the c-FLIPs expressed in the clones (as determined by Western blot test on cell extracts, upper section of figure 14a) correlates with the amount of cell viability in the presence of the apoptosis-inducing death receptor ligand TRAIL (Figure 15). The sensitivity of the Jurkat clone toward TRAIL-induced apoptosis was determined by through incubation with the concentrations given in the X-axis of recombinant soluble Flag-marked TRAIL and cross-linked anti-Flag antibody for 20 h at 37° and subsequent determination of the proliferation rate of the cells (Y-axis) by means of a cell proliferation assay (Cell titer 96 AQ, Promega). With increasing TRAIL concentration the parental non-transfected Jurkat clone (wt) and the non-transfected, non-FLIP_S-expressing clone JFS1 in this test showed a definite reduction in proliferation (sigmoid curve, reduction of optic density with decrease in proliferation). Clone JFS5, which in the Western Blot (Figure 14a above) showed an intermediate level of VSV-marked FLIPs, showed partial resistance to TRAIL, while clone JFS7, which expresses the VSV-marked FLIPs more vigorously, was practically completely resistant against TRAIL. The horizontal curve shows that no reduction of proliferation through death receptor ligand TRAIL occurred). The experimental curves also show that the two Jurkat clones JFL1 and JFL2 transfected with an

expression vector for human FLIP_L in spite of a very weak (clone JFL1) or weak (clone JFL2) expression of the VSV-marked FLIP_L (see Western blot test Figure 14a, upper part) were almost (JFL1) or nearly (JFL2) resistant against treatment with the death receptor ligand TRAIL.

Figure 16 shows the amino acid sequence of the viral FLIPs of HHV-8 (ORF 71) in the one-letter code of the amino acids. A DNA sequence coding for this amino acid sequence is found in the data base GenBank with the access number U90534 (OEF 71 in HHV-8).

Figure 17 shows the amino acid sequence of the viral FLIP of BHV-4 in the one-letter code of the amino acids. A DNA sequence coding for this amino acid sequence can be found in the data base GenBank under access code Z46385.

The present invention is further explained by means of exemplary embodiments.

First description of the experimental framework for all of the following exemplary embodiments shall be given.

The cell lines used were a human embryo kidney cell line (293T cells), a human leukemia T-cell line (Jurkat cells), or a human Burkitt lymphoma B cell line (Raji cells) and cultivated as described with Bodmer et al. (Immunity 6, 79-88 (1997)).

The monoclonal antibodies used for the immune precipitation and the "Western blotting" were anti-Flag antibodies and anti-Flag agarose (by Kodak International Bio- technologies), and anti-FADD antibodies (from Transduction Laboratories), and antibodies against the myc-Epitope (from the Sigma company, 9E10), against

the VSV-Epitope (from the Boehringer company) and against the HA-epitope.

A soluble component of the human TRAI protein, indeed with the amino acids 95-281, was produced from an EST (Expressed Sequence Tag) clone through a PCR-process. The clone is called 117926 and has been entered in the GenBank under access code T90422. The following sequences were used as oligonucleotides: the oligonucleotide JT403 5'-TCAGCTGCAGACCTCTGAGGAAAC-3' and the oligonucleotide JT469 5'-ACTAGTTAGCCAACTAAAAA-3'. The section was cloned in the vector pQE-16 (by the Qiagen company) in a PstI/speI intersection - depending on restriction enzyme abbreviations. The cloned sequence also includes a Flag sequence and a connecting element following this, with the amino acid sequence GPGQVQLQ. This is followed by PstI and SpeI intersections between the original BamHI/XbaI sites of the vector. Protein expression in the bacteria was induced with 0.5 mM IPTG. After 6 hours of incubation at 30°, the cells were harvested and lysed by means of sonication. The lysates were first extracted with 0.5% pre-condensed triton x-114 in order to eliminate the bacterial lipopolysaccharides, and finally the Flag/TRAIL protein was cleared with Me anti-Flag agarose by means of a chromatography column; after that elution with 50 mM citric acid and finally neutralization with a 1 M tris base and dialysis against PBS (phosphate buffered saline).

For isolating the cDNA clones of the human and murine FLIP, a DNA fragment of the human FLIP DNA sequence included in EST clone 309776, and obtained by PCR amplification was used as a ³²P marked probe. This fragment corresponds to the DNA sequence section numbered 394 to 903 in Figure 4a. The screening of the λ-ZAP cDNA database of activated human lymphocytes (Stratagene, available upon request through Hermann Eibel, U. of Freiburg,

Germany) for isolating the human forms of FLIP and the screening of a bank of murine cardiac cells (Stratagene) for isolating murine FLIP_L was carried out according to the manufacturer's directions.

The complete "Open Reading Frame" E8 of virus EHV-2 (ORF E8) was amplified from viral DNA by PCR methods, whereby a 5'primer with an EcoRI sequence extension and a 3'primer with a sequence extension for the restriction enzymes BamHI and EcoRI were used. The insertion (into the EcoRI intersection of the vector) occurred in the same reading frame as with the N-terminal Flag Epitope. The vector was derived from pCR-3-vector (from the Invitrogen company). The open reading frames procedure was used analogously with ORF 159 of virus MCV (from the Institut für Medizinische Virologie der Universität Heidelberg, Germany) and the Open Reading Frame ORF 71 of the virus HVS. The FLIP-coding ORFs of these viruses were also amplified through PCR methods and then inserted in the correct reading frame with the N-terminal Flag-Epitope into the EcoRI intersection of the vector derived from pCR-3. Further, the complete ORF of the human FLIP_S and the human FLIP_L and also a HindIII/XhoI fragment of the 3' terminal of the DNA sequence of the human FLIP_L were amplified by PCR methods and cloned in the correct reading frame in vectors derived from pCR-3, which add an N-terminal Flag or VSV Epitope to the gene products in question. In order to achieve a stable expression of FLAG E8, VSV-FLIP_S and VSV-FLIP_L in Jurkat and Raji cells, the constructs from Flag-E8, VSV-FLIP_S and VSV-FLIP_L were further cloned in the multiple cloning site (MCS) of the vector pSRαpuro (a gift from R. Sekaly, IRCM, Montreal, Canada). This vector exhibits puromycin resistance.

An expression vector for the cytoplasmic domain of the murine FAS receptor provided with the myc sequence was produced by insertion

of a PCR fragment corresponding to the amino acids 166-306, in the correct reading frame with the N-terminal myc-Epitope, and was inserted in the pCR-3 type vector. The expression vector for the human TRAMP or FADD in Vector pCR-3 can be found in the publication by Bodmer et al (Immunity 6, 79-88 (1997)).

Human Fas (with the nucleotide sequence -24 to +1009) was amplified from an EST clone by PCR methods (GenBank access code X63717) and then subcloned into the pCR-3 vector as a HindIII/XbaI fragment.

In order to obtain stable puromycin-resistant transfectants of Jurkat and Raji cells, the cells were washed with HeBS-buffer with a pH of 7.05 (0.8 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$), 20 mM Hepes, 137 mM NaCl, 5 mM KCl, and 5.5 mM D-glucose). Then 8×10^6 cells were resuspended in 800 μl , were mixed with 20 μg of the SR α puro plasmids (with and without Flag-E8 insertion) and finally exposed to a voltage of 250 V and a current of 960 μF . After 48 hours of transfection the cells were distributed on flat-bottom plates at a concentration of between 2,000 to 20,000 cells each well and stable transfectants selected by adding 5 $\mu\text{g}/\text{ml}$ puromycin (from Sigma Co).

In order to achieve a temporary transfection of 293T cells, the cells were distributed at a concentration of 1 to 2×10^6 cells/10cm plate or 3 to 2×10^5 cells/5cm plate and transfected the following day with the aid of the calcium phosphate precipitation method described in the literature. The precipitate was left on the cells for 8 hours and the cells were finally harvested 26 to 30 hours after transfection.

The 293 T cells of a 10 cm plate were lysed after transfection in 200 μl lysing buffer (with 1% NP40, 20 mM Tris-HCl, pH7.4, 150 mM

NaCl, whereby in addition 1mM EGTA, 1mM pefabloc-sc (from the Serva Co.) and in each case, 10 μ g/ml leupeptin and aprotinin (from the Sigma Co.) were added. Post-nuclear lysates were pre-purified for at least 1 hour before precipitation on Sepharose 6B (from Pharmacia). Subsequently immuno-precipitation was undertaken with 3 μ . anti-Flag Agarose, either for two hours or overnight. The precipitates were washed in lysing buffer for a total of four times, with the lysing buffer during the first two washings containing 1% NP40 and during the last two washings 0.1% NP40. The precipitates were then heated in probe buffers and analyzed with SDS-PAGE and Western blotting. The blots were then saturated with 5% milk in PBS with 0.5% Tween. They were then incubated at room temperature for one hour using monoclonal anti-Flag-antibody at a concentration of 5 μ g/ml, with monoclonal anti-myc antibody (9E10) at a concentration of 5 μ g/ml, or with monoclonal anti-HA antibody at 1 μ g/ml, whereby a second antibody marked with Peroxidase was added (second antibody from the Jackson Laboratories). The detection of the proteins was enhanced by chemi-luminescence (Amersham International). Jurkat, Raji or 293 cell clones were tested for their expression of transfected proteins. This was done with anti-Flag, anti-VSV, anti-FADD, anti-myc, or anti-HA Western blot analysis of post-nuclear cell lysates of equivalent protein contents. The metabolic marking of Raji cells with ^{35} S, the anti-CD95 immune precipitation and their 2D-gel electrophoresis were conducted as described above (Kischkel, F.C. et al., EMBO J. 14, 5579-5588 (1995)).

The analysis of the apoptosis induced by FasL (DD95L) was undertaken as follows. Puromycin-resistant Jurkat clones (ca. 3×10^5 /500 μ l) were incubated for 3 hours at a temperature of 37°C with 50 μ l of the supernatant of neuro-2a-cells transfected with a FasL expression vector, or with a control supernatant of

cells transfected with a sham vector (Rensing-Ehl, A. *et al.* Eur. J. Immunol. 25, 2253-2258 (1995)). The Jurkat cells are washed with FACS buffer (2% icFCS and 0.02% acid) and fixed in 70% ice cold ethanol. After washing for another time with FACS buffer treatment with RNase for 5 minutes at a temperature of 37°C followed (50 µg/ml RNase A in 100mM Tris HCl, pH 7.4, 100mM NaCl, 5mM EDTA). Staining for analysis of the DNA content proceeded with 250 µg/ml propidium iodide in PBS/1% NP40. Apoptotic cell fraction was analyzed and quantified in a Becton-Dickinson FACScan-apparatus using the Lysis II software. The susceptibility of Raji clones to anti-APO1 induced apoptosis was analyzed by means of cell incubation (5×10^5 /ml) with varying concentrations of monoclonal anti-APO1 antibody (in medium for 16 hours at 37°C). Quantification of the DNA fragmentation as a measure of the extent of the apoptosis in this case was mainly carried out as described by Nicoletti, I. *et al.*, J. Immunol. Methods 139, 271-279 (1991). In sum it may be said that the cells were washed once with PBS and were carefully resuspended in 0,1% sodium citrate and 0.1% triton x-100 with 50 g/ml propidium iodide. After incubation at 4° in darkness for a duration of at least 24 hours, the percentage of the apoptotic cell nuclei was determined by FACScan® (Becton-Dickinson, Heidelberg, Germany).

For the quantification of the apoptosis with the transient transfected 293T cells, cells from a 5 cm plate were lysed in 200 µl incubation buffer, and lysates from 25,000 cells were analyzed for presence of histone DNA complexes by means of a Cell Death Detection ELISA (Boehringer Mannheim) according to manufacturer's specifications. The survival of clones transfected with E8 or with human FLIP, as well as control Jurkat clones after cell death induction after adding TRAIL was tested after a 20-hour incubation of ca. 50,000 cells per well at the indicated concentrations of recombinant TRAIL with a FLAG appended and 1

$\mu\text{g/ml}$ of monoclonal antibody anti-Flag, and the proliferating cells were subsequently quantified with a cell titer AQ proliferation test (Promega), likewise according to manufacturer's specifications.

The viral in vitro cultures and the Northern blot analysis of transcripts were carried out as with Fickenscher et al., (J. Virol. 70, 6012-6019 (1996)). The effect of an HVS-infection on the cell death of owl monkey kidney cells (OMK) initiated through CD95L was tested by distributing the cells to 96 well plates at a concentration of ca. 10^4 cells/well. Two days later half of the wells were infected with viruses to such an extent that the proportion of the infectious agent was about one virus per cell. Recombinant sCD95L (sFasL) (as described by Bodmer, J.L. et al., Immunity 6, 79-88 (1997)) was added at a concentration of 0.3 $\mu\text{g/ml}$ after the infection at different points in time. The samples were examined 20 hours later for presence of histone-DNA complexes, as described above.

1. Experimental Exemplary Embodiment

In order to test the inhibitory effect of the viral FLIP proteins on apoptosis and to show that proteins with death effector domains might also have an inhibitory effect on apoptosis, 239T cells with expression vectors for coding FLIP protein (from virus EHV-2) marked with Flag for FADD or N-terminal were used. The promoters for the expression vectors in each case were CMV vectors. After transfection of the cells cell extracts were examined by the so-called Western blot method. The proteins present in the cell lysates were separated in one direction according to their isoelectric points, and in the other direction of the two-dimensional probe were recorded according to size. Through the appropriate antibodies the expression of the desired

proteins may then be examined. In the case at hand anti-Flag antibodies (attached to the N-terminal end of the FLIP protein) and anti-Fadd antibody were used to identify a stable transfection of the 293T cells. The corresponding illustrations are found in the lower part of Figure 5. In addition 293T cells were also transfected with a myc-CD95 construct (this is an apoptosis receptor). All in all five different transfection clones were produced. The transfection pattern of the various clones is shown in the upper part of Figure 5a. For instance, the fourth from the last column shows 293T cell transfectants which have been transfected with Flag-E8, FaDD and my-CD95. After testing for a stable transfection by means of the corresponding proteins the mutual association of the individual proteins was investigated by means of the Western blots and with the aid of c-immune precipitation experiments shown in the lower section of Figure 5a. This is done by immunoprecipitation with anti-Flag E8 antibodies. In the Western blot recording the proteins FaDD or myc-CD95 are recognizable only if they have previously been precipitated with the anti-Flag antibody as a Coprecipitate. thus the detection of FADD and/or myc-CD95 is possible only if at the time of the antibody attachment the Flag-E8 construct was likewise associated with FADD and/or myc-CD95. Therefore the two first experiments (column 1, 2) in Figure 5a, in which there was no Flag-E8 transfection, serve as control experiments. The last three columns (in each case Flag-E8, i.e. containing viral FLIP protein) of Figure 5a show the binding behavior of viral FLIP to either FADD and/or myc CD95. Because this involves a denaturized gel, no associations show up in the Western blots. Instead, the linkage of the two proteins to the viral FLIP protein in the cell extract is demonstrated indirectly by the presence of FADD and/or myc-CD95 after immune precipitation.

Figure 5b shows an analogous experiment, in that here in a

construct made up of Flag and the FLIP gene of the MCV virus (Open Reading Frame 159L) the 293 T-cells have been transfected. The experimental procedure is analogous to the procedure in Figure 5a and described in detail in exemplary embodiment 7. For the immune precipitation see the method of Bodmer et al. (immunity 6, 79-88 (1997)).

In Figure 5b as well the result reveals that an association between the proteins myc-CD95 and Flag/-FLIP (159L) only exists if the 293T cells with all three expression vectors have been stably transfected. This may be seen in the right-hand column of Figure 5b. On the other hand the two viral FLIP constructs (E8 or 159L) do not show any association with the CD95 receptor if the cells are not FADD-positive (Figure 5a, third column, Figure 5b, fourth column). In the reverse case the viral FLIP protein does not prevent the association of FADD with myc-cD95 (Figure 5a, fourth column, Figure 5b, fifth column), because after the co-immune precipitation in this case FADD and also myc-CD95 may be detected by means of the Western blot method.

2. Experimental Exemplary Embodiment:

The goal of the second experimental embodiment was an investigation of the incorporation of the FLIP protein (in this case the E8 FLIP protein) in the so-called DISC complex, which during activation of the apoptotic signal cascade is associated with the cytoplasmic portion of the CD95 receptor. The E8 FLIP gene served to transfect Raji clones in a stable fashion. Here expression vectors were also employed, with the promoter being an S α promoter. The stably transfected Raji clones (RE8/11 and RE8/19) were compared with control clones which had only been transfected with the vector, but without insertion of the FLIP

Gene. These are called RCo/1 and RCo/3. In order to identify a stable transfection, the expression of the transfected gene was investigated by means of Western blot analysis analogous to the first exemplary embodiment. In Figure 6a in each case a band of the FLIP protein can be detected with the transfected clones RE8/11 and RE8/19, but clones RCo/1 and RCo/3, the control clones, do not exhibit any expression of the E8 protein. Here too a co-immune precipitation was undertaken, with anti-CD95 antibody. The co-immune precipitates then were separated by 2D gel electrophoresis under denaturization conditions (one of the axes records the SDS-PAGE and the other direction records the results by iso-electric focussing). An anti-Flag antibody, served to treat the blot and at the same time was directed against the viral FLIP protein.

Figure 6b presents the result of this experiment. It is shown that only in the case Raji clone stably transfected with Flag E8 a positive signal for an approximately 23 kD protein with a pI value of about 5.0 could be observed. With the control clone without E8 expression (upper illustration in Figure 6b) no corresponding signal can be detected. The E8 Flag construct gives rise to an expectation of a pI value of 5.0 and a molecular weight of approximately 23 kD.

3. Experimental Exemplary Embodiment

This experimental exemplary embodiment seeks to demonstrate that the E8 FLIP protein has an effect on the constitutive structure of the DISC complex. DISC formation was therefore analyzed in detail. This was shown in a comparison involving the control clone RCo/3 and the clone RE8/19 which was stably transfected by means of Flag E8 FLIP (Figure 7a). Anti-APO antibodies with ³⁵S-markers were used for this. The antibody has an agonistic effect

on the death receptor Apo-1 (CD95). It was received from P.H. Krammer (DKFZ, Heidelberg, Germany). In the following the immune precipitate received using the above antibody was analyzed by 2D gel electrophoresis and the gel was evaluated by autoradiograph. The method used has by the way been described by Kischel et al. (EMBO Journal 14, 5579-5588 (1995)).

In addition the proteolytical activity in the disc complex against the FLICE protein was examined in Raji cells transfected with E8 and in control Raji cells. For this purpose the cells were either treated with anti-Apo-1 antibody for five minutes or they remained untreated. With the anti-Apo-1 antibody, immune precipitation was then carried out, and then the proteolytical activity of the immune precipitate from the anti-body treated and the untreated cells tested against FLICE. Proteolysis in vitro of the FLICE protein marked by ³⁵S was initiated by incubation with the immune precipitate. Subsequently the specific fission products of the FLICE protein (p43, p26, p17, p12 and p9) were examined by autoradiography on an SDA emulsion.

The result of the investigations with the Raji control clones and the Raji clones transfected with E8 shows characteristic differences. Above all, the typical DISC proteins CAP4 (=FLICE) and CAP3 (a FLICE derivative) are missing in the DISC complex of the Raji clones transfected with E8. This means that an orderly structure of the DISC complex as with the control cells, no longer exists while the E8 protein is present (Figure 7a). Functionally there is another marked difference with regard to the fission activity of the FLICE protein, insofar as in the case of the RE8/19 clones treated with anti-Apo-1 antibody, in contrast to the control clones, the specific fission products of the FLICE protein can almost no longer be detected (Figure 7b).

4. Experimental Exemplary Embodiment

To further prove the significance of the viral FLIP protein for inhibiting apoptosis, cell death of several cells through various agonists was analyzed in the absence and presence of viral FLIP protein. For this purpose at first the number of apoptotic cells was measured in relation to the number of the anti-Apo-1 antibodies. Quantification of the apoptotic cells occurred as explained above. Here too the apoptotic cells of control clones RCo/1 and RCo/3 were analyzed in a comparison with the E8 FLIP transfected Raji B cell clones. The induction of the apoptotic signal cascade took place through agonistic anti-Apo-1 antibodies (Figure 8a).

In addition cell extracts of E8 transfectants (JE8/1, JE8/5, JE8/10, and JE8/13), and of control cells (Jco/2, Jco/3, Jco/4) Jurkat clones were analyzed. For this purpose the Flag-E8-FLIP expression was determined by means of anti-Flag antibody on Western blots. As described above, after an incubation of 3 hours at 37°, and induced by CD95L, cell death was measured (with the aid of supernatant of neuronal cells). As described above in connection with Figure 8, the cells marked with propidium iodide were examined by FaCscan flow-through cytometer as to their apoptotic reaction.

The over-expression of CD95 receptor in human embryo kidney cells (293T cells) was determined as another apoptotic agent. Single transfectants of 293T cells were produced with an expression vector coding for CD95, as well as double transfectants with an expression vector coding for E8 or CD95. During over expression of CD95 (2 μ g), massive cell death is observed. Hereby the relative quantity of DNA histone complexes liberated into the cytoplasm is measured. In a typical CD95 transfection experiment

about 50-90% of cells succumb to apoptosis due to over-expression of the CD95 receptor. For comparison a CD95 single transfectant was also treated with the protease inhibitor z-VAD-fmk (25 μ M).

The result of all three experiments shows that after stimulation of the apoptotic reaction, perhaps by means of an agonistic anti-Apo-1 antibody (Figure 8a), by CD95L or over-expression of the CD95 receptor, the apoptotic reaction may be blocked or at least to a large extent reduced if the stimulated cells have previously been stably transfected with viral FLIP. In analogous experiments single and double transfectants of 293T-cells were transfected with expression vectors for TRAMP (Bodmer et al. Immunity 6, 79-88, (1997), each time with different quantities of expression vectors either for E8-FLIP (EHV-2) or 159L-FLIP (MCV) or 71-FLIP (HVS). As controls false transfectants (mock) without TRAMP or FLIP protein expression were chosen. Incremental amounts of expression vectors were used with the FLIP proteins, while the amounts of TRAMP expression vectors was kept constant (3 μ g) in all experiments. The result (summarized in Figure 9) shows that increasing amounts of viral FLIP protein expression vectors can markedly reduce the amount of apoptosis caused by over-expression of TRAMP receptors.

5. Experimental Exemplary Embodiment:

This exemplary embodiment served to investigate when viral FLIP protein is expressed during the viral replication cycle. The HVS-71-FLIP protein was chosen for this purpose. OMK cells (Owl Monkey Kidney) were used as host cells for the viral infection. Northern blot analysis was used to analyze the transcription of permissive OMK cells with an HVS infection of the C488 strain. An mRNA determination of the viral FLIP gene in the cells infected with the HVS virus was undertaken one, two, three or four days

after infection. Non-virus-infected OMK cells were examined as controls. Two additional mRNA analyses were performed, on the one hand with HVS-infected T-cells of the line P-1079 and on the other with OMK cells that were infected with the HVS strain A11. With the last-mentioned cell line the samples were examined by Northern blot test four days after infection with the virus strain.

Figure 11a shows the result that the permissive OMK cells that had been infected with a cytopathological HVS strain (C488 or A11), on the fourth day after infection showed a numerous presence of a 5kb mRNA fragment. This mRNA fragment serves to translate the viral FLIP protein. Therefore the FLIP transcript appears one day before massive cellular lysis. This may be seen in Figure 11b.

6. Experimental Exemplary Embodiment

To determine the binding of human FLIP_S and FLIP_L to CD95 via the adapter molecule FADD, cotransfection experiments were conducted in human embryo kidney cells (293 cells), which constitutively express SV40 large T antigen and therefore exhibit a more vigorous protein expression of expression vectors with an SV40 (293T cells).

The expression vector pCR-3 of Invitrogen has this property. Therefore DNA fragments coding for human proteins or protein segments FLIP_S, FLIP_L and FLIP_P were cloned for expression in 293T cells in a modified version of the vector pCR-3, which provides these proteins or protein segments with an N-terminal Flag-epitope, while the DNA site coding for the cytoplasmic protein segment of CD95 was inserted in an analogous expression vector with an N-terminal myc epitope. As shown in Figure 12, various

combinations of expression vectors were transfected for the cytoplasmic part of CD95, for FADD, FLIP_S, FLIP_L and FLIP_P transfected in 293T, and the expression of the gene products coded by the various expression vectors was controlled by specific anti-Flag, anti-FADD, or anti-myc antibody in the Western blot. In addition the Flag-marked FLIP_S, FLIP_L and FLIP_P present in cell lysates of appropriately transfected 293T cells, were, as described above, immune-precipitated by means of anti-Flag agarose, and these immune precipitates were then analyzed for association of FADD or the myc-marked CD95 protein segment in the anti-FADD or anti-myc Western blot.

Result

It was determined that the human proteins FLIP_S and FLIP_L, but not a protein segment of the human FLIP_L, which only includes the caspase-homologous inactive protease domain (FLIP_P), bind to the adapter molecule FADD. This binding does not inhibit the attachment of FADD to the cytoplasmic protein segment of the CD95 death receptor and as a result the human proteins FLIP_S and FLIP_L can bind to the cytoplasmic part of the death receptor CD95.

7. Experimental Exemplary Embodiment

In order to establish that human FLIP_S and FLIP_L bind to FLICE, co-transfection experiments were undertaken in 293T cells described above (see Figure 13). The expression vector for human FLICE (a gift from M. Peter, Heidelberg) provides this protein with an N-terminal HA epitope, while the expression vectors for FLIP_S, FLIP_L and FLIP_P as described above, provide these proteins or protein segments with an N-terminal Flag epitope. The expression of the gene products coded by the appropriate expression vectors in Western blot with specific anti-Flag or

anti HA antibodies. the expression of the gene products coded by the various expression vectors was controlled by specific with specific anti-Flag or anti-HA antibodies in the Western blot. The Flag-marked FLIP_S, FLIP_L and FLIP_P present in cell lysates of appropriately transfected 293T cells, were immune-precipitated by means of anti-Flag agarose, and these immune precipitates were then analyzed in the anti-HA Western blot for association of HA-FLICE with FLIP_S, FLIP_L and FLIP_P.

Result

It was determined that the human proteins FLIP_S and FLIP_L bind to the caspase FLICE. Both the N-terminal protein segment with the two death effector domains in FLIP_S and the C-terminal protein segment FLIP_P, including the caspase homologous inactive protease domain, contribute to the binding of FLIP_L to FLICE.

8. Experimental Exemplary Embodiment

In order to establish the inhibiting effect of FLIP_S and FLIP_L on the apoptosis induced by the death receptor CD95, a human Jurkat T-cell line and a human Raji B-cell line were transfected with an expression vector for human FLIP_S and FLIP_L provided with an N-terminal VSV epitope. An expression vector with an S α promoter lending puromycin resistance to the transfected cells was used for stable transfection of these cells (The Vector was a gift from R. Sekaly, ICRM, Montreal, Canada). The cells were transfected by electroporation of $8 \cdot 10^6$ cells at 250V and 960 μ F in HeBS buffer solution with 20 μ g of the plasmid to be transfected. After growing in culture medium without puromycin as described above, they were seeded in flat bottom cell culture plates with 96 wells after selection in culture medium with 5 μ g/ml puromycin at 2000-20,000 cells each well. Within 2-3 weeks

Puromycin resistant clones grew up and were then tested for expression of VSV-marked FLIP_S or FLIP_L in Western blot. Clones with different expression levels of FLIP_S or FLIP_L were then tested for their resistance toward apoptosis induced by sFasL (see Figure 14). To achieve this the clones were incubated in culture medium with Flag-marked sFasL and 1 μ g/ml cross-linked anti-Flag antibody in the concentrations indicated in Figure 14 for 20 h and at 37°C, and then the cell viability of the cells thus treated was determined through a cell proliferation assay (cell titer 96 AQ, Promega).

Result

It was determined that the human T-cell line Jurkat and the human B-Cell line Raji acquire a resistance to the apoptosis induced by the CD95 death receptor by expression of the human FLIP_S or FLIP_L. The expression of the longer form of the human FLIP (FLIP_L) offers more efficient protection against the apoptosis induced through CD95 than the shorter form of the human FLIP (FLIP_S), which does not include the caspase-homologous inactive protease domain.

8. Experimental Exemplary Embodiment

In order to establish the inhibiting effect of FLIP_S and FLIP_L on the apoptosis induced by the death receptor ligand TRAIL, a human Jurkat T-cell line was, as described in the above exemplary embodiment, transfected with the expression vector for human FLIP_S and FLIP_L provided with an N-terminal VSV epitope and clones raised under puromycin selection, which then were tested for VSV-marked human FLIP_S or FLIP_L in Western blot (see upper part of Figure 14a). Clones with different expression levels of FLIP_S or FLIP_L were then tested for their resistance toward apoptosis induced by TRAIL (see Figure 15). To achieve this the

clones were incubated in culture medium for 20 h and at 37°C with Flag-marked TRAIL and 1 μ g/ml cross-linked anti-Flag antibody in the concentrations indicated in Figure 15, and then the cell viability of the cells thus treated was determined through a cell proliferation assay (cell titer 96 AQ, Promega).

Result

It was determined that the human T-cell line Jurkat acquires a resistance to the apoptosis induced by the death receptor ligand TRAIL through expression of the human FLIP_S or FLIP_L. The expression of the longer form of the human FLIP (FLIP_L) offers more efficient protection against the apoptosis induced through CD95 than the shorter form of the human FLIP (FLIP_S), which does not include the caspase-homologous inactive protease domain.

Tissue homeostasis is maintained through an even balance between cell growth and apoptosis. While apoptotic signal transduction is responsible for the cell death of superfluous or infected cells, cell growth balances out certain cell losses. With numerous infectious diseases or with malignant tumors, this balance has been upset. Tumor diseases are characterized by either a site-specific or site-diffuse, accelerated proliferation of cells. With tumor cells regulation of cell division has been lost. With tumor cells an effective apoptosis no longer takes place. Thus there are clear experimental indications that tumor cells, such as melanomas or hepatomas, do not react with cell death to binding with CD95L. This is possible through "down-regulation" of the CD95 expression or a blockage within the signal transduction path (Hahne, M. et al., Science 274, 1363 (1996) Strand, S. et al., Nature Med 2, 1361-1366 (1996)).

The effect of viral FLIP proteins is based on an inhibition of the apoptotic signal transduction mechanism as described above. Viruses have integrated the presumably originally cellular genetic material with their genome in order to escape the virus-specific immune response of the immune system. Due to the presence of the viral FLIP proteins as an inhibitor of apoptosis the immune reaction is incapable of killing off the virus-infected immune cell and thereby to interrupt the reproductive cycle of the virus. The integration of the FLIP protein into the viral genome thus promotes viral spread, i.e. the consistent infection of the host.

The integration of the virus or the expression of the FLIP protein may, however, also have a transformative effect. In this connection it is interesting that of all things, numerous herpes viruses have transformational properties, i.e. properties that allow the normally regulated cell to become a tumor cell. The identification of the inhibiting viral FLIP proteins according to the invention makes a decisive contribution to understanding the connection between transformations and the origin of tumors, which hereby is being disclosed.

Viral FLIP protein also plays a key role in understanding viral tumor origination. Numerous data confirm the correlation between virus persistence and the origination of tumors. Thus Virus MCV produces slow-growing epidermal neoplasms, which may escape immune resistance for a long time. The virus HVS causes tumors in certain primate groups, and there are indications through dermatological studies that the virus HHV-8 also operates as an infectious co-factor for the Kaposi sarcoma, which particularly occurs with AIDs patients, as well as for certain forms of primary lymphoma with regard to the transformation process.

Interestingly, all γ -herpes viruses that code for a FLIP protein also have a bcl-2 homologue. The anti-apoptotic bcl-2, however, blocks cell death which is initiated by external influences, such as deprivation of growth factors, γ -radiation or cytotoxic substances. In contrast to the gene products of the viral DNA sequences in accordance with the invention, their anti-apoptotic effect with lymphocyte cell lines through stimulation of the CD95 receptor is less developed. Therefore the above-mentioned viruses have two genotypes with complementary properties, for instance, one bcl-2 homologue and one vFLIP protein. According to the invention, by combining these two genotypes, for instance two different expression vectors carrying these genotypes, an anti-apoptotic property toward many environmental influences may be achieved for the cell. According to the invention, making a recombinant protein with effective domains of bcl-2 homologues and effective domains of vFLIP proteins available is also conceivable.

While the genes or gene products according to the invention make a considerable contribution to the immortalizing process of tumor cells and thus function as tumor agents, on the other hand, with the aid of the sequences according to the invention, or their gene products according to the invention, an immortalizing process can be initiated if so desired. This is true in particular for illnesses which, due to an unregulated stimulation of the apoptosis mechanism, lead to frequent or massive cell loss. In this connection auto-immune diseases must be mentioned in particular (for instance, rheumatoid arthritis or lupus erythematosus, or multiple sclerosis). The appropriate application of the DNA sequences according to the invention, of their gene products according to the invention, the expression vectors derived from these, or host cells transformed with these expression vectors could prevent such a cell death.

In particular an immortalization of the cells affected by autoimmune disease must be considered. In the form of gene-therapeutic procedures these cells could be transfected with the DNA sequences according to the invention, for instance also *in vitro*, and the cells could then be re-transplanted.

In the case of HIV infections an application of the subjects of the invention is another possibility. FLIP proteins with their potential for inhibiting the apoptotic signal transduction mechanism could then be integrated into cells, preferably T-cells, *ex vivo*, and after retransplantation of the cells, after extra-cellular stimulation of the cells *in vivo*, be kept from dying off. These cells could survive in the patient without limit and exercise their immunological functions. Thus, in case of HIV infections, immune cells might be saved from mass destruction.

Laboratory application is another possibility. Lab medicine and/or the biomedical field has for decades been hampered by the problem that certain cell lines do not survive several generations of laboratory cultivation. The limited cell division of certain cell lines does not permit any thorough examination of their cellular or physiological properties *in vitro*. By means of transfection of cell lines with the DNA sequences according to the invention, immortality may be achieved for these cell lines.

An important area of application thus awaits the current invention in the field of laboratory or clinical research, whereby a decisive contribution to simplifying cell cultivation methods may be expected.

Patent Claims

1. DNA sequence, characterized in that it codes for a gene product inhibiting cell apoptosis, or a gene product segment inhibiting cell apoptosis, with at least one death effector domain, including all functionally homologous derivatives of the DNA sequence or all functionally homologous alleles of the DNA sequence.
2. DNA sequence according to Claim 1, characterized in that a significance level of $p < 10^{-2}$ results if the sequence of the death effector domain is compared with a search profile according to figure 1a or a search profile according to figure 1b.
3. DNA sequence according to Claims 1 or 2, characterized in that its gene product has two death effector domains.
4. DNA sequence according to one of the previous Claims, characterized in that its gene product binds to a protein of the apoptosis signal transduction path.
5. DNA sequence according to one of the previous Claims, characterized in that its gene product binds to the cytoplasmic segment of a membranous, cellular receptor of the apoptosis signal transduction path.
6. DNA sequence according to Claim 5, characterized in that its gene product binds to a receptor of the class of TNF receptors.
7. DNA sequence according to one of the previous Claims, characterized in that its gene product binds to soluble, intracellular proteins of the apoptosis signal transduction path.

8. DNA sequence according to one of the previous Claims, characterized in that its gene product binds to a protease of the caspase type.
9. DNA sequence according to one of the previous Claims, characterized in that its gene product binds to a protein of the "DISC" complex.
10. DNA sequence according to one of the previous Claims, characterized in that its Gene product binds to the protein FLICE.
11. DNA sequence according to one of the previous Claims, characterized in that it occurs in viral, prokaryotic or eukaryotic DNA hereditary informational molecules.
12. DNA sequence according to Claim 11, characterized in that its gene product includes an amino acid sequence in accordance with the "GenBank" access code U60315 (MCV 159L).
13. DNA sequence according to Claim 11, characterized in that its gene product includes an amino acid sequence in accordance with the "GenBank" access code U60315 (MCV 160L).
14. DNA sequence according to Claim 11, characterized in that it occurs in the hereditary information of a virus of the γ -Herpes type of viruses.
15. DNA sequence according to Claim 14, characterized in that its gene product includes an amino acid sequence according to the "GenBank" access code U20824 (E8).
16. DNA sequence according to Claim 14, characterized in that its gene product includes an amino acid according to Figure 16.

17. DNA sequence according to one of the previous Claims, characterized in that its gene product includes an amino acid sequence according to the "GenBank" access code X64346 (ORF 71).
18. DNA sequence according to Claim 14, characterized in that its gene product includes an amino acid sequence according to Figure 17.
19. DNA sequence according to Claim 11, characterized in that it occurs in the hereditary information of mammalian cells.
20. DNA sequence according to Claim 19, characterized in that its gene product includes an amino acid sequence according to one of Figures 4a, 4b, or 4c.
21. DNA sequence according to Claim 19 or 20, characterized in that it includes a nucleotide sequence according to one of Figures 4a, 4b, or 4c.
22. DNA sequence according to Claim 19, characterized in that its gene product includes two death effector domains and one caspase-homologous domain which is non-functional.
23. DNA sequence according to one of Claims 1 to 22, characterized in that it includes a promoter functionally attached to the claimed DNA sequence.
24. DNA sequence according to Claim 23, characterized in that it includes further regulatory elements for transcription and/or translation.
25. An expression vector, characterized in that it includes a DNA sequence according to one of Claims 1 to 24.

26. A procedure for isolating gene products with at least one death effector domain, characterized in that host cells with an expression vector are transformed according to Claim 25 and are cultivated under appropriate conditions, promoting the expression, and the gene product is finally purified out of the culture.

27. A procedure for the expression of gene products with at least one death effector domain, characterized in that host cells are transformed with an expression vector according to Claim 25.

28. Host cell, characterized in that it has been transformed with an expression vector according to Claim 25.

29. Host cell according to Claim 28, characterized in that it is a mammalian cell.

30. Host cell according to Claims 28 or 29, characterized in that it is a human cell.

31. Host cell according to Claim 30, characterized in that it is an immune system cell, in particular a T- or B-lymphocyte.

32. Purified gene product or segment of this gene product, characterized in that it has at least one death effector domain capable of inhibiting cell apoptosis, including all functionally homologous derivatives.

33. Purified gene product or segment of this gene product, characterized in that the gene product or segment has a significance level of $p < 10^{-2}$ in a comparison according to Figures 1a or 1b by means of a search profile.

34. Purified gene product or segment of this gene product, characterized in that it is coded by a DNA sequence according to one of Claims 1 through 24.

WO 98/44104

PCT/EP98/01857

ID MATRIX.

AC ZZ99999;

DE Based on FADD, FLICE and Mch4.

MA /GENERAL_SPEC: ALPHABET='ABCDEFGHIJKLMNPQRSTUVWXYZ'; LENGTH=79;

MA /DISJOINT: DEFINITION=PROTECT; N1=6; N2=74;

MA /NORMALIZATION: MODE=1; FUNCTION=LINEAR; R1=.8551; R2=.01636739; TEXT='NScore';

MA /CUT_OFF: LEVEL=0; SCORE=467; N_SCORE=8.5; MODE=1;

MA /CUT_OFF: LEVEL=-1; SCORE=344; N_SCORE=6.5; MODE=1;

MA /DEFAULT: D=-20; I=-20; B1=-50; E1=-50; MI=-105; MD=-105; IM=-105; DM=-105;

MA /I: B1=0; BI=-105; BD=-105;

MA /M: SY='P'; M=3,-5,-23,-1,-3,-20,-13,-15,-13,-12,-10,-12,-8,12,-8,-16,1,-3,-13,-30,-19,-7;

MA /M: SY='F'; M=-20,-28,-22,-36,-28,71,-30,-13,0,-26,8,0,-20,-30,-34,-18,-20,-10,-2,14,39,-28;

MA /M: SY='R'; M=-12,-14,-24,-14,-5,-12,-19,-7,-15,8,-3,-4,-6,-21,1,33,-7,-5,-11,-23,-9,-5;

MA /M: SY='V'; M=-7,-5,-19,-7,-2,-13,-20,-11,-1,-2,-9,-5,-1,-21,-8,3,-4,-4,7,-30,-14,-6;

MA /M: SY='L'; M=-10,-11,-22,-16,-11,-4,-21,-9,4,-6,16,15,-8,-23,-7,-6,-18,-8,-2,-23,-5,-9;

MA /M: SY='L'; M=-10,-30,-20,-30,-20,10,-30,-20,20,-30,50,20,-30,-30,-20,-30,-10,10,-20,0,-20;

MA /M: SY='Y'; M=-18,-17,-28,-17,-15,13,-27,33,-4,-14,4,4,-14,-27,-7,-9,-19,-13,-11,5,49,-15;

MA /M: SY='E'; M=-6,5,-25,8,14,-25,-17,-6,-12,-4,-17,-11,2,-10,11,-8,7,-2,-14,-31,-14,12;

MA /M: SY='T'; M=-9,-30,-24,-35,-27,3,-35,-27,37,-29,29,19,-25,-25,-21,-25,-22,-9,26,-21,-1,-27;

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Fig. 1a

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Forts. Fig. 1a

MA /M: SY='S'; M=3,8,-17,11,0,-25,10,-10,-27,-10,-30,-22,10,-12,-3,-12,26,8,-17,-37,-22,-1;
 MA /M: SY='E'; M=-2,4,-24,10,31,-27,-2,-6,-28,0,-24,-20,3,-6,8,-6,13,-2,-24,-32,-21,20;
 MA /M: SY='N'; M=-3,7,-23,5,10,-27,7,-3,-26,-3,-26,-17,14,-12,10,-5,12,-2,-25,-31,-20,10;
 MA /M: SY='L'; M=-8,-30,-20,-32,-24,6,-32,-24,28,-28,37,18,-28,-22,-22,-24,-8,21,-22,-2,-24;
 MA /M: SY='D'; M=-3,17,-21,24,4,-29,9,-8,-31,-8,-30,-24,12,-12,-4,-12,18,2,-21,-36,-22,0;
 MA /M: SY='S'; M=1,-9,-16,-9,-8,-18,0,-13,-15,-6,-21,-13,-1,-17,-6,2,16,5,-3,-32,-18,-8;
 MA /M: SY='E'; M=-4,9,-22,10,26,-27,-11,0,-24,2,-24,-16,11,-8,19,-1,14,1,-24,-33,-18,22;
 MA /M: SY='D'; M=-15,35,-28,44,29,-33,-11,2,-33,3,-27,-25,22,-9,6,-5,2,-8,-30,-37,-20,17;
 MA /M: SY='L'; M=-8,-30,-18,-30,-22,8,-30,-22,22,-28,43,18,-30,-30,-22,-20,-26,-8,17,-22,-2,-22;
 MA /M: SY='K'; M=-1,-4,-23,-7,8,-20,-17,-8,-18,13,-14,-3,-4,-11,5,13,-2,-1,-12,-23,-12,5;
 MA /M: SY='D'; M=-5,20,-21,23,20,-27,-7,-2,-27,-1,-27,-22,19,-9,5,-6,16,2,-23,-37,-20,12;
 MA /M: SY='L'; M=-12,-28,-20,-32,-22,21,-28,-16,16,-26,37,24,-26,-28,-20,-18,-26,-10,8,-14,6,-20;
 MA /M: SY='K'; M=-10,-6,-30,-8,2,-24,-24,-14,-14,34,-20,-4,-4,-12,4,18,-12,-10,-10,-20,-8,2;
 MA /M: SY='F'; M=-20,-30,-20,-40,-30,80,-30,-20,0,-30,10,0,-20,-30,-40,-20,-20,-10,0,10,30,-30;
 MA /M: SY='L'; M=-10,-30,-20,-30,-20,10,-30,-20,20,-30,50,20,-30,-30,-20,-20,-30,-10,10,-20,0,-20;
 MA /M: SY='C'; M=-7,-21,44,-25,-21,-8,-25,-23,-9,-27,5,-5,-19,-31,-21,-23,-9,-5,-2,-37,-17,-21;
 MA /M: SY='L'; M=-11,-16,-27,-18,-6,-13,-27,-12,2,1,6,6,-13,-19,5,6,-16,-10,-4,-20,-5,-2;
 MA /M: SY='D'; M=-11,18,-30,29,20,-34,12,-6,-37,-3,-27,-24,7,-10,0,-10,0,-13,-30,-31,-23,10;
 MA /M: SY='R'; M=-10,-10,-24,-8,4,-8,-19,-2,-14,0,-6,-7,-7,-18,1,11,-3,-4,-13,-18,3,0;
 MA /M: SY='I'; M=-6,-30,-19,-34,-28,2,-34,-28,35,-26,22,16,-26,-24,-24,-18,-6,35,-24,-4,-28;

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Forte. Fig. 1a

MA /M: SY='P'; M=-2,-12,-29,-7,-3,-27,-2,-17,-23,-11,-30,-20,-8,44,-8,-17,7,-2,-24,-32,-27,-8;
 MA /M: SY='K'; M=-10,7,-28,4,10,-30,-16,-3,-26,34,-28,-10,11,-12,16,21,-5,-8,-24,-24,-12,13;
 MA /I: I=-5; MI=-25; MD=-25; IM=-25;
 MA /M: SY='R'; M=-12,-8,4,-10,-4,-18,-7,-24,17,-18,-10,-4,-18,1,30,-8,-8,-14,-22,-12,-4; D=-5;
 MA /I: I=-5; MI=-25; MD=-25; IM=-25; DM=-25;
 MA /M: SY='K'; M=-8,0,-24,0,8,-24,-16,-8,-24,40,-24,-8,0,-8,8,24,-8,-8,-16,-16,-8,8; D=-5;
 MA /I: I=-5; MI=-25; MD=-25; IM=-25; DM=-25;
 MA /M: SY='L'; M=-8,-19,-18,-19,-9,0,-22,-11,9,-17,28,13,-19,-21,-3,-11,-19,-8,1,-16,-2,-6; D=-5;
 MA /I: I=-5; MI=-25; MD=-25; IM=-25; DM=-25;
 MA /M: SY='E'; M=-10,15,-24,25,41,-26,-14,0,-26,6,-18,-18,4,-2,12,-2,0,-8,-24,-26,-16,26; D=-5;
 MA /I: I=-5; MI=-25; IM=-25; DM=-25;
 MA /M: SY='R'; M=-12,3,-28,7,4,-25,-18,-9,-26,13,-23,-15,0,4,1,17,-2,2,-19,-27,-15,0;
 MA /M: SY='V'; M=-6,-2,-21,3,3,-17,-20,-15,-1,-10,-9,-7,-6,-15,-7,-14,2,-2,5,-32,-14,-3;
 MA /M: SY='M'; M=-6,-8,-22,-12,-1,-19,-16,-1,-6,5,-8,18,-6,-14,17,2,-2,-4,-9,-24,-8,8;
 MA /M: SY='S'; M=-1,16,-15,13,1,-21,-6,-7,-21,-6,-26,-20,19,-12,-2,-8,24,17,-15,-38,-18,0;
 MA /M: SY='A'; M=18,-12,-17,-15,-12,-17,13,-18,-14,-16,-8,-9,-7,-16,-12,-18,6,-3,-8,-24,-19,-12;
 MA /M: SY='L'; M=-6,-24,-18,-24,-16,4,-24,-18,13,-26,35,13,-23,-26,-16,-18,-17,-4,6,-24,-4,-16;
 MA /M: SY='D'; M=-12,28,-24,39,9,-29,-10,-2,-26,-4,-22,-13,11,-12,0,-10,5,-4,-19,-37,-17,5;
 MA /M: SY='L'; M=-10,-30,-20,-34,-26,20,-32,-24,23,-28,29,14,-26,-28,-26,-22,-22,-8,19,-16,4,-26;
 MA /M: SY='F'; M=-18,-30,-20,-38,-28,66,-30,-20,4,-30,18,4,-22,-30,-36,-20,-22,-10,2,4,24,-28;

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Forts. Fig. 1a

MA /M: SY='Q'; M=6,-6,-21,-9,6,-20,-17,-12,-6,-7,-11,-6,-4,-10,7,-11,6,4,-6,-26,-13,5;
 MA /M: SY='H'; M=-14,-12,-24,-14,-1,3,-23,11,-10,-5,-5,4,-9,-20,-5,4,-11,-11,-7,-19,3,-4;
 MA /M: SY='L'; M=-10,-28,-20,-30,-20,8,-28,-16,20,-26,44,27,-28,-28,-16,-18,-28,-10,10,-20,0,-18;
 MA /M: SY='E'; M=-10,-10,-26,-6,18,-14,-24,-7,-6,-8,11,1,-13,-15,9,-7,-13,-10,-12,-24,-9,13;
 MA /M: SY='E'; M=1,2,-26,5,28,-28,-16,-8,-26,22,-22,-14,-2,-6,11,8,-2,-8,-21,-24,-16,19;
 MA /M: SY='Q'; M=-12,0,-30,2,22,-33,-20,3,-25,20,-22,-7,0,-10,35,23,-4,-10,-26,-22,-12,28;
 MA /M: SY='N'; M=-10,11,-24,9,-6,-22,3,-7,-20,-3,-21,-15,14,-20,-8,2,-1,-8,-13,-31,-18,-8;
 MA /M: SY='M'; M=-13,-1,-26,0,-5,-15,-23,-11,0,-3,2,8,-7,-17,-5,-8,-15,-10,-3,-25,-7,-6;
 MA /M: SY='L'; M=-10,-30,-22,-32,-22,8,-32,-22,26,-30,44,20,-28,-28,-20,-22,-28,-10,14,-20,0,-22;
 MA /M: SY='E'; M=-6,12,-26,21,26,-30,2,-6,-32,-1,-26,-22,6,-8,5,-8,7,-6,-26,-32,-22,15;
 MA /M: SY='E'; M=-11,3,-31,12,44,-29,-20,-3,-29,10,-21,-19,-3,9,15,7,-3,-10,-29,-29,-20,28;
 MA /M: SY='G'; M=4,7,-27,13,6,-30,26,-11,-35,-9,-28,-22,6,-13,-5,-13,7,-9,-27,-29,-24,1;
 MA /M: SY='H'; M=-14,24,-26,20,6,-26,-11,28,-28,7,-27,-15,28,-16,4,4,-1,-9,-28,-34,-8,4;
 MA /M: SY='L'; M=-7,-20,-21,-21,-14,-3,-26,-20,5,-21,19,5,-20,-2,-16,-17,-13,6,0,-24,-8,-16;
 MA /M: SY='S'; M=-7,4,-21,5,1,-6,-5,-12,-22,-11,-17,-16,2,-14,-9,-12,7,6,-15,-25,-10,-3;
 MA /M: SY='F'; M=-14,-28,6,-35,-27,27,-32,-24,8,-30,16,5,-23,-30,-29,-24,-21,-10,6,-15,5,-27;
 MA /M: SY='L'; M=-10,-30,-20,-30,-20,10,-30,-20,20,-30,50,20,-30,-30,-20,-30,-10,10,-20,0,-20;
 MA /M: SY='K'; M=-1,-2,-26,-2,14,-26,-16,-7,-26,26,-22,-12,-2,-11,8,25,-4,-8,-18,-22,-14,10;
 MA /M: SY='E'; M=-14,14,-30,24,41,-30,-18,0,-32,12,-22,-20,4,-6,14,11,-2,-10,-28,-30,-18,27;
 MA /M: SY='L'; M=-8,-30,-18,-30,-22,8,-30,-22,22,-28,43,18,-30,-30,-22,-20,-26,-8,17,-22,-2,-22;

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Form. Fig. 1a

MA /M: SY='C'; M=-10,-26,34,-30,-24,-2,-30,-24,1,-30,23,5,-26,-34,-24,-24,-22,-10,2,-32,-12,-24;
 MA /M: SY='A'; M=13,-13,-19,-19,-11,4,-15,-11,-1,-9,-6,-12,-17,-11,-8,-5,-6,-6,9,-11;
 MA /M: SY='T'; M=-4,-7,-21,-11,-4,-18,-19,-10,-7,-3,-13,-5,-1,-14,7,3,9,10,-6,-27,-11,0;
 MA /M: SY='I'; M=-8,-30,-23,-35,-27,3,-35,-27,38,-28,26,18,-25,-22,-25,-21,-8,29,-22,-2,-27;
 MA /M: SY='R'; M=-12,4,-22,-3,-6,-16,-15,-3,-14,9,-17,-9,15,-22,-2,27,-3,-4,-9,-29,-14,-6;
 MA /M: SY='R'; M=-14,-8,-32,-6,6,-28,-20,-4,-26,22,-24,-10,-4,6,15,33,-8,-10,-24,-22,-14,7;
 MA /I: I=-6; MI=-32; MD=-32; IM=-32;
 MA /M: SY='H'; M=-9,-5,-17,-5,-1,-9,-14,21,-10,2,-3,1,-2,-12,1,2,-9,-9,-10,-15,3,-1; D=-6;
 MA /I: I=-6; MI=-32; IM=-32; DM=-32;
 MA /M: SY='D'; M=-11,21,-26,30,12,-32,-12,-6,-32,18,-30,-20,10,-10,4,6,4,-4,-22,-32,-16,8;
 MA /M: SY='L'; M=-10,-30,-20,-30,-20,10,-30,-20,20,-30,50,20,-30,-30,-20,-30,-10,10,-20,0,-20;
 MA /M: SY='L'; M=-10,-30,-20,-30,-20,10,-30,-20,20,-30,50,20,-30,-30,-20,-30,-10,10,-20,0,-20;
 MA /M: SY='R'; M=-13,-8,-30,-10,3,-25,-23,-4,-14,17,-15,-2,-3,-15,21,27,-8,-10,-15,-20,-8,10;
 MA /M: SY='H'; M=-13,0,-25,-8,-7,-15,-20,12,-10,-1,-13,-5,10,-18,-1,11,-3,0,-11,-28,-5,-7;
 MA /M: SY='I'; M=-10,-28,-24,-32,-25,8,-34,-19,30,-25,25,15,-24,-26,-20,-22,-22,-9,20,-13,12,-25;
 MA /M: SY='B'; M=-13,25,-25,25,14,-22,-12,0,-21,-3,-15,-16,23,-15,1,-6,-1,-6,-23,-35,-17,7;
 MA /M: SY='D'; M=-14,25,0,29,5,-31,-14,-6,-33,4,-28,-22,15,-17,-4,-4,-2,-8,-24,-38,-20,1;
 MA /I: E1=0; IE=-105; DE=-105;

//

WO 98/44104

PCT/EP98/01857

ID MATRIX.

AC ZZ99999;

DE Based on FADD, FLICE, Mch4 and viral inhibitors.

MA /GENERAL_SPEC: ALPHABET='ABCDEFGHIJKLMNPQRSTUVWXYZ'; LENGTH=78;

MA /DISJOINT: DEFINITION=PROTECT; N1=6; N2=73;

MA /NORMALIZATION: MODE=1; FUNCTION=LINEAR; R1=.8310; R2=.02233434; TEXT='NScore';

MA /CUT_OFF: LEVEL=0; SCORE=343; N_SCORE=8.5; MODE=1;

MA /CUT_OFF: LEVEL=-1; SCORE=253; N_SCORE=6.5; MODE=1;

MA /DEFAULT: D=-20; I=-20; B1=-50; E1=-50; MI=-105; MD=-105; IM=-105; DM=-105; MM=1; M0=-1;

MA /I: B1=0; BI=-105; BD=-105;

MA /M: SY='P'; M=-3,-7,-26,-4,1,-21,-18,-13,-11,-7,-13,-7,-9,15,-3,-10,-3,-3,-12,-28,-17,-3;

MA /M: SY='Y'; M=-14,-18,-23,-21,-17,27,-25,3,-5,-17,-1,-3,-14,-20,-16,-13,-9,-1,-7,2,33,-18;

MA /M: SY='R'; M=-13,-12,-26,-13,-6,-11,-15,-1,-18,9,-10,-4,-6,-21,1,24,-10,-9,-12,-18,-3,-5;

MA /M: SY='T'; M=-8,-7,-14,-7,-3,-12,-22,-11,-8,-7,-4,-5,-7,-14,-5,-6,-4,1,-5,-26,-7,-4;

MA /M: SY='L'; M=-7,-16,-18,-20,-14,3,-24,-16,5,-16,14,8,-14,-22,-12,-9,4,5,-22,-3,-13;

MA /M: SY='L'; M=-9,-28,-19,-31,-23,9,-29,-19,23,-25,35,24,-27,-28,-19,-19,-24,-8,18,-21,-1,-21;

MA /M: SY='Y'; M=-6,-20,-15,-22,-16,2,-25,-4,2,-14,7,4,-17,-25,-10,-8,-14,-8,1,-14,9,-14;

MA /M: SY='H'; M=-4,9,-16,9,6,-23,-13,10,-20,-7,-18,-12,8,-15,3,-9,3,-6,-18,-32,-11,3;

MA /M: SY='I'; M=-6,-28,-20,-33,-26,2,-33,-27,33,-26,23,15,-24,-25,-22,-23,-17,-3,29,-24,-4,-26;

MA /M: SY='N'; M=-2,6,-20,4,-3,-17,2,-8,-19,-10,-17,-14,10,-16,-6,-11,8,4,-16,-28,-12,-5;

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Fig. 16

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Forts. Fig. 16

MA /M: SY='E'; M=-7,12,-24,12,14,-21,-5,-3,-25,-1,-22,-17,13,-12,4,-2,5,-3,-23,-29,-16,9;
 MA /M: SY='N'; M=-7,9,-23,6,13,-18,-7,3,-21,0,-22,-12,15,-12,6,-3,7,-3,-22,-30,-14,9;
 MA /M: SY='L'; M=-9,-30,-21,-33,-24,11,-32,-23,26,-29,37,18,-27,-28,-22,-22,-25,-9,18,-19,1,-24;
 MA /M: SY='D'; M=-6,17,-14,21,0,-27,2,-10,-29,-9,-26,-22,11,-14,-7,-12,11,6,-19,-35,-20,-3;
 MA /M: SY='S'; M=4,3,-18,5,2,-22,-7,-10,-18,-6,-16,-14,1,-13,-1,-7,11,3,-10,-31,-17,0;
 MA /M: SY='E'; M=-8,12,-25,16,25,-28,-13,3,-28,9,-25,-18,10,-8,12,5,6,-4,-24,-32,-16,18;
 MA /I: MD=-29;
 MA /M: SY='D'; M=-15,31,-29,44,37,-34,-14,1,-34,5,-25,-25,13,-6,9,-5,1,-9,-30,-35,-20,23;
 MA /I: I=-8; MI=-10; MD=-29; IM=-10; DM=-29;
 MA /M: SY='L'; M=-9,-18,-20,-18,-12,-2,-26,-12,9,-18,21,11,-18,-23,-12,-12,-16,-3,6,-24,-4,-13;
 MA /I: DM=-29;
 MA /M: SY='E'; M=-10,-5,-27,-4,15,-14,-21,-5,-18,10,-14,-8,-4,-12,5,14,-5,-6,-16,-20,-5,9;
 MA /M: SY='S'; M=-1,-4,-9,-4,0,-18,-16,-12,-10,-7,-13,-9,-3,-17,-3,-6,6,3,-2,-32,-16,-2;
 MA /M: SY='L'; M=-11,-27,-21,-31,-22,12,-28,-14,20,-24,34,26,-26,-27,-17,-18,-25,-9,11,-16,6,-20;
 MA /M: SY='K'; M=-12,-15,-10,-17,-10,-10,-27,-13,-7,6,-2,0,-13,-22,-7,5,-16,-10,-6,-19,0,-10;
 MA /M: SY='F'; M=-17,-27,-20,-35,-26,62,-28,-17,1,-27,11,1,-19,-29,-33,-19,-17,-8,0,4,26,-26;
 MA /M: SY='L'; M=-6,-28,-12,-31,-22,9,-29,-22,17,-29,36,14,-26,-28,-21,-22,-24,-9,10,-20,-2,-22;
 MA /M: SY='C'; M=0,-19,37,-25,-20,-10,-23,-23,-7,-25,2,-5,-17,-28,-20,-23,-6,-4,-1,-35,-17,-20;
 MA /M: SY='R'; M=-9,2,-19,-1,-2,-21,-13,-2,-18,6,-14,-5,4,-18,2,7,-7,-9,-16,-27,-12,-1;
 MA /M: SY='D'; M=-11,16,-29,23,14,-31,-2,-5,-29,0,-26,-20,9,-2,2,-7,-1,-10,-25,-31,-21,7;

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Forts. Fig. 16

MA /M: SY='Y'; M=-10,-13,-13,-14,-10,5,-23,-9,-7,-12,3,-3,-13,-23,-12,-5,-10,-4,-5,-15,7,-12;
 MA /M: SY='I'; M=-4,-24,-22,-32,-24,4,-30,-24,29,-25,20,13,-18,-23,-20,-23,-17,-7,20,-20,-2,-24;
 MA /M: SY='P'; M=-7,-14,-35,-7,1,-29,-5,-17,-24,-7,-29,-19,-12,54,-8,-15,-3,-9,-27,-29,-27,-7;
 MA /I: MD=-18;
 MA /M: SY='K'; M=-3,1,-22,-1,3,-24,-7,-6,-23,13,-25,-13,7,-7,7,12,6,-2,-19,-26,-15,4; D=-4;
 MA /I: MD=-18;
 MA /M: SY='R'; M=-7,-2,-2,-4,1,-15,-13,-1,-18,5,-14,-9,2,-14,0,9,-1,-4,-13,-23,-10,0; D=-4;
 MA /I: I=-5; MI=0; MD=-18; IM=0; DM=-18;
 MA /M: SY='L'; M=0,-10,-12,-9,-4,-5,-10,-8,0,-9,5,0,-9,1,-4,-8,-5,-2,-3,-13,-6,-5; D=-4;
 MA /I: DM=-18;
 MA /M: SY='E'; M=1,3,-8,5,11,-18,1,-7,-19,-4,-17,-14,1,-7,0,-7,7,-2,-14,-22,-15,5; D=-4;
 MA /I: DM=-18;
 MA /M: SY='E'; M=-6,2,-19,5,12,-18,-13,-7,-18,6,-16,-12,0,4,3,3,4,-15,-22,-12,6; D=-4;
 MA /I: DM=-18;
 MA /M: SY='S'; M=-4,-8,-19,-7,-4,-10,-16,-14,-2,-9,-11,-7,-5,-1,-7,-12,4,0,-2,-24,-11,-7; D=-4;
 MA /I: DM=-18;
 MA /M: SY='K'; M=-7,-7,-14,-10,-1,-22,-19,-9,-15,9,-16,-1,-5,-10,8,6,-1,1,-11,-26,-12,3;
 MA /M: SY='T'; M=-4,5,-1,-2,-5,-17,-15,-12,-16,-8,-18,-14,10,-16,-6,-7,12,16,-10,-35,-16,-6;
 MA /M: SY='F'; M=3,-19,-11,-23,-19,6,-8,-18,-5,-21,4,-2,-15,-23,-20,-19,-7,-4,-1,-16,-2,-19;
 MA /M: SY='L'; M=-3,-18,-9,-19,-10,-5,-23,-18,3,-17,12,2,-17,-23,-13,-12,-8,-1,7,-27,-10,-12;

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Forte, Fig. 16

MA /M: SY='D'; M=-9,22,-25,29,11,-29,-12,3,-25,0,-21,-15,11,-13,5,-4,1,-7,-20,-33,-15,8;
 MA /M: SY='L'; M=-4,-29,-25,-33,-24,15,-25,-24,9,-25,16,4,-26,-22,-21,-22,-12,4,16,7,-22;
 MA /M: SY='F'; M=-6,-25,-17,-30,-23,27,-25,-22,10,-24,12,4,-20,-26,-26,-19,-11,-4,13,-14,5,-23;
 MA /M: SY='S'; M=-4,-2,-16,-3,-2,-18,-11,-9,-16,-3,-17,-12,1,-11,-1,-2,4,-1,-14,-27,-11,-3;
 MA /M: SY='A'; M=5,-16,-10,-21,-12,5,-18,-11,-5,-15,1,2,-13,-20,-13,-13,-5,-3,-1,-19,-4,-12;
 MA /M: SY='L'; M=-10,-29,-21,-31,-21,8,-29,-18,22,-28,45,25,-28,-28,-18,-19,-28,-10,11,-20,0,-19;
 MA /M: SY='E'; M=-8,-2,-18,2,27,-22,-21,-6,-17,-1,-10,-11,-5,-10,11,-3,-2,-7,-18,-29,-16,18;
 MA /M: SY='E'; M=-5,16,-25,17,19,-28,-12,-2,-27,11,-25,-18,12,-10,9,5,3,-5,-23,-30,-17,14;
 MA /M: SY='M'; M=-7,-13,-23,-14,-4,-11,-23,0,-4,-4,2,5,-12,-19,4,1,-10,-5,-4,-20,-1,-1;
 MA /M: SY='N'; M=-4,3,-24,2,-2,-22,0,3,-22,-2,-17,-11,7,-18,-4,2,-3,-10,-17,-27,-13,-4;
 MA /M: SY='M'; M=-4,-11,-13,-12,-7,-12,-21,-14,-2,-6,3,4,-12,-19,-8,-9,-9,-5,0,-27,-10,-8;
 MA /M: SY='L'; M=-9,-25,-22,-25,-15,1,-29,-20,16,-18,29,13,-24,-25,-16,-14,-23,-9,11,-22,-3,-16;
 MA /M: SY='S'; M=-2,5,-23,8,6,-26,12,-10,-29,-6,-26,-20,5,-12,-3,-7,13,1,-21,-31,-21,2;
 MA /M: SY='P'; M=-6,-9,-20,-6,8,-23,-16,-13,-21,1,-17,-13,-10,13,-2,-2,-6,-6,-19,-27,-19,1;
 MA /M: SY='D'; M=-1,5,-23,8,2,-24,-3,-10,-18,-9,-14,-12,1,-14,1,-12,3,-3,-15,-29,-16,1;
 MA /M: SY='N'; M=-8,14,-15,9,-2,-21,-11,0,-21,-2,-24,-16,17,-17,-3,-5,2,-3,-20,-23,-12,-3;
 MA /M: SY='L'; M=-9,-18,-21,-18,-13,0,-26,-14,7,-17,16,9,-19,-19,-9,-14,-15,-4,5,-20,-1,-11;
 MA /I: I=-5; MI=-27; MD=-27; IM=-27;
 MA /M: SY='S'; M=-4,3,-18,6,0,-14,-8,-10,-14,-8,-16,-13,1,1,-5,-11,8,5,-11,-25,-12,-3; D=-5;
 MA /I: I=-5; MI=-27; MD=-27; IM=-27; DM=-27;

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Form Fig. 16

MA /M: SY=L'; M=-5,-21,1,-26,-20,11,-24,-18,10,-21,12,5,-18,-22,-20,-18,-14,-6,10,-15,1,-20; D=-5;
 MA /I: I=-5; MI=-27; MD=-27; IM=-27; DM=-27;
 MA /M: SY=L'; M=-9,-26,-18,-27,-18,12,-26,-18,18,-26,38,16,-24,-25,-18,-18,-24,-9,9,-15,2,-18; D=-5;
 MA /I: I=-5; MI=-27; IM=-27; DM=-27;
 MA /M: SY=K'; M=2,-9,-22,-12,3,-17,-18,-10,-7,6,-8,4,-10,-14,1,4,-6,-7,-3,-23,-12,1;
 MA /M: SY=E'; M=-11,9,-27,14,28,-27,-18,10,-28,7,-21,-15,4,-8,13,4,2,-3,-24,-30,-12,19;
 MA /M: SY=L'; M=-9,-27,4,-31,-23,2,-29,-20,14,-26,28,18,-26,-30,-19,-21,-23,-9,10,-26,-6,-22;
 MA /M: SY=L'; M=-11,-28,-4,-31,-23,15,-29,-20,11,-29,35,15,-27,-31,-23,-21,-26,-10,6,-20,0,-22;
 MA /M: SY=Y'; M=-7,-11,-18,-14,-6,4,-21,0,-16,2,-11,-7,-9,-19,-6,3,-9,-6,-12,-8,14,-7;
 MA /M: SY=T'; M=0,-11,-20,-15,-7,-16,-20,-13,-1,-5,-8,-2,-7,-15,3,0,3,5,1,-25,-11,-4;
 MA /M: SY=T'; M=-4,-27,-20,-31,-23,1,-29,-24,27,-26,24,14,-23,-24,-20,-23,-16,-6,21,-23,-4,-23;
 MA /M: SY=R'; M=-10,-4,-18,-7,-6,-23,2,0,-26,3,-22,-12,5,-20,2,18,-2,-10,-20,-26,-15,-4;
 MA /M: SY=R'; M=-18,-9,-31,-8,2,-23,-20,-2,-29,29,-22,-10,-1,-12,11,57,-10,-10,-21,-21,-11,3;
 MA /I: I=-6; MI=-32; MD=-32; IM=-32;
 MA /M: SY=M'; M=-7,-17,-20,-19,-13,3,-20,-4,2,-11,4,7,-13,-12,-10,-8,-11,-7,0,-15,3,-12; D=-6;
 MA /I: I=-6; MI=-32; IM=-32; DM=-32;
 MA /M: SY=D'; M=-16,38,-29,53,15,-37,-5,-3,-38,3,-30,-27,16,-11,0,-6,1,-9,-28,-37,-20,7;
 MA /M: SY=L'; M=-10,-30,-20,-31,-21,9,-31,-21,22,-30,46,20,-29,-29,-20,-21,-29,-10,13,-20,0,-21;
 MA /M: SY=L'; M=-4,-25,-11,-27,-18,0,-27,-20,14,-24,28,12,-24,-27,-16,-19,-18,-7,11,-24,-6,-17;
 MA /M: SY=R'; M=-12,-8,-27,-9,1,-21,-22,-8,-17,22,-15,-4,-4,-16,10,28,-8,-5,-12,-21,-9,4;

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Forts. Fig. 16

MA /M: SY='N'; M=-5,2,-21,-4,-4,-17,-15,0,-9,-2,-12,0,7,-16,4,2,2,1,-9,-28,-10,-1;
 MA /M: SY='I'; M=-9,-28,-22,-31,-23,3,-32,-22,25,-22,24,14,-24,-26,-19,-14,-20,-8,21,-20,0,-23;
 MA /M: SY='F'; M=-11,-7,-21,-10,-7,9,-21,-11,-6,-10,-4,-4,-4,-21,-9,-9,-7,-6,-5,-19,-1,-6;
 MA /M: SY='D'; M=-6,9,-17,10,1,-25,-2,-3,-24,-5,-19,-14,7,-16,-2,-8,-1,-6,-18,-30,-16,-1;
 MA /I: E1=0; IE=-105; DE=-105;
 //

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Fig. 2

EHV-2	(E8)	1
HHV-8	(71)	1
HVS	(71)	1
BHV-4	(ORF)	1
MCV	(159L)	7
MCV	(160L)	5
FLICE		1
Mch4		18

	DED	
EHV-2 (E8)	24	L
HHV-8 (71)	25	L
HVS (71)	25	L
BHV-4 (ORF)	26	L
MCV (159L)	25	L
MCV (160L)	25	L
FLICE	25	L
Mch4	43	L

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Forte, Fig. 2

DEED

EHV-2 (E8)	46
HHV-8 (71)	43
HVS (71)	44
BHV-4 (ORF)	43
MCV (159L)	44
MCV (160L)	44
FLICE	49
Mch4	67

[illegible]

— DE —

EHV-2 (E8)	68
HHV-8 (71)	68
HVS (71)	69
BHV-4 (ORF)	68
MCV (159L)	66
MCV (160L)	66
FLICE	74
Mch4	92

[illegible]

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Form Fig. 2

DED II

EHV-2 (E8) 140
HHV-8 (71) 138
HVS (71) 140
BHV-4 (ORF) 140
MCV (159L) 138
MCV (160L) 134
FLICE 147
Mch4 158
FADD 50
PEA-15 50

E D E N E C E N E E E E L E
E N E K E N D A K R K Q N S H
E D H A G A V G K Q N D L S H
E L L L L L L L L L L L L L L
L L L L L L L L L L L L L L L
L G G L G L G S S G E D E P D
G D T . D S Q G N L K D N L
K D C N S D K N L T E
L D C D L L L L L L L L L L
F D A C F L L L L L L L L L
F L M K E A V K E R E H
H S K T A T R D L L L L L L
L L L L L L L L L L L L L L
T Q Q A C A F E I S R P D
G R S G H R C N V P R R R P
A V S M L Y . K H D
A D D D D D S K H D

DED II

EHV-2 (E8) 165
HHV-8 (71) 163
HVS (71) 163
BHV-4 (ORF) 165
MCV (159L) 163
MCV (160L) 159
FLICE 171
Mch4 182
FADD 75
PEA-15 75

L L L L L L L L L L L L L L
L Q A C S V I N R T M
N Q T M C Q V A I N R T M
Q V Q T . K N V E S A N D E D V D



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Form Fig. 3

DED I	
FLIPL _ HS	55
FLIPL _ MM	60
VFLIP _ EHV2	56
FLICE _ HS	59
Mch4 _ HS	76

DED II	
FLIPL _ HS	77
FLIPL _ MM	82
VFLIP _ EHV2	77
FLICE _ HS	83
Mch4 _ HS	99

DED II	
FLIPL _ HS	100
FLIPL _ MM	105
VFLIP _ EHV2	100
FLICE _ HS	108
Mch4 _ HS	122

DEED

FLIPL	HS	124
FLIPL	MM	129
VFLIP	EHV2	125
FLICE	HS	132
Mch4	HS	145

DEED

FLIPL	HS	149
FLIPL	MM	154
VFLIP	EHV2	150
FLICE	HS	157
Mch4	HS	167

FLIPL_HS	174	SVQAGTSYRNVLOAAIOK.SLKDP
FLIPL_MM	179	SSQGA.RSNMNTLQASLPKLSIK..
FLICE_HS	181	FS.....
Mch4_HS	190	

19/63 Forks. Fig. 3

FLIPS	MITPYAHCPDLKILGNCSM
FLIPL	HS
FLIPL	MM
FLICE	HS
Mch4	HS
198	SNNFRILHNGRSKEQRLKQLGAQQE
201	.YNSRILONGRSKEPRFVYRDSORT
183	.KERSSSLSEGSPEFSNGEFLCGVM
190	.REKAIQIVTTPVDKEAFSYQGEE

FLIPL	HS	FLIPC	Caspase
FLIPL	MM		
FLICE	HS		
Mch4	HS		
223	PVKKSIOESSEAFLPQSIPER		YKMK
225	LVKTSIOESSEAFLPPIRER		YRMO
207	TLISDSPREOSQ..TLDKVOMK		
214	LVSQILDVKTFLEAL..PRAAVYRMN		

FLIPL	HS	Caspase	
FLIPL	MM		
FLICE	HS		
Mch4	HS		
248	SKPLGLCLIIDCIGNE		
250	SKPLGLCLIIDCIGND		
230	SKPLGLCLIIDCIGNF		
237	RNHRGLCVIVNNHSFT		

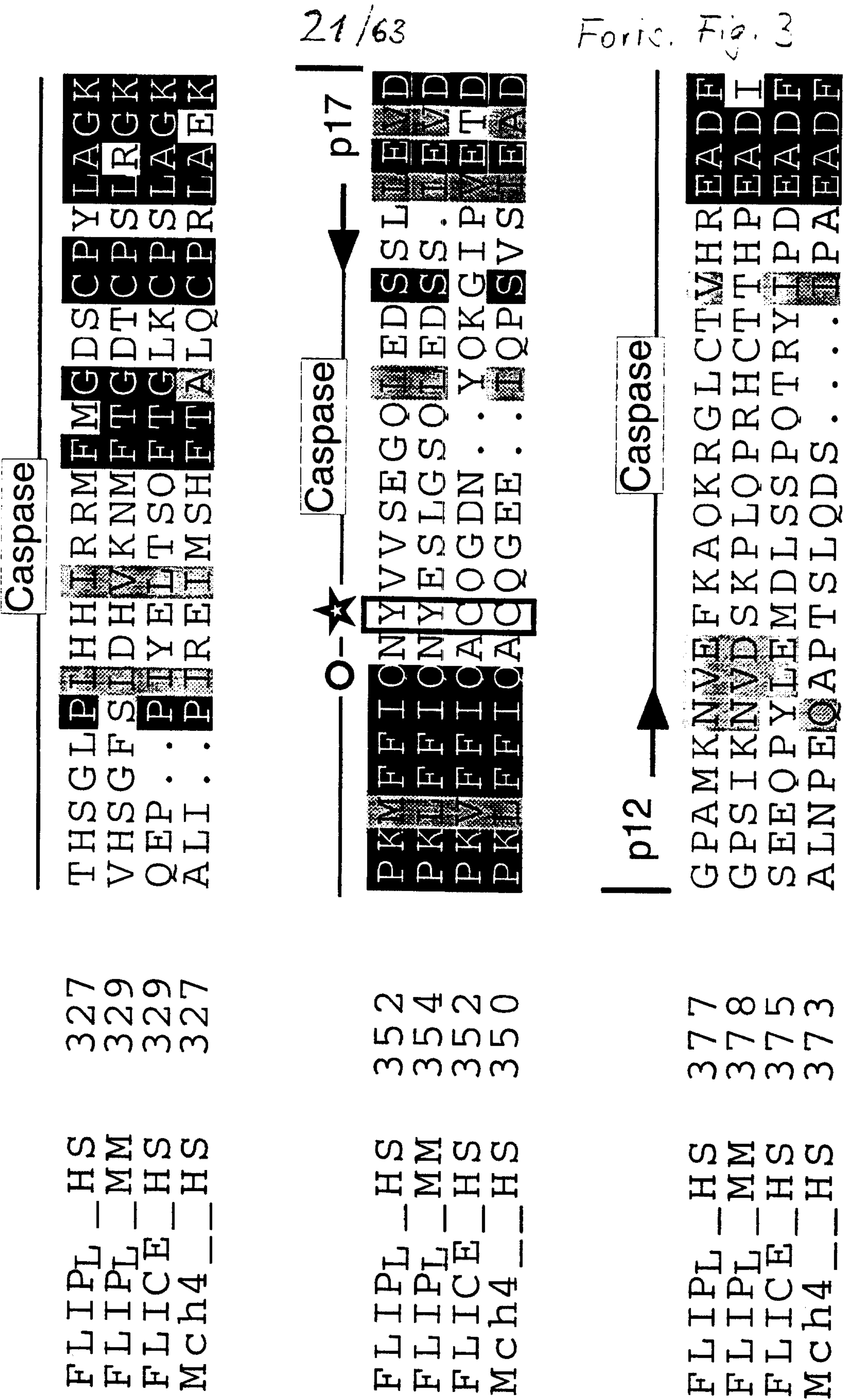
FLIPL	HS	264	O Caspase TELRLDFTSLG TKYLOETFSLG HSIRDRNGTHLDA SLKDRQGTHKDA
FLIPL	MM	266	O Caspase YEVQKFLH YHQLFLF GATTTTFFELHF EITSHVFQWLGF
FLICE	HS	255	
Mch4	HS	253	

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FLIPL	HS	284	Caspase ...L S M H G I S Q I I G Q F A C M P E
FLIPL	MM	286	Caspase ...P K S H D I T O I I R R Y A S M A O
FLICE	HS	280	Caspase E I K P H D D C T V E Q I Y E I I K I Q L M D
Mch4	HS	277	Caspase T V H I H N N V T K V E M V I I Q K Q K C N P A

Fork Fig. 3

FLIPL	HS	302	Caspase ☆☆ H R D Y D S F V C V L V S R G S Q S V Y C V D O
FLIPL	MM	304	Caspase ☆☆ H Q D Y D S F A C V L V S L G S Q S M C R D O
FLICE	HS	304	Caspase ☆☆ H S N M D C F C C C T L T H G D K G I Y G T D G E
Mch4	HS	302	Caspase ☆☆ H A D G D C F C C C T L T H G R F G A V Y S S D E



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Forts. Fig. 2

Caspase			
FLIPL	HS	402	FWSLCTADMSLLEOSHSSPSLYLCOCK
FLIPL	MM	403	FWSLCTADVSHLEKPSSSSVYLOK
FLICE	HS	400	LLGLMATVNNCVSYRNP AEGSWYLOS
Mch4	HS	394	LLGLATVPGYVSFRHVFEESWYLOS

Caspase			
FLIPL	HS	427	LSOKLRQERKRPLLDHIEHNGMY
FLIPL	MM	428	LSOKLRQGRRRPLVDLHVFLMDKVY
FLICE	HS	425	LWOSLRERCPR.GDDLTIT EVNY
Mch4	HS	419	LCHSLRKLVPR.HFDDLSITAVND

Caspase			
FLIPL	HS	452	DWNSRVSAK.FKYYVWLQHTLRKK
FLIPL	MM	453	AWN SGVSSK.FKYSLSLOHTLRKK
FLICE	HS	449	EVSNKDDKK.NMGKQMPQPTFLRKK
Mch4	HS	443	DVSRVDDKKQGTKKQMPQPAFLRKK

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Form. Fig. 3

Caspase		
FLIP _L —HS	475	LLSYT.....
FLIP _L —MM	476	LLAPT.....
FLICE—HS	474	LLFPSD.....
Mch4—HS	468	LLFVPPLDALSI

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24/63 Fig. 4a

Human c-FLIP_s Nucleotides: 1 to 1190

10	20	30	40
* *	* *	* *	* *
AAGCTCGAAA	TTAACCCTCA	CTAAAGGGAA	CAAAAGCTGG
50	60	70	80
* *	* *	* *	* *
AGCTCCACCG	CGGTGGCGGC	CGCTCTAGAA	CTAGTGGATC
90	100	110	120
* *	* *	* *	* *
CCCCGGGCTG	CAGGAATTCG	GCACGAGCGG	CACGAGTAGA
130	140	150	160
* *	* *	* *	* *
CTTCTATAGA	TCCCTTTCTA	TAGAACTTAA	TCTACTTAAG
170	180	190	200
* *	* *	* *	* *
TCAGGGAGAC	CACCCAGAAG	GAAAGAGCCC	ATACTTTCAA
210	220	230	240
* *	* *	* *	* *
TCTTAGGCAT	AAGTTAGCTT	GATAAGATTT	TCAGAAAAAT
250	260	270	280
* *	* *	* *	* *
TCCCTTTTAA	CCACAGAACT	CCCCCACTGG	AAAGGATTCT

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25/63 Forks Fig. 4a

300 310 320

* * * *

GAAAGAAATG AAGTCAGCCC TCAGAAATGA AGTTGACTGC

330 340 350 360

* * * *

CTGCTGGCTT TCTGTTGACT GGCCCGGAGC TGTACTGCAA

370 380 390 400

* * * *

GACCCTTGTG AGCTTCCCTA GTCTAAGAGT AGGATGTCTG

M S

410 420 430 440

* * * *

CTGAAGTCAT CCATCAGGTT GAAGAAGCAC TTGATACAGA

A E V I H Q V E E A L D T D

450 460 470 480

* * * *

TGAGAAGGAG ATGCTGCTCT TTTTGTGCCG GGATGTTGCT

E K E M L L F L C R D V A

490 500 510 520

* * * *

ATAGATGTGG TTCCACCTAA TGTCAGGGAC CTTCTGGATA

I D V V P P N V R D L L D

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26/63 *Foris. Fig. 4a*

530	540	550	560
* *	* *	* *	* *
TTTTACGGGA	AAGAGGTAAG	CTGTCTGTCTG	GGGACTTGGC
I L R E	R G K	L S V	G D L A
570	580	590	600
* *	* *	* *	* *
TGAACTGCTC	TACAGAGTGA	GGCGATTTGA	CCTGCTCAAA
E L L	Y R V	R R F D	L L K
610	620	630	640
* *	* *	* *	* *
CGTATCTTGA	AGATGGACAG	AAAAGCTGTG	GAGACCCACC
R I L	K M D R	K A V	E T H
650	660	670	680
* *	* *	* *	* *
TGCTCAGGAA	CCCTCACCTT	GTTTCGGACT	ATAGAGTGCT
L L R N	P H L	V S D	Y R V L
690	700	710	720
* *	* *	* *	* *
GATGGCAGAG	ATTGGTGAGG	ATTTGGATAA	ATCTGATGTG
M A E	I G E	D L D K	S D V
730	740	750	760
* *	* *	* *	* *
TCCTCATTA	TTTTCCTCAT	GAAGGATTAC	ATGGGCCGAG
S S L	I F L M	K D Y	M G R

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27/63 Forks Fig 4a

770	780	790	800
* *	* *	* *	* *
GCAAGATAAG	CAAGGAGAAG	AGTTTCTTGG	ACCTTGTGGT
G K I S	K E K	S F L	D L V V
810	820	830	840
* *	* *	* *	* *
TGAGTTGGAG	AAACTAAATC	TGGTTGCCCC	AGATCAACTG
E L E	K L N	L V A P	D Q L
850	860	870	880
* *	* *	* *	* *
GATTTATTAG	AAAAATGCCT	AAAGAACATC	CACAGAATAG
D L L	E K C L	K N I	H R I
890	900	910	920
* *	* *	* *	* *
ACCTGAAGAC	AAAAATCCAG	AAGTACAAGC	AGTCTGTTCA
D L K T	K I Q	K Y K	Q S V Q
930	940	950	960
* *	* *	* *	* *
AGGAGCAGGG	ACAAGTTACA	GGAATGTTCT	CCAAGCAGCA
G A G	T S Y	R N V L	Q A A
970	980	990	1000
* *	* *	* *	* *
ATCCAAAAGA	GTCTCAAGGA	TCCTTCAAAT	AACTTCAGGA
I Q K	S L K D	P S N	N F R

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28/63 Foris. Fig. 4a

1010	1020	1030	1040
* *	* *	* *	* *
TGATAACMCC	CTATGCCCCAT	TGCCCTGATC	TGAAAATTCT
M I T P	Y A H	C P D	L K I L
1050	1060	1070	1080
* *	* *	* *	* *
TGGAAATTGT	TCCATGTGAT	TAACATGGAA	CBGCCTCTAC
G N C	S M>		
1090	1100	1110	1120
* *	* *	* *	* *
TTAATCATTC	TGAATGATTA	AATCGTTTCA	TTTTCTAAAT
1130	1140	1150	1160
* *	* *	* *	* *
GTGTAAAAAA	AAAAAAAAAA	AAAAAACTC	GAGGGGGGGC
1170	1180	1190	
* *	* *	* *	
CCGTACCCAA	TTCGCCCTAT	AGTGAGTCGT	

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29/63 Fig. 46
Human c-FLIP_L nucleotides: 1 to 2143

10	20	30	40
* *	* *	* *	* *
TAGGGGTGGG	GACTCGGCCT	CACACAGTGA	GTGCCGGCTA
50	60	70	80
* *	* *	* *	* *
TTGGACTTTT	GTCCAGTGAC	AGCTGAGACA	ACAAGGACCA
90	100	110	120
* *	* *	* *	* *
CGGGAGGAGG	TGTAGGAGAG	AAGCGCCGCG	AACAGCGATC
130	140	150	160
* *	* *	* *	* *
GCCCAGCACC	AAGTCCGCTT	CCAGGCTTTC	GGTTTCTTTG
170	180	190	200
* *	* *	* *	* *
CCTCCATCTT	GGGTGCGCCT	TCCCGGCGTC	TAGGGGAGCG
210	220	230	240
* *	* *	* *	* *
AAGGCTGAGG	TGGCAGCGGC	AGGAGAGTCC	GGCCGCGACA
250	260	270	280
* *	* *	* *	* *
GGACGAACTC	CCCCACTGGA	AAGGATTCTG	AAAGAAATGA

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30/63 Forc. Fig. 46

290	300	310	320
* *	* *	* *	* *
AGTCAGCCCT	CAGAAATGAA	GTTGACTGCC	TGCTGGCTTT
330	340	350	360
* *	* *	* *	* *
CTGTTGACTG	GCCCGGAGCT	GTACTGCAAG	ACCCTTGTGA
370	380	390	400
* *	* *	* *	* *
GCTTCCCTAG	TCTAAGAGTA	GGATGTCTGC	TGAAGTCATC
		M S A	E V I
410	420	430	440
* *	* *	* *	* *
CATCAGGTTG	AAGAAGCACT	TGATACAGAT	GAGAAGGAGA
H Q V	E E A L	D T D	E K E
450	460	470	480
* *	* *	* *	* *
TGCTGCTCTT	TTTGTGCCGG	GATGTTGCTA	TAGATGTGGT
M L L F	L C R	D V A	I D V V
490	500	510	520
* *	* *	* *	* *
TCCACCTAAT	GTCAGGGACC	TTCTGGATAT	TTTACGGGAA
P P N	V R D	L L D I	L R E

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31/63 *Foris. Fig. 4b*

530	540	550	560
* *	* *	* *	* *
AGAGGTAAGC	TGTCTGTCGG	GGACTTGGCT	GAACTGCTCT
R G K	L S V G	D L A	E L L
570	580	590	600
* *	* *	* *	* *
ACAGAGTGAG	GCGATTTGAC	CTGCTCAAAC	GTATCTTGAA
Y R V R	R F D	L L K	R I L K
610	620	630	640
* *	* *	* *	* *
GATGGACAGA	AAAGCTGTGG	AGACCCACCT	GCTCAGGAAC
M D R	K A V	E T H L	L R N
650	660	670	680
* *	* *	* *	* *
CCTCACCTTG	TTTCGGACTA	TAGAGTGCTG	ATGGCAGAGA
P H L	V S D Y	R V L	M A E
690	700	710	720
* *	* *	* *	* *
TTGGTGAGGA	TTTGGATAAA	TCTGATGTGT	CCTCATTAAT
I G E D	L D K	S D V	S S L I
730	740	750	760
* *	* *	* *	* *
TTTCCTCATG	AAGGATTACA	TGGGCCGAGG	CAAGATAAGC
F L M	K D Y	M G R G	K I S

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32/63 *Expts. fig. 4b*

770	780	790	800
* *	* *	* *	* *
AAGGAGAAGA	GTTTCTTGGA	CCTTGTGGTT	GAGTTGGAGA
K E K	S F L D	L V V	E L E
810	820	830	840
* *	* *	* *	* *
AACTAAATCT	GGTTGCCCCA	GATCAACTGG	ATTTATTAGA
K L N L	V A P	D Q L	D L L E
850	860	870	880
* *	* *	* *	* *
AAAATGCCTA	AAGAACATCC	ACAGAATAGA	CCTGAAGACA
K C L	K N I	H R I D	L K T
890	900	910	920
* *	* *	* *	* *
AAAATCCAGA	AGTACAAGCA	GTCTGTTCAA	GGAGCAGGGA
K I Q	K Y K Q	S V Q	G A G
930	940	950	960
* *	* *	* *	* *
CAAGTTACAG	GAATGTTCTC	CAAGCAGCAA	TCCAAAAGAG
T S Y R	N V L	Q A A	I Q K S
970	980	990	1000
* *	* *	* *	* *
TCTCAAGGAT	CCTTCAAATA	ACTTCAGGCT	CCATAATGGG
L K D	P S N	N F R L	H N G

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33/63 Forks. Fig. 46

1010	1020	1030	1040
* *	* *	* *	* *
AGAAGTAAAG	AACAAAGACT	TAAGGAACAG	CTTGGCGCTC
R S K	E Q R L	K E Q	L G A
1050	1060	1070	1080
* *	* *	* *	* *
AACAAGAACC	AGTGAAGAAA	TCCATTCAGG	AATCAGAAGC
Q Q E P	V K K	S I Q	E S E A
1090	1100	1110	1120
* *	* *	* *	* *
TTTTTTGCCT	CAGAGCATAC	CTGAAGAGAG	ATACAAGATG
F L P	Q S I	P E E R	Y K M
1130	1140	1150	1160
* *	* *	* *	* *
AAGAGCAAGC	CCCTAGGAAT	CTGCCTGATA	ATCGATTGCA
K S K	P L G I	C L I	I D C
1170	1180	1190	1200
* *	* *	* *	* *
TTGGCAATGA	GACAGAGCTT	CTTCGAGACA	CCTTCACTTC
I G N E	T E L	L R D	T F T S
1210	1220	1230	1240
* *	* *	* *	* *
CCTGGGCTAT	GAAGTCCAGA	AATTCTTGCA	TCTCAGTATG
L G Y	E V Q	K F L H	L S M

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34/63 Forks Fig. 46

1250	1260	1270	1280
* *	* *	* *	* *
CATGGTATAT	CCCAGATTCT	TGGCCAATTT	GCCTGTATGC
H G I	S Q I L	G Q F	A C M
1290	1300	1310	1320
* *	* *	* *	* *
CCGAGCACCG	AGACTACGAC	AGCTTTGTGT	GTGTCCTGGT
P E H R	D Y D	S F V	C V L V
1330	1340	1350	1360
* *	* *	* *	* *
GAGCCGAGGA	GGCTCCCAGA	GTGTGTATGG	TGTGGATCAG
S R G	G S Q	S V Y G	V D Q
1370	1380	1390	1400
* *	* *	* *	* *
ACTCACTCAG	GGCTCCCCCT	GCATCACATC	AGGAGGATGT
T H S	G L P L	H H I	R R M
1410	1420	1430	1440
* *	* *	* *	* *
TCATGGGAGA	TTCATGCCCT	TATCTAGCAG	GGAAGCCAAA
F M G D	S C P	Y L A	G K P K
1450	1460	1470	1480
* *	* *	* *	* *
GATGTTTTTT	ATTCAGAACT	ATGTGGTGTC	AGAGGGCCAG
M F F	I Q N	Y V V S	E G Q

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35/63 Forts. Fig. 4b

1490	1500	1510	1520
* *	* *	* *	* *
CTGGAGGACA	GCAGCCTCTT	GGAGGTGGAT	GGGCCAGCGA
L E D	S S L L	E V D	G P A
1530	1540	1550	1560
* *	* *	* *	* *
TGAAGAATGT	GGAATTCAAG	GCTCAGAAGC	GAGGGCTGTG
M K N V	E F K	A Q K	R G L C
1570	1580	1590	1600
* *	* *	* *	* *
CACAGTTCAC	CGAGAAGCTG	ACTTCTTCTG	GAGCCTGTGT
T V H	R E A	D F F W	S L C
1610	1620	1630	1640
* *	* *	* *	* *
ACTGCGGACA	TGTCCCTGCT	GGAGCAGTCT	CACAGCTCAC
T A D	M S L L	E Q S	H S S
1650	1660	1670	1680
* *	* *	* *	* *
CATCCCTGTA	CCTGCAGTGC	CTCTCCCAGA	AACTGAGACA
P S L Y	L Q C	L S Q	K L R Q
1690	1700	1710	1720
* *	* *	* *	* *
AGAAAGAAAA	CGCCCACTCC	TGGATCTTCA	CATTGAACTC
E R K	R P L	L D L H	I E L

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36/63 *Ex. 13, Fig. 4b*

1730	1740	1750	1760
* *	* *	* *	* *
AATGGCTACA	TGTATGATTG	GAACAGCAGA	GTTTCTGCCA
N G Y	M Y D W	N S R	V S A
1770	1780	1790	1800
* *	* *	* *	* *
AGGAGAAATA	TTATGTCTGG	CTGCAGCACA	CTCTGAGAAA
K E K Y	Y V W	L Q H	T L R K
1810	1820	1830	1840
* *	* *	* *	* *
GAAACTTATC	CTCTCCTACA	CATAAGAAAC	CAAAAGGCTG
K L I	L S Y	T	
1850	1860	1870	1880
* *	* *	* *	* *
GGCGTAGTGG	CTCACACCTG	TAATCCCAGC	ACTTTGGGAG
1890	1900	1910	1920
* *	* *	* *	* *
GCCAAGGNGG	GCAGATCACT	TCAGGTCAGG	AGTTCGAGAC
1930	1940	1950	1960
* *	* *	* *	* *
CAGCCTGGCC	AACATGGTAA	ACGCTGTCCC	TAGTAAAAAT
1970	1980	1990	2000
* *	* *	* *	* *
ACAAAAATTA	GCTGGGTGTG	GGTGTGGGTA	CCTGTATTCC

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2010 2020 2030 2040
* * * * *
CAGTTACTTG GGAGGCTGAG GTGGGAGGAT CTTTTGAACC

2050 2060 2070 2080
* * * * *
CAGGAGTTCA GGGTCATAGC ATGCTGTGAT TGTGCCTACG

2090 2100 2110 2120
* * * * *
AATAGCCACT GCATACCAAC CTGGGCAATA TAGCAAGATC

2130 2140
* * * *
CCATCTCTTT AAAAAAAAAA AAA

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38/63 Fig. 46
Mouse c-FLIP_L nucleotides: 1 to 2452

10	20	30	40
* *	* *	* *	* *
GAATTCCGAG	CCTCTCAAGC	GGCCACTTAG	GGCCGGACAG
50	60	70	80
* *	* *	* *	* *
AGTGTCTCTA	TTGCAAGAAC	TCTGAGAGAA	ATGAAGAGAG
90	100	110	120
* *	* *	* *	* *
TCCTCAGCAA	TGATGTTGGC	TTCTCGTGGT	TCCCAGAGCC
130	140	150	160
* *	* *	* *	* *
CTGCTTAATG	GATGGAGACT	GGACGAGAAC	CTGGCTGCTG
170	180	190	200
* *	* *	* *	* *
TGGTTCTGAA	CATGGCCCAG	AGCCCTGTGT	CTGCCGAGGT
	M A Q	S P V	S A E V
210	220	230	240
* *	* *	* *	* *
CATTCACCAG	GTGGAAGAGT	GTCTTGATGA	AGACGAGAAG
I H Q	V E E	C L D E	D E K

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39/63 Forts. Fig. 4 c

250	260	270	280
* *	* *	* *	* *
GAGATGATGC	TCTTCCTGTG	TAGAGATGTG	ACTGAGAACC
E M M	L F L C	R D V	T E N
290	300	310	320
* *	* *	* *	* *
TGGCTGCACC	TAACGTCAGG	GACCTCCTGG	ATAGCTTAAG
L A A P	N V R	D L L	D S L S
330	340	350	360
* *	* *	* *	* *
TGAGAGAGGC	CAGCTCTCTT	TTGCTACCTT	GGCTGAATTG
E R G	Q L S	F A T L	A E L
370	380	390	400
* *	* *	* *	* *
CTCTACAGAG	TGAGGCGGTT	TGACCTTCTC	AAGAGGATCT
L Y R	V R R F	D L L	K R I
410	420	430	440
* *	* *	* *	* *
TGAAGACAGA	CAAAGCAACC	GTGGAGGACC	ACCTGCGCAG
L K T D	K A T	V E D	H L R R
450	460	470	480
* *	* *	* *	* *
AAACCCTCAC	CTGGTTTCTG	ATTATAGGGT	CCTGCTGATG
N P H	L V S	D Y R V	L L M

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40/63 Forks. Fig. 4c

490	500	510	520
* *	* *	* *	* *
GAGATTGGTG	AGAGCTTAGA	TCAGAACGAT	GTATCCTCCT
E I G	E S L D	Q N D	V S S
530	540	550	560
* *	* *	* *	* *
TAGTTTTCCT	TACAAGGGAT	TACACAGGCA	GAGGCAAGAT
L V F L	T R D	Y T G	R G K I
570	580	590	600
* *	* *	* *	* *
AGCCAAGGAC	AAGAGTTTCT	TGGATCTGGT	GATTGAATTG
A K D	K S F	L D L V	I E L
610	620	630	640
* *	* *	* *	* *
GAGAAACTGA	ATCTAATTGC	TTCAGACCAA	TTGAATTTGT
E K L	N L I A	S D Q	L N L
650	660	670	680
* *	* *	* *	* *
TAGAAAAATG	CCTGAAGAAC	ATCCACAGAA	TAGACTTGAA
L E K C	L K N	I H R	I D L N
690	700	710	720
* *	* *	* *	* *
CACAAAGATC	CAGAAGTACA	CCCAGTCCAG	CCAAGGAGCA
T K I	Q K Y	T Q S S	Q G A

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41/63 Forrs. Fig. 4c

730	740	750	760
* *	* *	* *	* *
AGATCAAATA	TGAATACTCT	CCAGGCTTCG	CTCCCAAAT
R S N	M N T L	Q A S	L P K
770	780	790	800
* *	* *	* *	* *
TGAGTATCAA	GTATAACTCA	AGGCTCCAGA	ATGGGCGAAG
L S I K	Y N S	R L Q	N G R S
810	820	830	840
* *	* *	* *	* *
TAAAGAGCCA	AGATTTGTGG	AATACCGTGA	CAGTCAAAGA
K E P	R F V	E Y R D	S Q R
850	860	870	880
* *	* *	* *	* *
ACACTGGTGA	AGACATCCAT	CCAGGAATCA	GGAGCTTTTT
T L V	K T S I	Q E S	G A F
890	900	910	920
* *	* *	* *	* *
TACCTCCGCA	CATCCGTGAA	GAGACTTACA	GGATGCAGAG
L P P H	I R E	E T Y	R M Q S
930	940	950	960
* *	* *	* *	* *
CAAGCCCCTA	GGAATCTGCT	TGATCATTGA	TTGTATTGGC
K P L	G I C	L I I D	C I G

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42/63 Forts. Fig. 4c

970	980	990	1000
* *	* *	* *	* *
AACGACACAA	AATATCTTCA	AGAGACCTTC	ACTTCCCTGG
N D T	K Y L Q	E T F	T S L
1010	1020	1030	1040
* *	* *	* *	* *
GCTATCATAT	CCAGCTTTTC	TTGTTTCCCA	AGTCACATGA
G Y H I	Q L F	L F P	K S H D
1050	1060	1070	1080
* *	* *	* *	* *
CATAACCCAG	ATTGTTCGCC	GATATGCAAG	TATGGCCCAA
I T Q	I V R	R Y A S	M A Q
1090	1100	1110	1120
* *	* *	* *	* *
CATCAAGACT	ATGACAGCTT	TGCATGTGTT	CTGGTGAGCC
H Q D	Y D S F	A C V	L V S
1130	1140	1150	1160
* *	* *	* *	* *
TAGGAGGCTC	CCAAAGCATG	ATGGGCAGAG	ATCAAGTTCA
L G G S	Q S M	M G R	D Q V H
1170	1180	1190	1200
* *	* *	* *	* *
CTCAGGGTTC	TCCTTGGATC	ATGTCAAGAA	CATGTTCACG
S G F	S L D	H V K N	M F T

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43/63 Foris. Fig. 4c

1210	1220	1230	1240
* *	* *	* *	* *
GGGGACACGT	GCCCTTCTCT	CAGAGGGAAG	CCAAAGCTCT
G D T	C P S L	R G K	P K L
1250	1260	1270	1280
* *	* *	* *	* *
TTTTTATTCA	GAACTATGAG	TCGTTAGGTA	GCCAGTTGGA
F F I Q	N Y E	S L G	S Q L E
1290	1300	1310	1320
* *	* *	* *	* *
AGATAGCAGC	CTGGAGGTAG	ATGGGCCATC	AATAAAAAAT
D S S	L E V	D G P S	I K N
1330	1340	1350	1360
* *	* *	* *	* *
GTGGACTCTA	AGCCCCTGCA	ACCCAGACAC	TGCACAACCTC
V D S	K P L Q	P R H	C T T
1370	1380	1390	1400
* *	* *	* *	* *
ACCCAGAAGC	TGATATCTTT	TGGAGCCTGT	GCACAGCAGA
H P E A	D I F	W S L	C T A D
1410	1420	1430	1440
* *	* *	* *	* *
CGTATCTCAC	TTGGAGAAGC	CCTCCAGCTC	ATCCTCTGTG
V S H	L E K	P S S S	S S V

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44/63 Forts. Fig. 4c

1450	1460	1470	1480
* *	* *	* *	* *
TATCTGCAGA	AGCTCTCCCA	GCAGCTGAAG	CAAGGCAGGA
Y L Q	K L S Q	Q L K	Q G R
1490	1500	1510	1520
* *	* *	* *	* *
GACGCCCACT	CGTGGACCTC	CACGTTGAAC	TCATGGACAA
R R P L	V D L	H V E	L M D K
1530	1540	1550	1560
* *	* *	* *	* *
AGTGTATGCG	TGGAACAGTG	GTGTTTCGTC	TAAGGAGAAA
V Y A	W N S	G V S S	K E K
1570	1580	1590	1600
* *	* *	* *	* *
TACAGCCTCA	GCCTGCAGCA	CACTCTGAGG	AAGAAACTCA
Y S L	S L Q H	T L R	K K L
1610	1620	1630	1640
* *	* *	* *	* *
TCCTGGCTCC	TACGTGAGAA	CCCCAGACCG	TTGGTGTTCT
I L A P	T>		
1650	1660	1670	1680
* *	* *	* *	* *
TGGTATATCA	TCCAGGGTGG	CGGCTTGGAG	CAGAGCTTGG

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45/63 Forts. Fig. 4c

1690	1700	1710	1720
* *	* *	* *	* *
CGGTTACGGC	TGCTTCTGGC	TGCTTCTGGC	TCTGCCGTGA
1730	1740	1750	1760
* *	* *	* *	* *
GTCCTGGCCT	AGGGTTCTCC	TGTGCACAGG	CATGAGCCGT
1770	1780	1790	1800
* *	* *	* *	* *
AACCCTGTGC	CTGGGAAACG	TCTCACTCCC	GCCGCCGTGC
1810	1820	1830	1840
* *	* *	* *	* *
CTTTACCTCT	CTAAACTTCC	CTACTTACAT	TCCTTAGTCG
1850	1860	1870	1880
* *	* *	* *	* *
GATGTTTTC	CAGAGTGTGG	AGAACAGTAA	GACATAAACC
1890	1900	1910	1920
* *	* *	* *	* *
TATTGTTTGT	TTGTTTTTTT	GGGGGGGAGG	TTATCTACCA
1930	1940	1950	1960
* *	* *	* *	* *
AGTTATACCA	AGTTATTGTA	TGGGTGTATA	GTGTATAGTG

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46/63 Forts. Fig. 4c

1970	1980	1990	2000
* *	* *	* *	* *
GTTCAAGATT	TGACACTGAA	TGTAACCTGA	GACTTACCTG
2010	2020	2030	2040
* *	* *	* *	* *
AGTTTGTCAT	GCGACTGGGT	AAATTGTTTC	TATGGCACAT
2050	2060	2070	2080
* *	* *	* *	* *
CTAATCATTT	AATAAGTAAT	TACCTCATTA	AGTACCCATT
2090	2100	2110	2120
* *	* *	* *	* *
GCTTCAGGAC	TTTCACATTG	GCCACCAATT	TCTGTGACCC
2130	2140	2150	2160
* *	* *	* *	* *
AGCTCCACAT	TTATATTCTC	TTTCTGCAAA	ACCAAATTTC
2170	2180	2190	2200
* *	* *	* *	* *
ATTATGTCTG	TTTAATATCT	ACAGTCTAAT	GCTTTGTAAG
2210	2220	2230	2240
* *	* *	* *	* *
ACATCTAGAT	AGAAAAATAG	TTACCCATGA	GCACAGAAGG

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2250 2260 ^{47/63} 2270 ^{Forts. Fig. 4c} 2280
 * * * * * *
 GCTGGCCTGA CCCTCACCAG CTGTGCAGTG GCTTCGGTGA

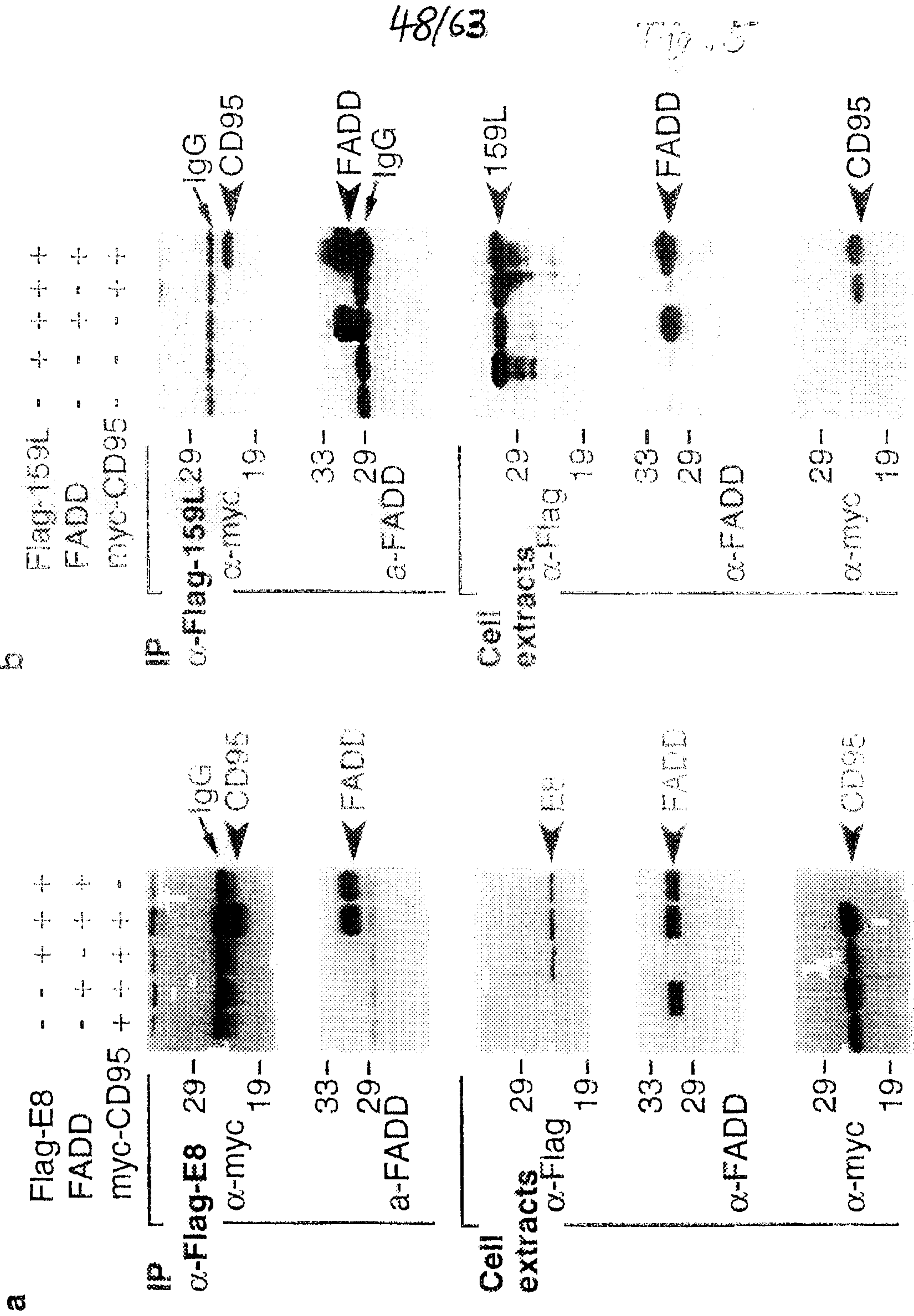
2290 2300 2310 2320
 * * * * * *
 AGGAGAATGA GCCCTACTCC TTGAAGGTTG TAGTGCTTGG

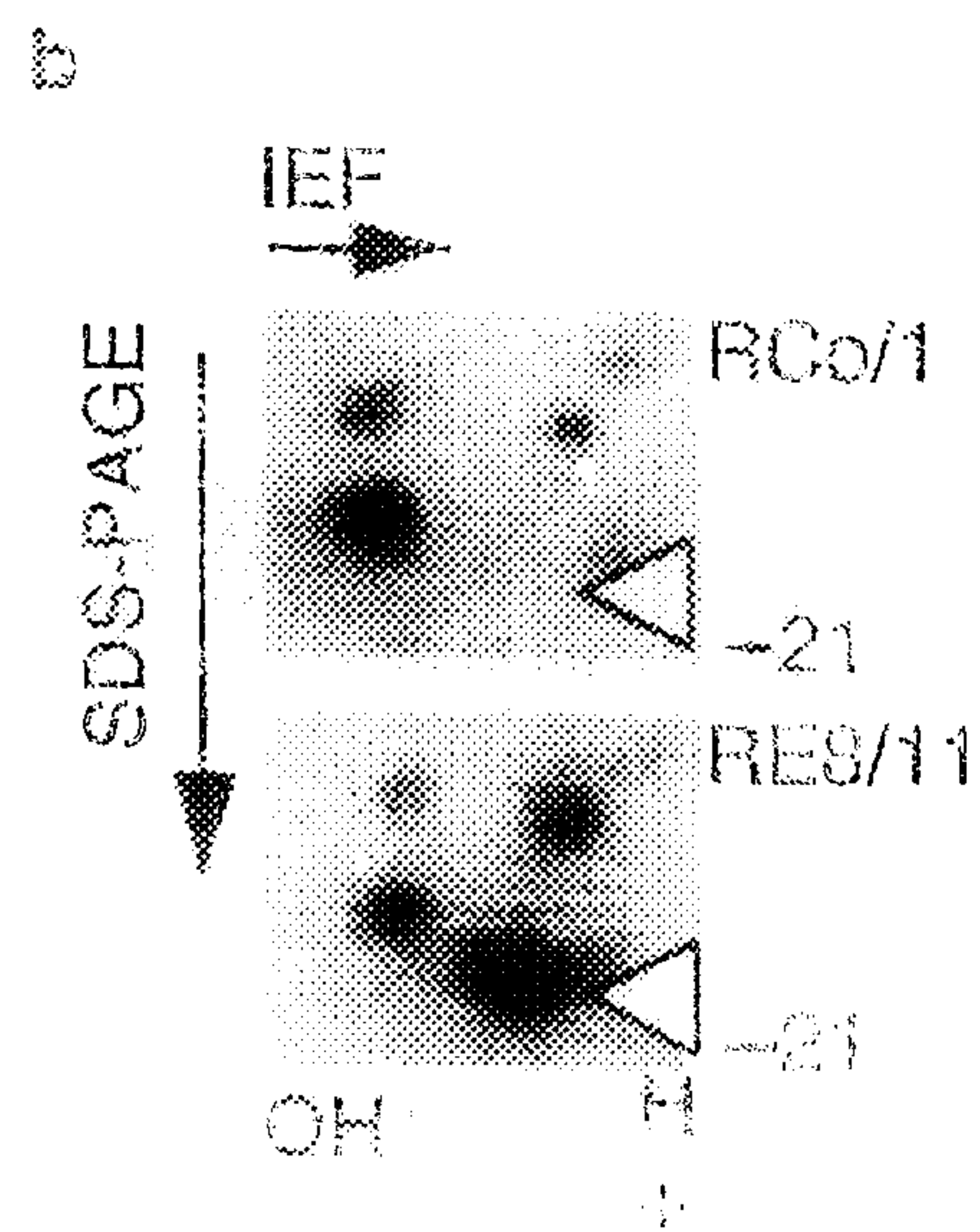
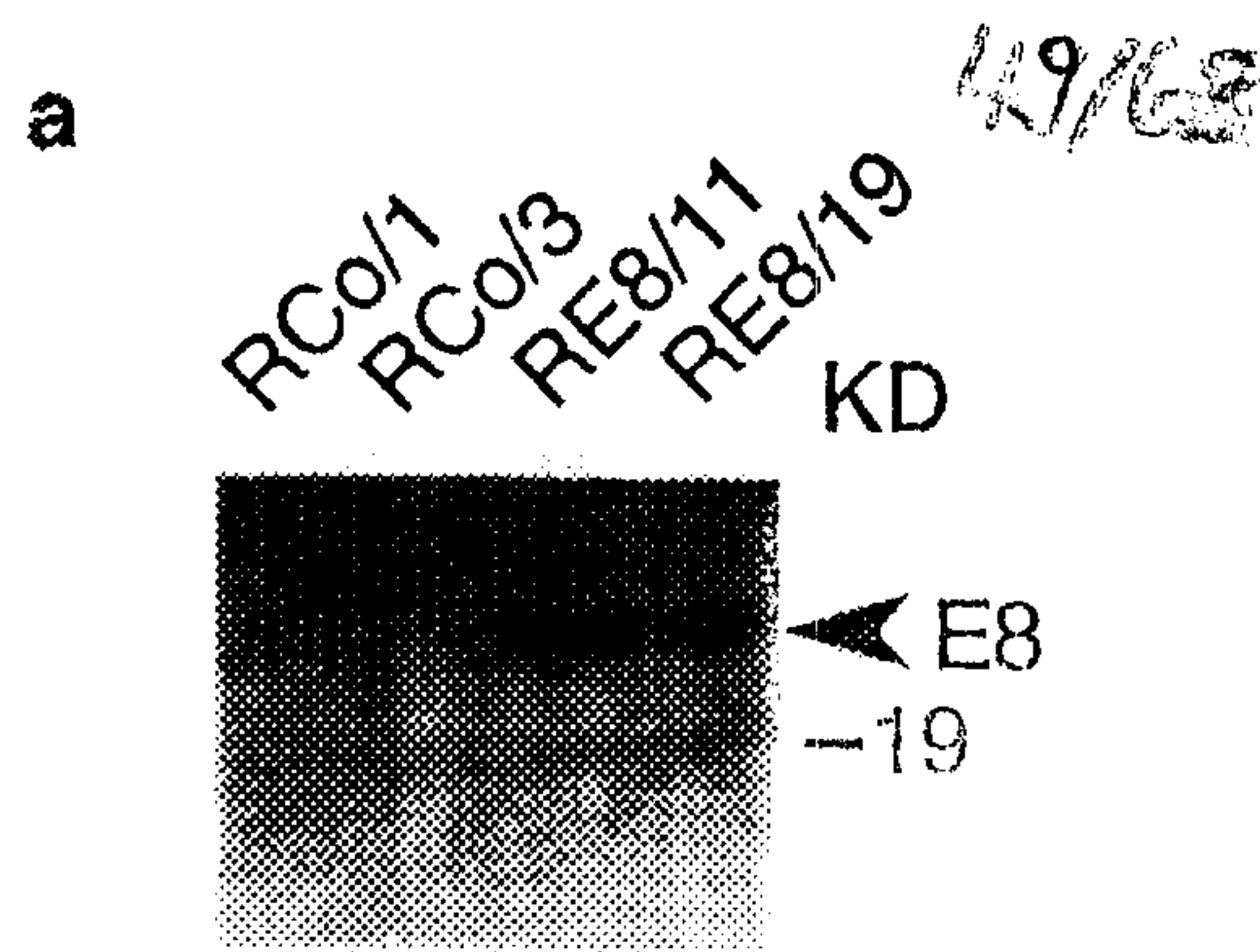
2330 2340 2350 2360
 * * * * * *
 GAGAGCAGTC TGTACCTTGC CTGGGCAGCA CAGTAGAGCC

2370 2380 2390 2400
 * * * * * *
 AGCCCCAAGA ACACAACAGT GAGTGGGGGA GCTTGCCCTG

2410 2420 2430 2440
 * * * * * *
 GTTGGCTCAG GATCAGGAAC AGGAGGGATG ACGGAATTCC

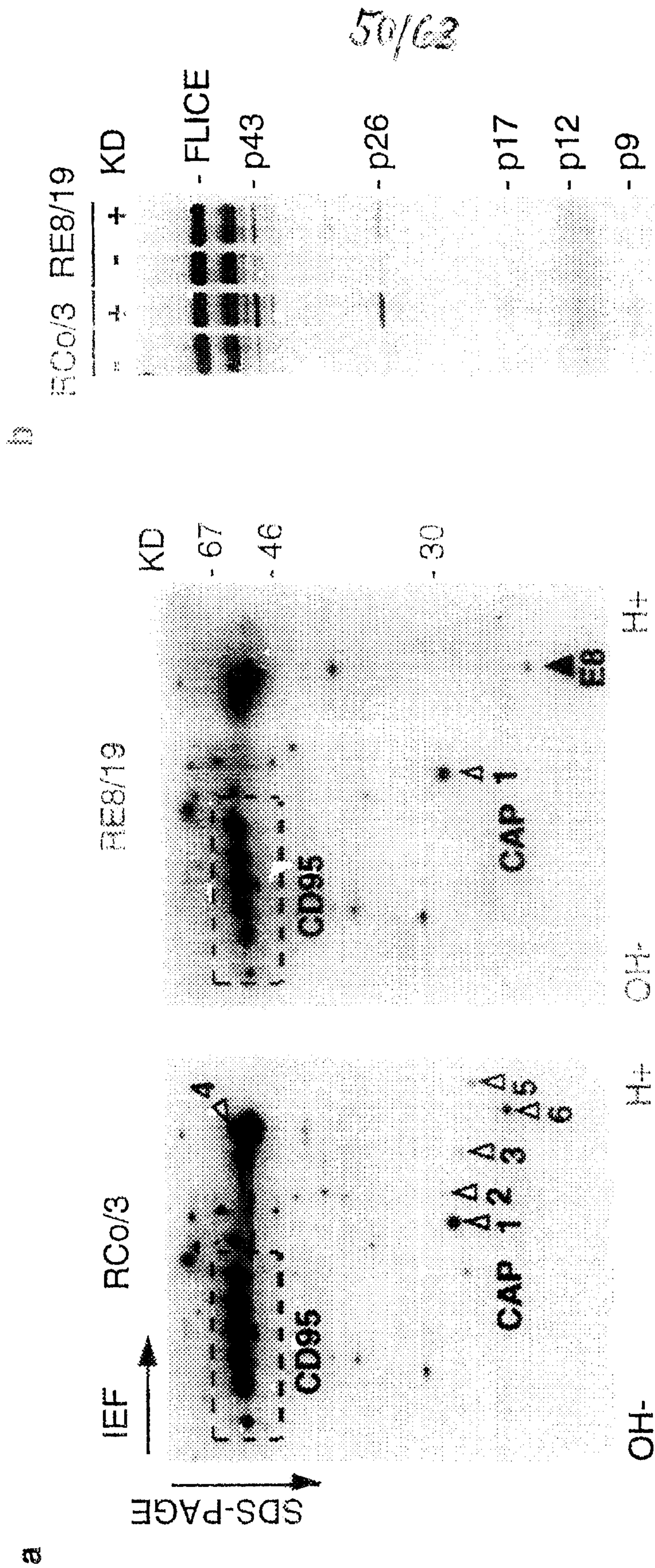
2450
 * *
 TGCAGCCCGG GG





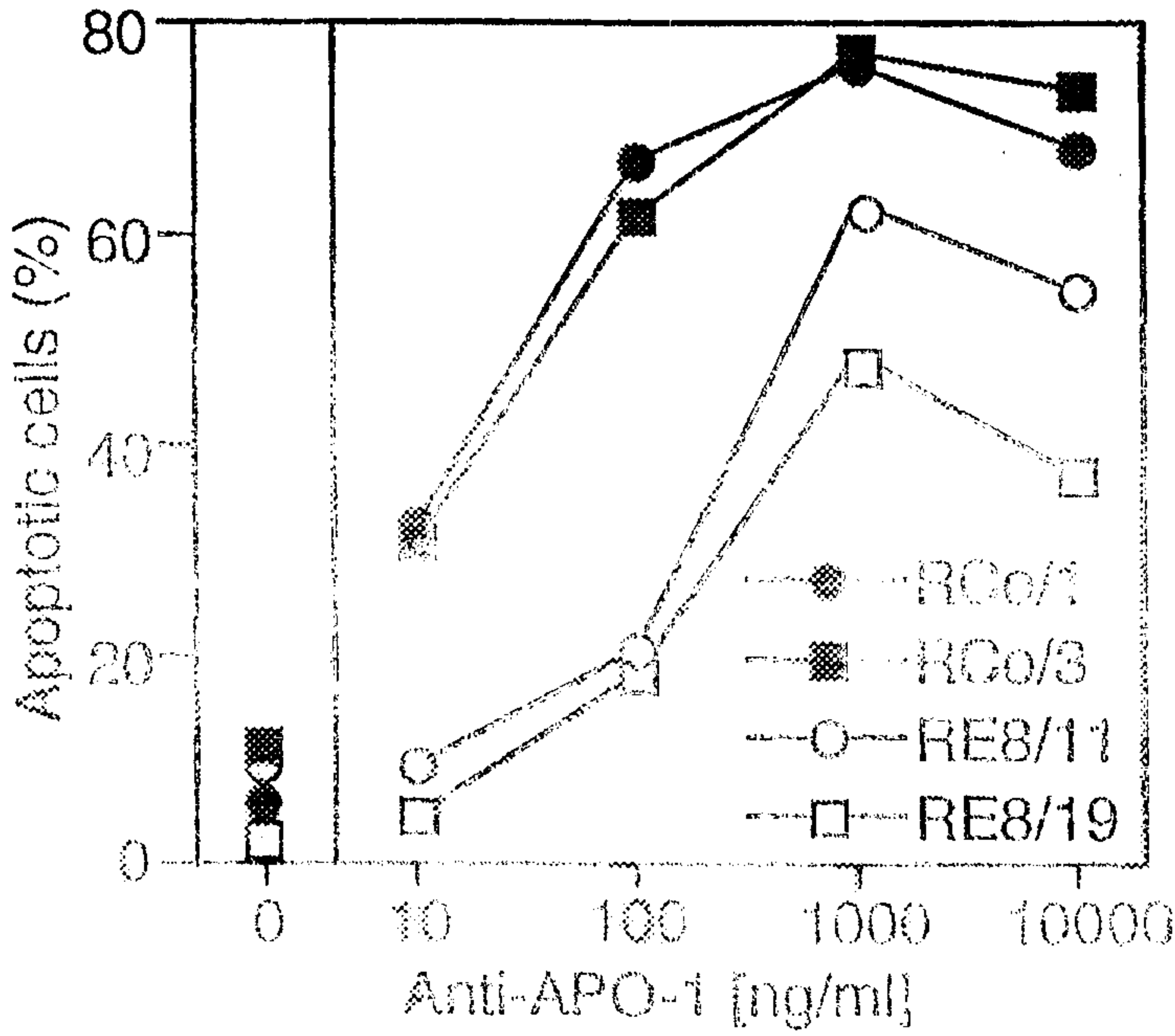
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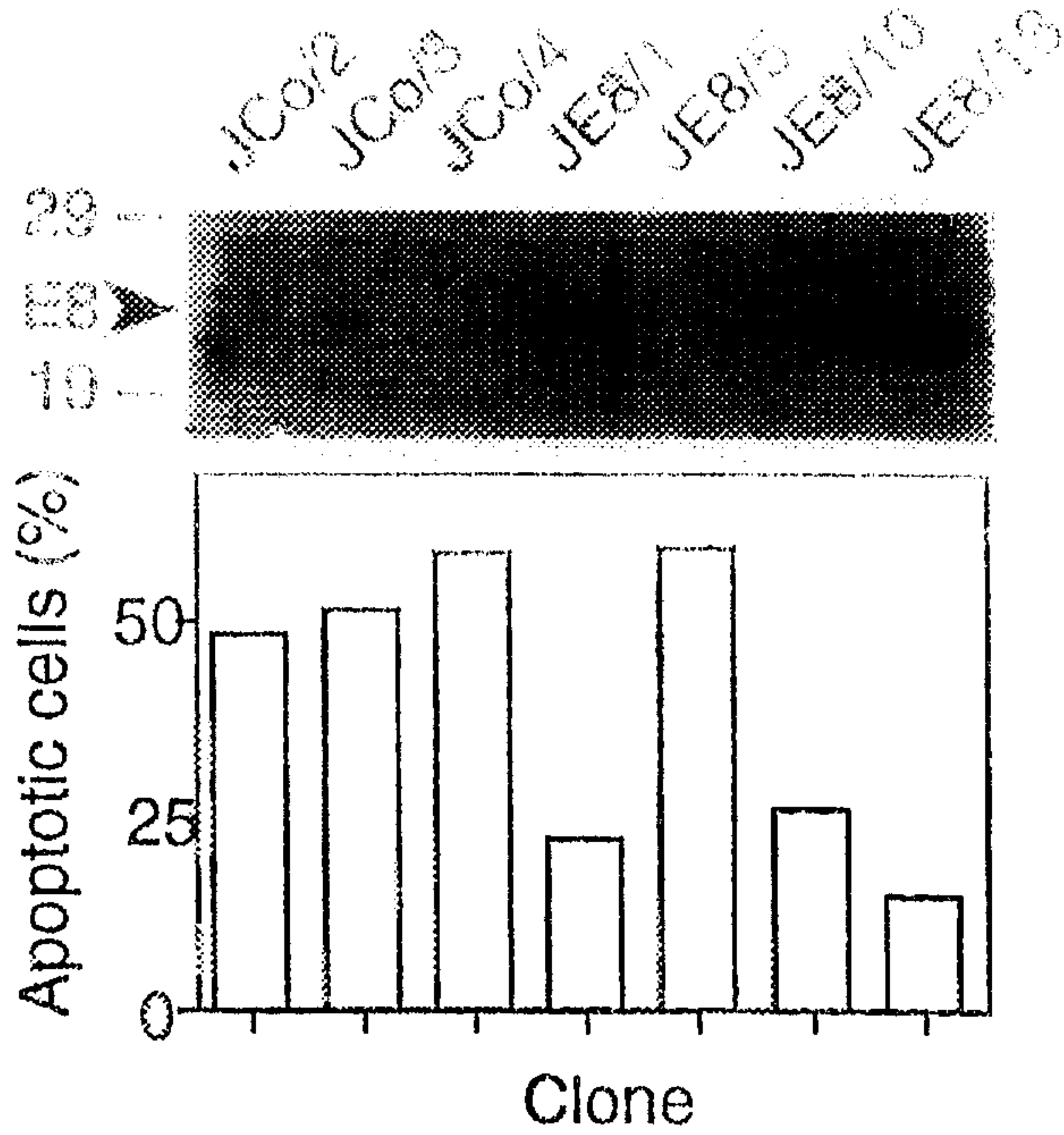


SI/62 Fig. 8

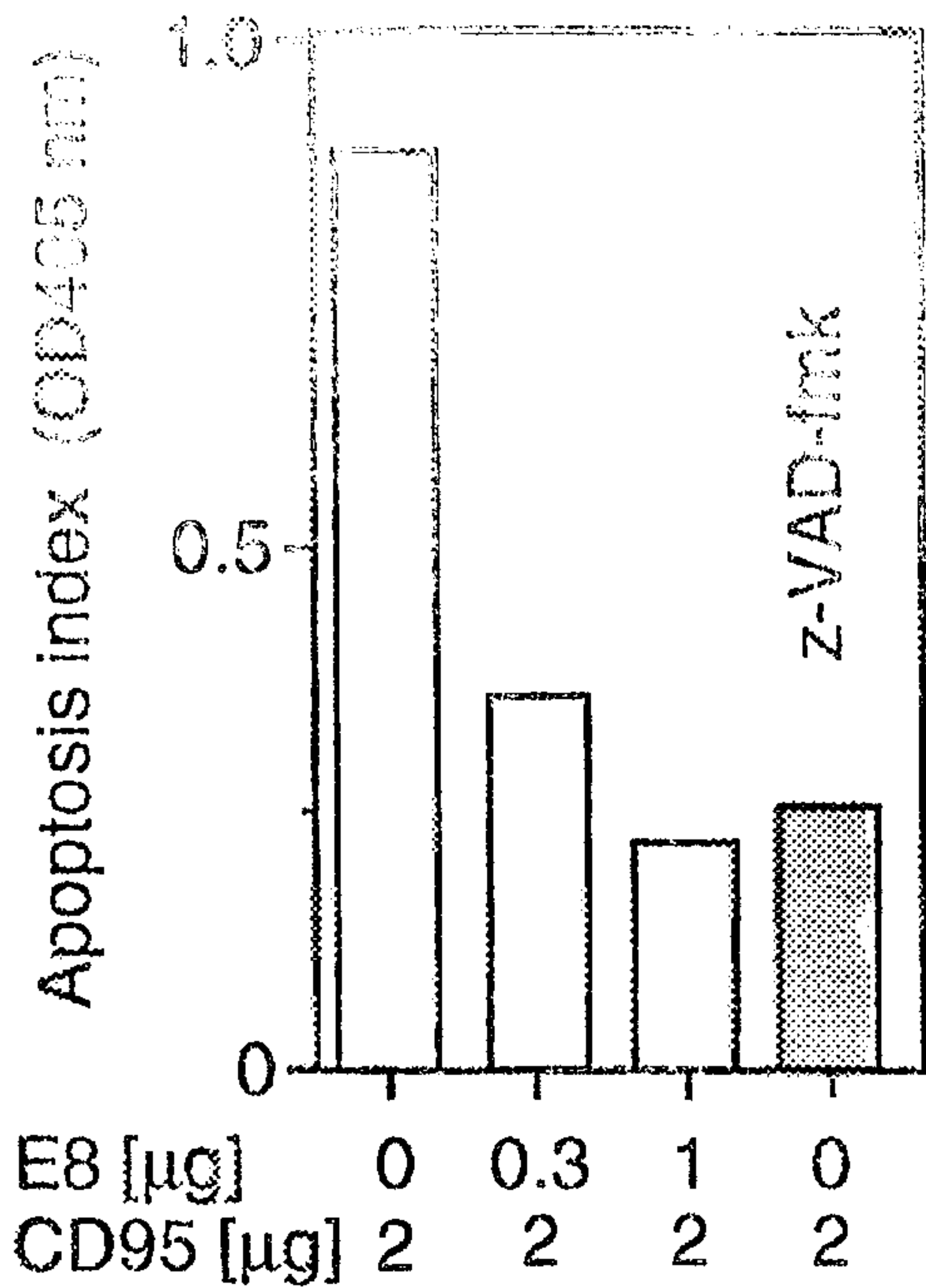
a



b



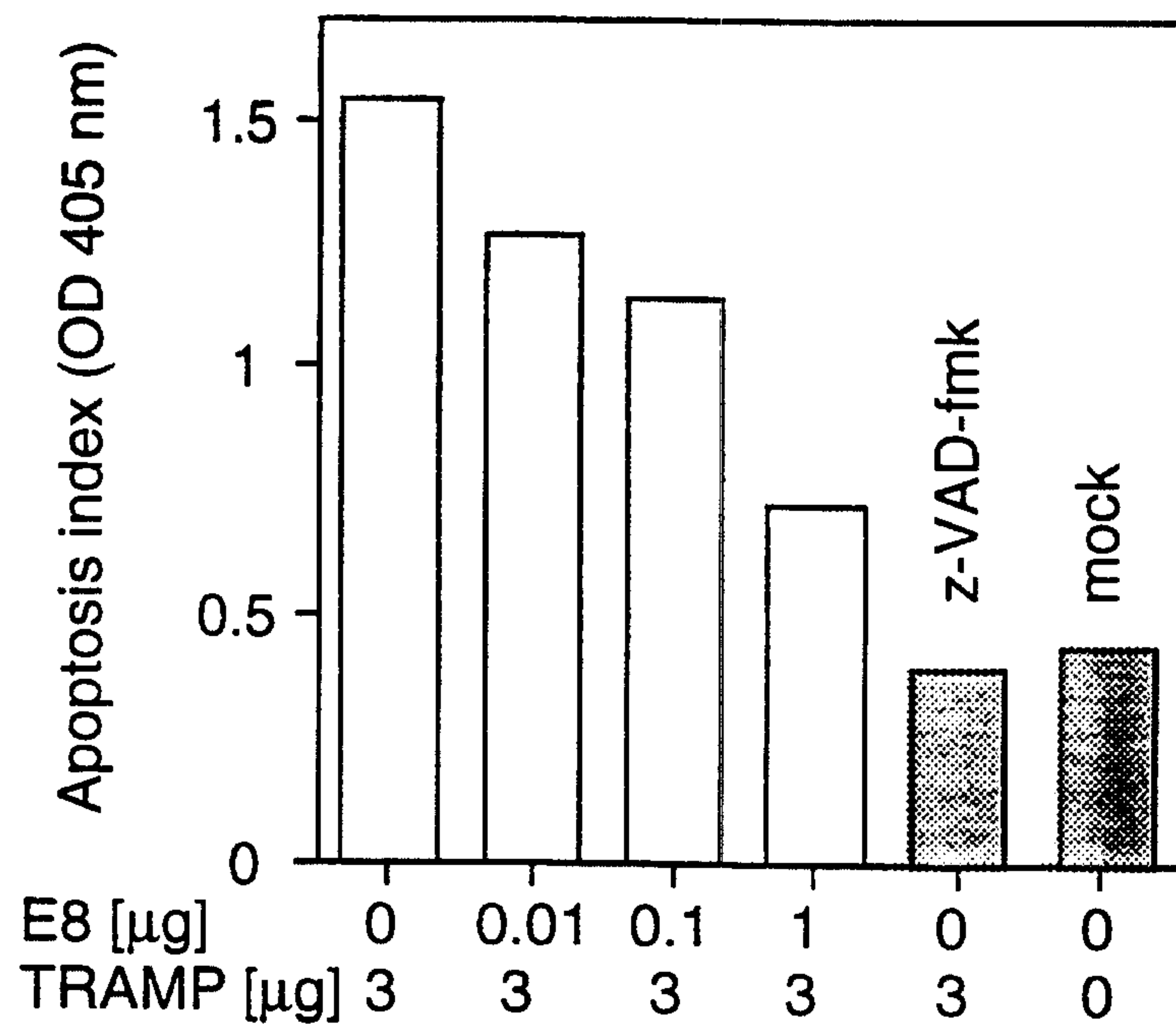
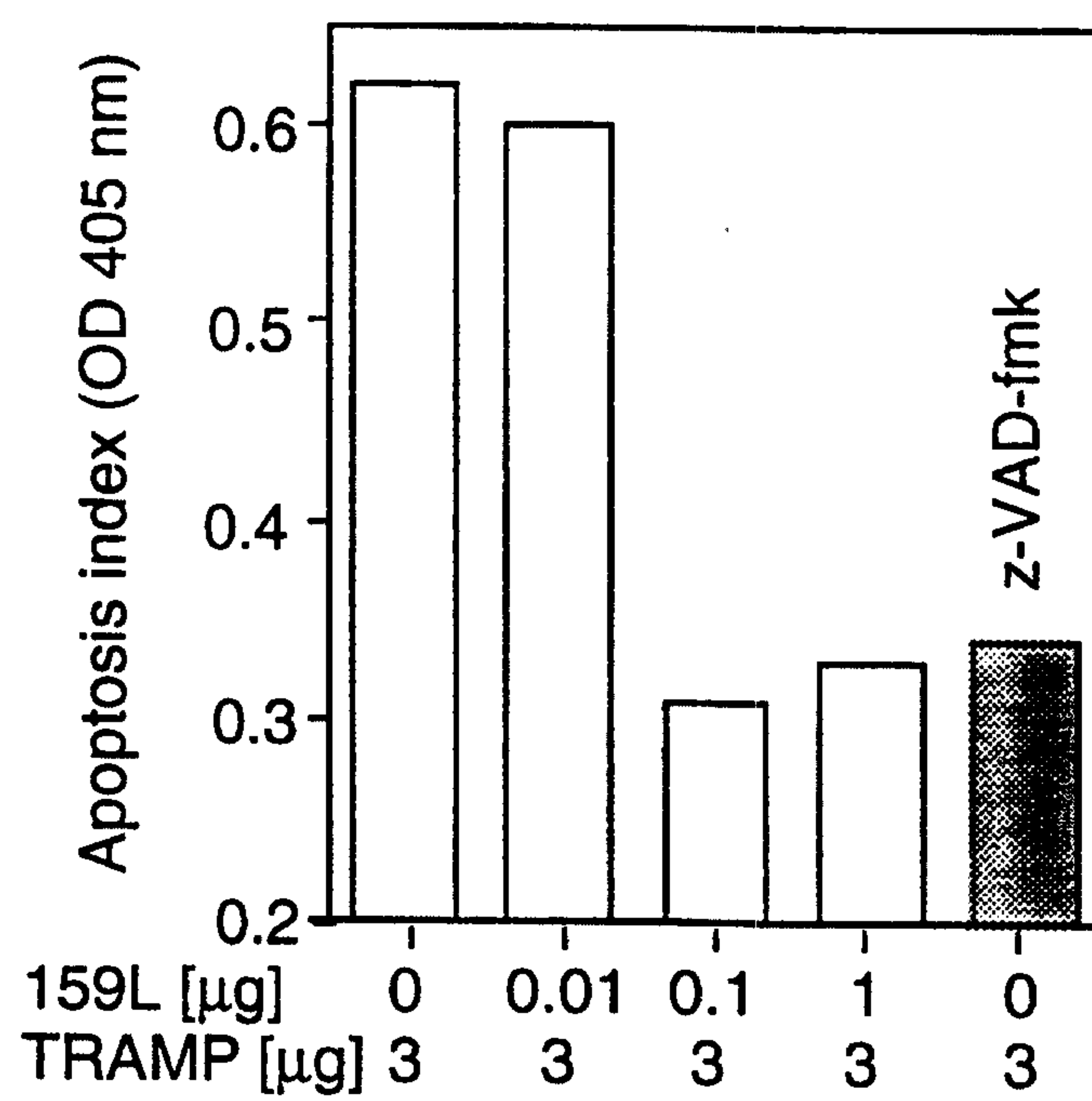
c



a

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Fig 3

**b**

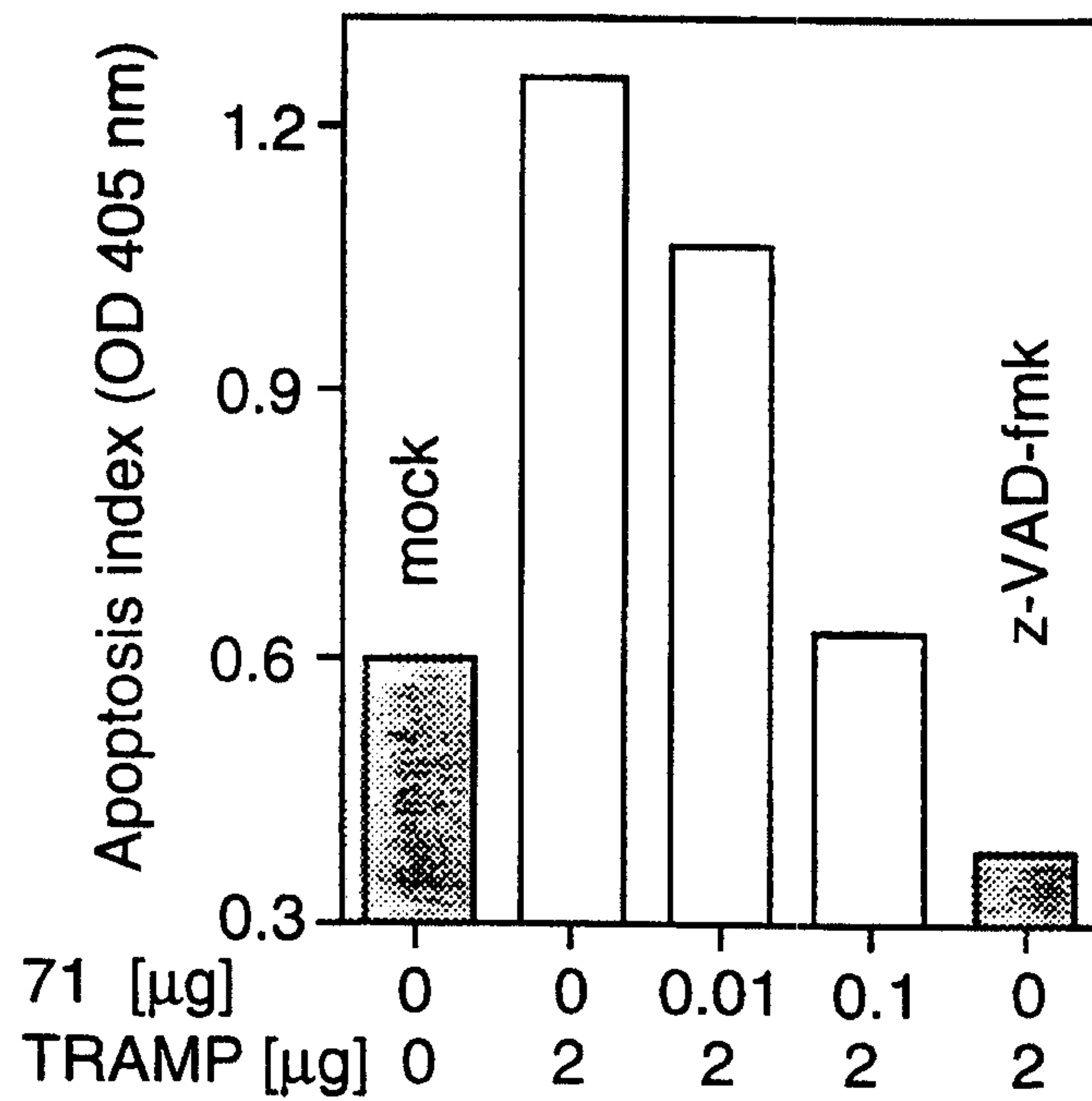
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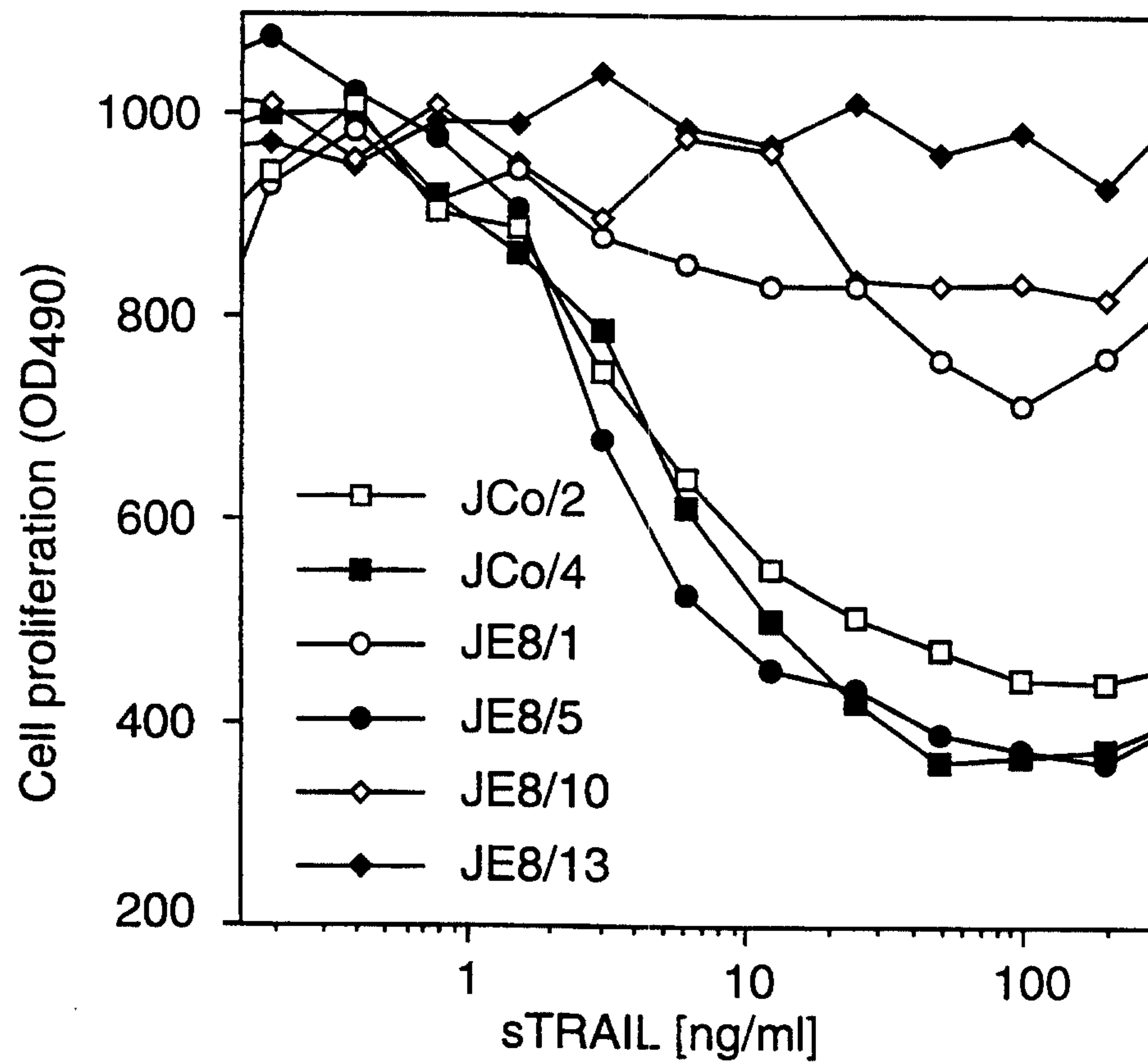
Forts. Fig. 9

c



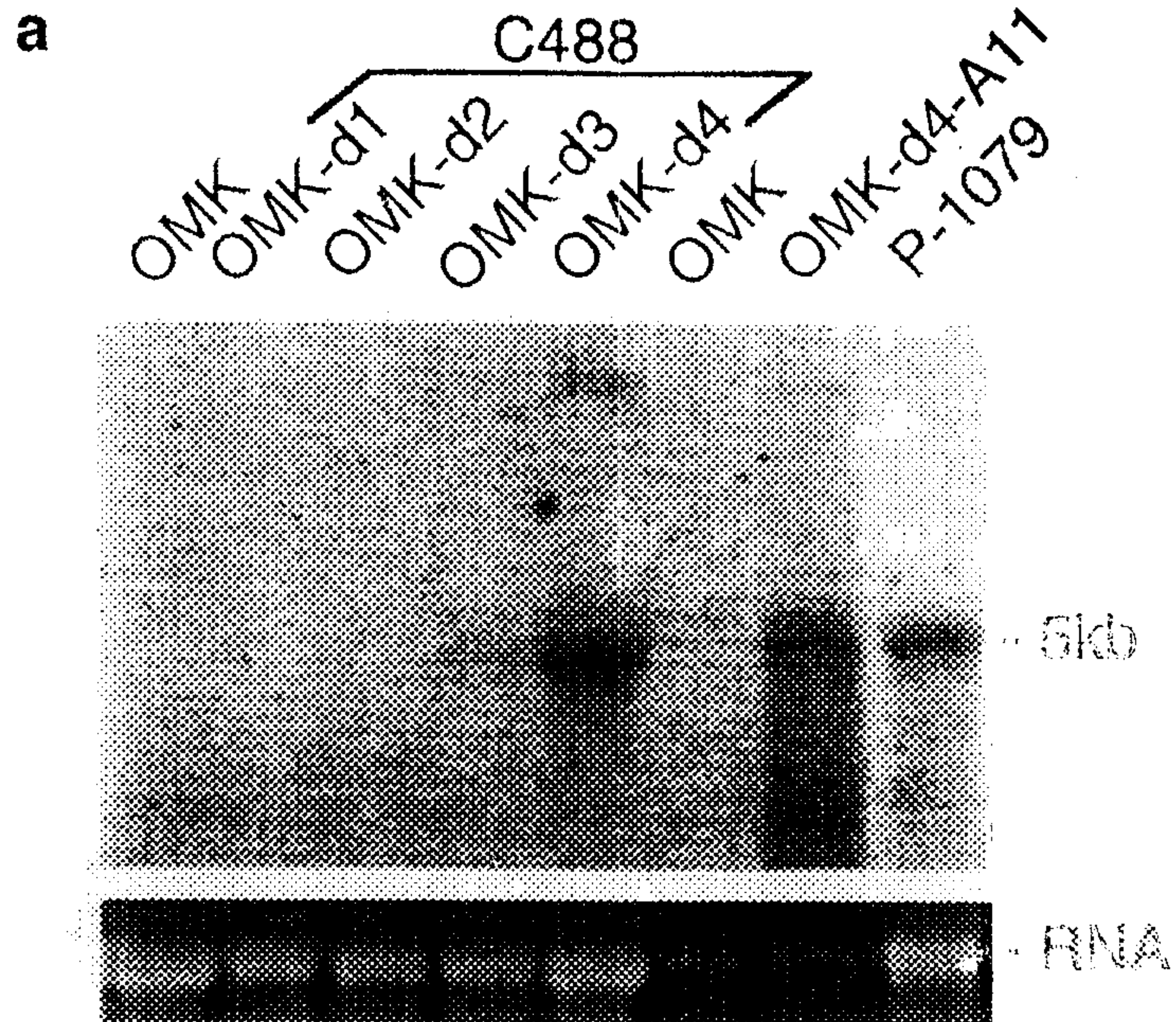
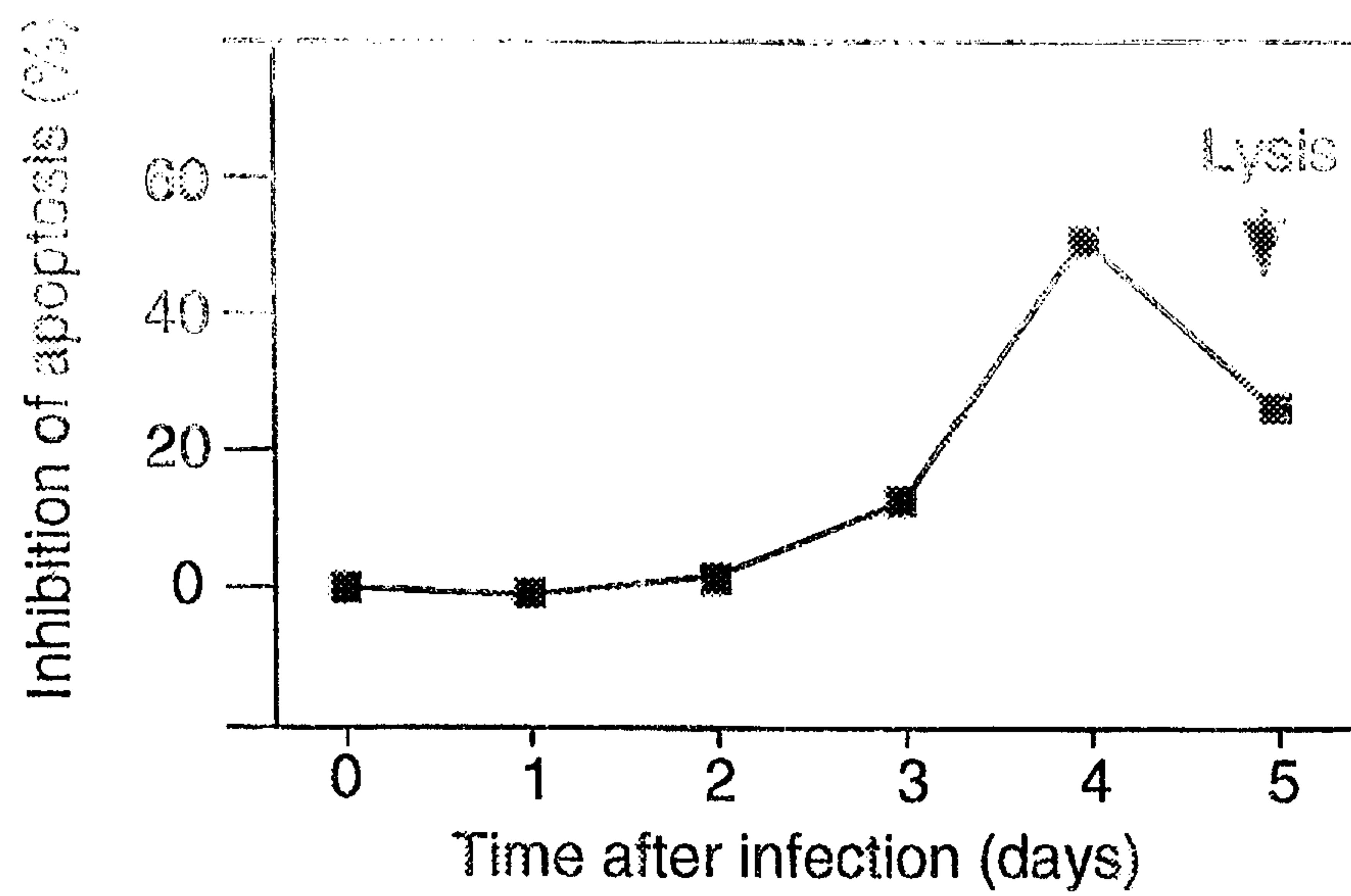
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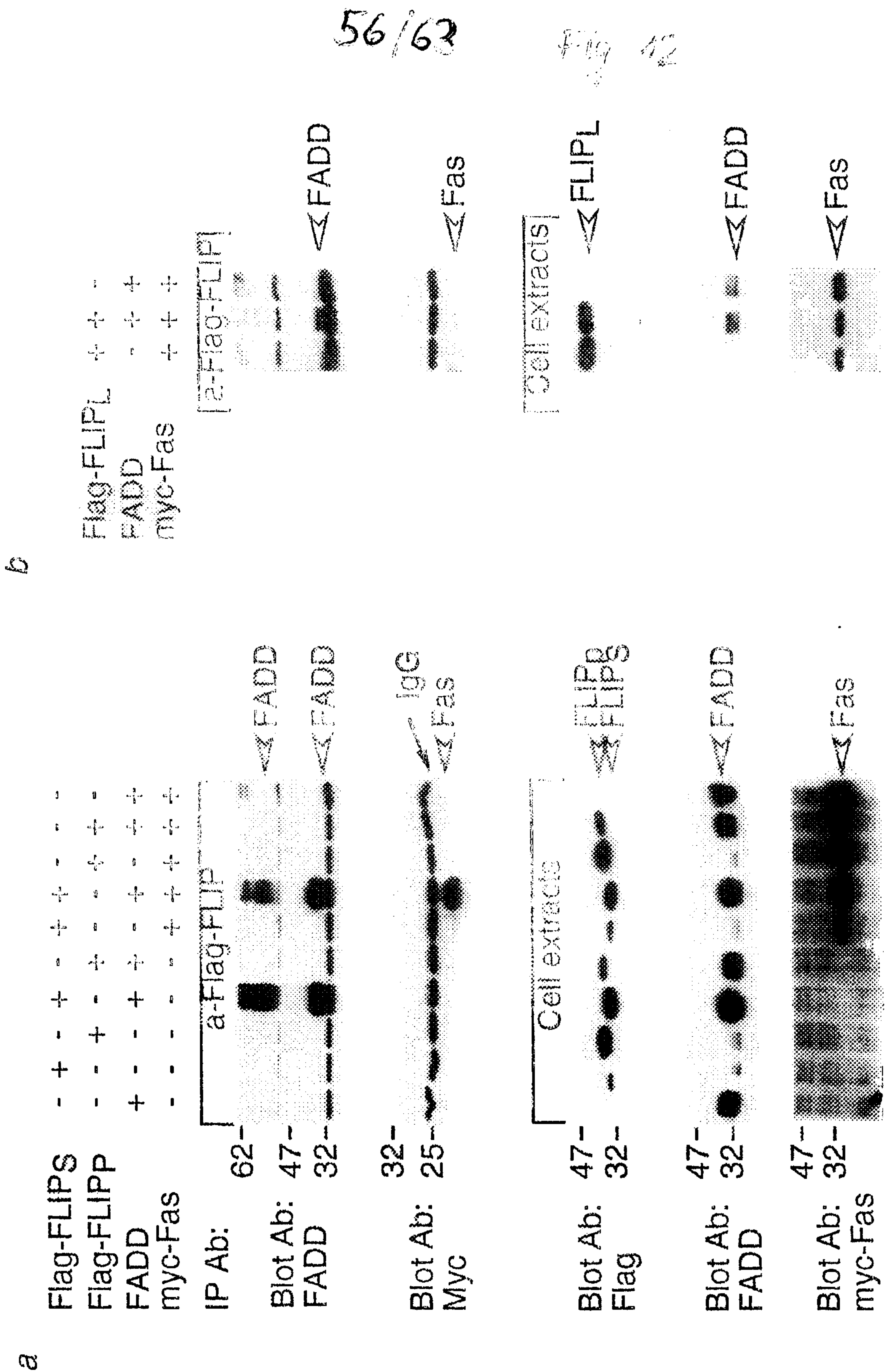
Fig. 10



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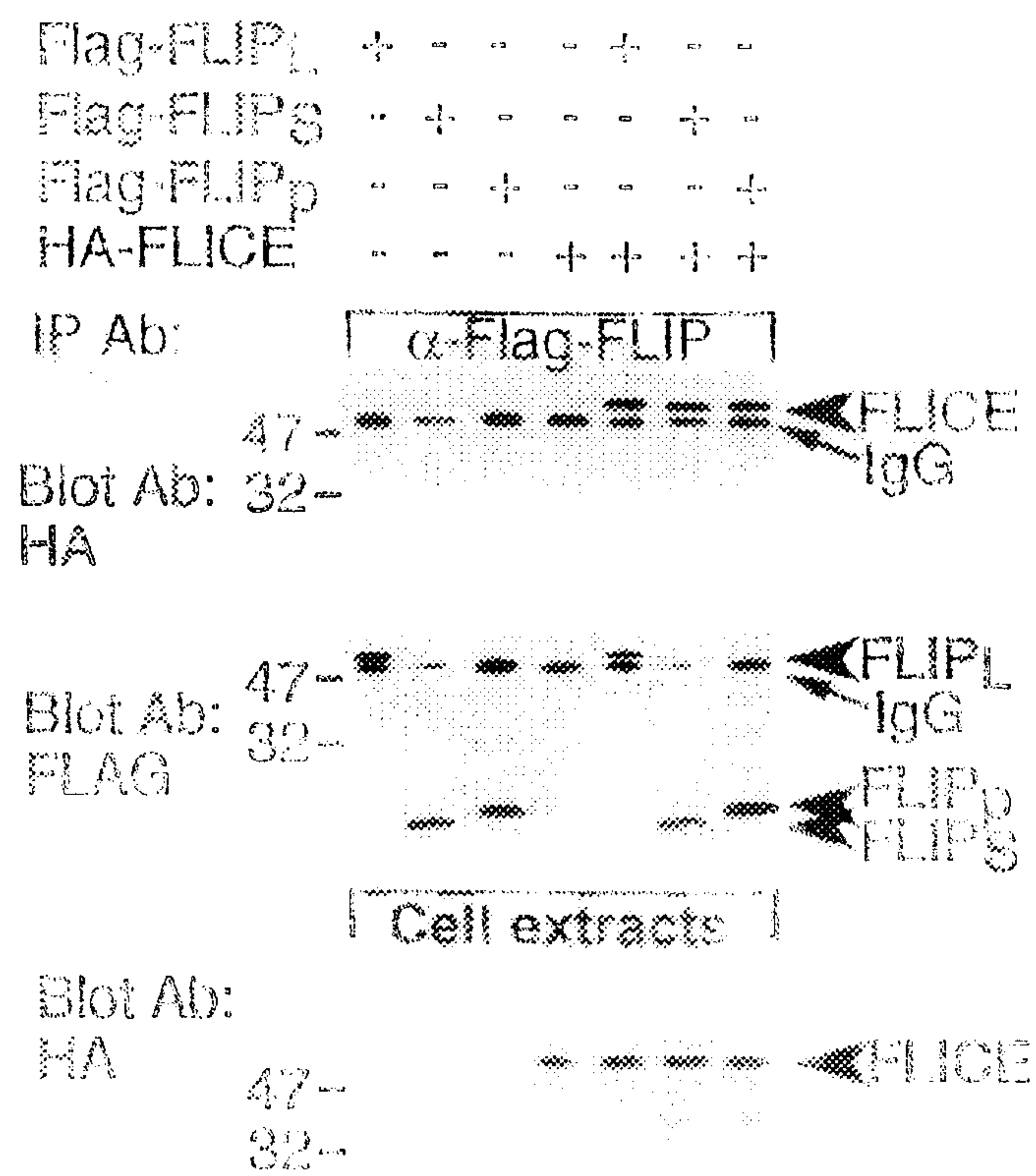
Fig. 11

**b**



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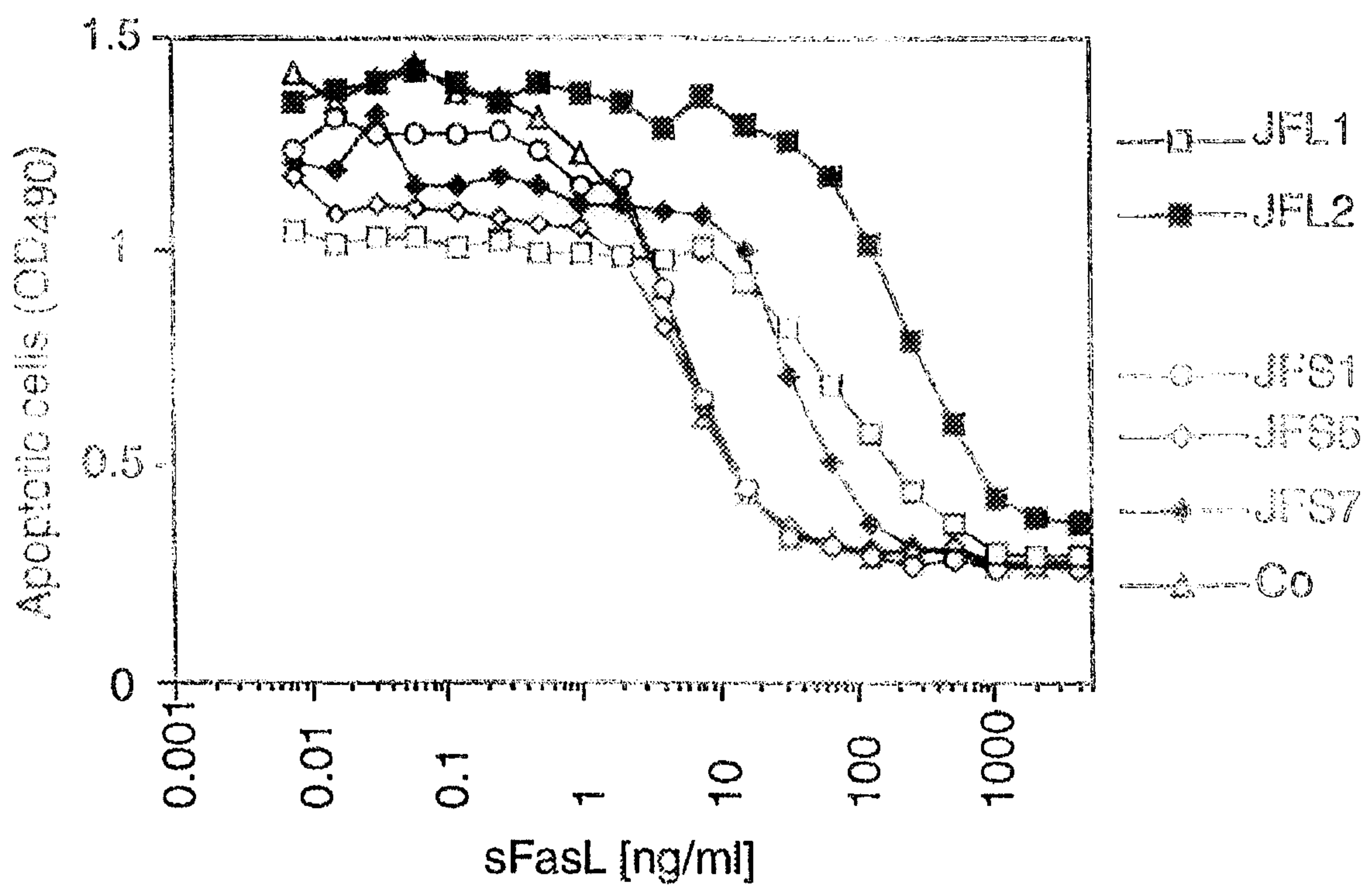
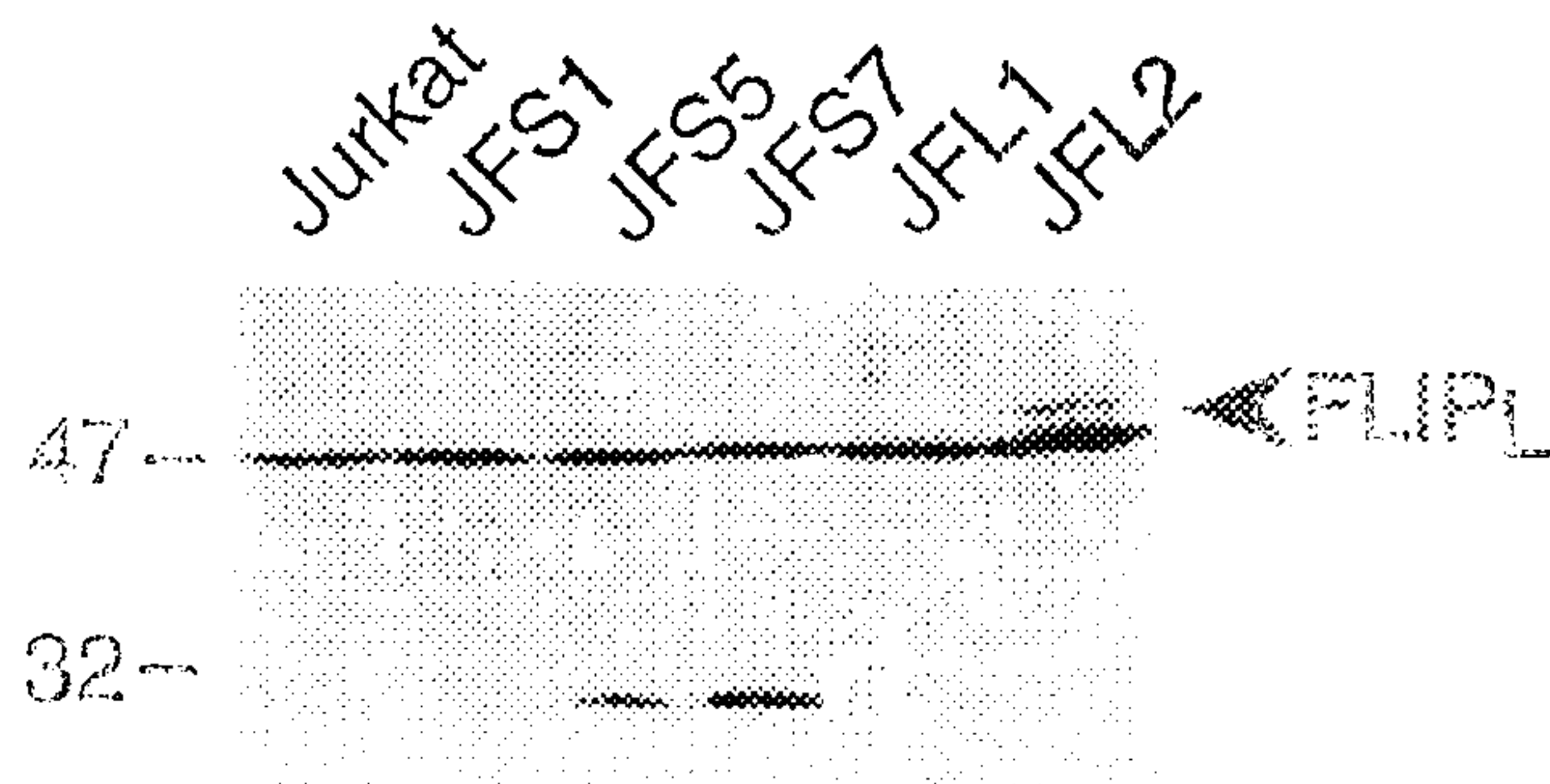
Fig. 43



a

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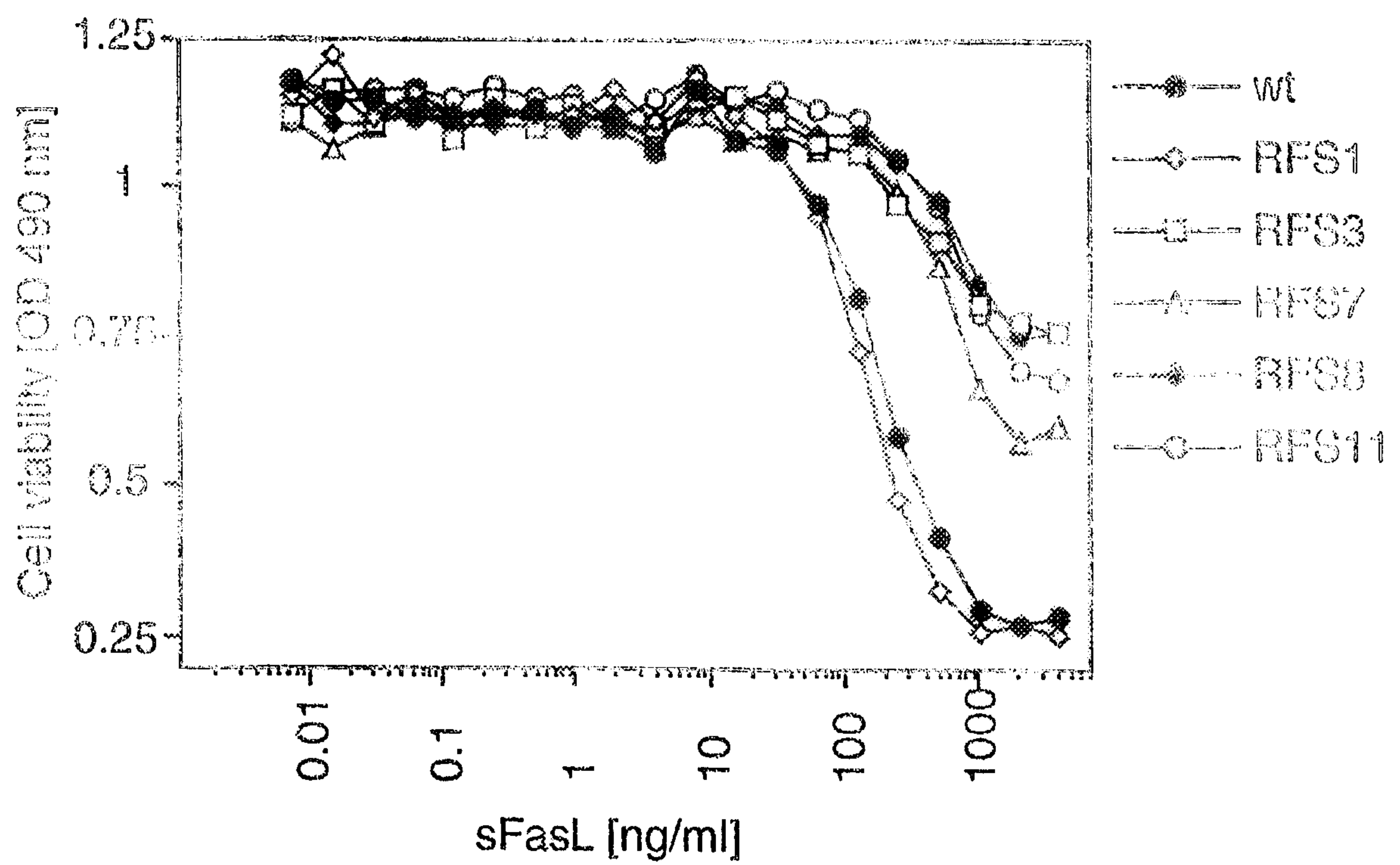
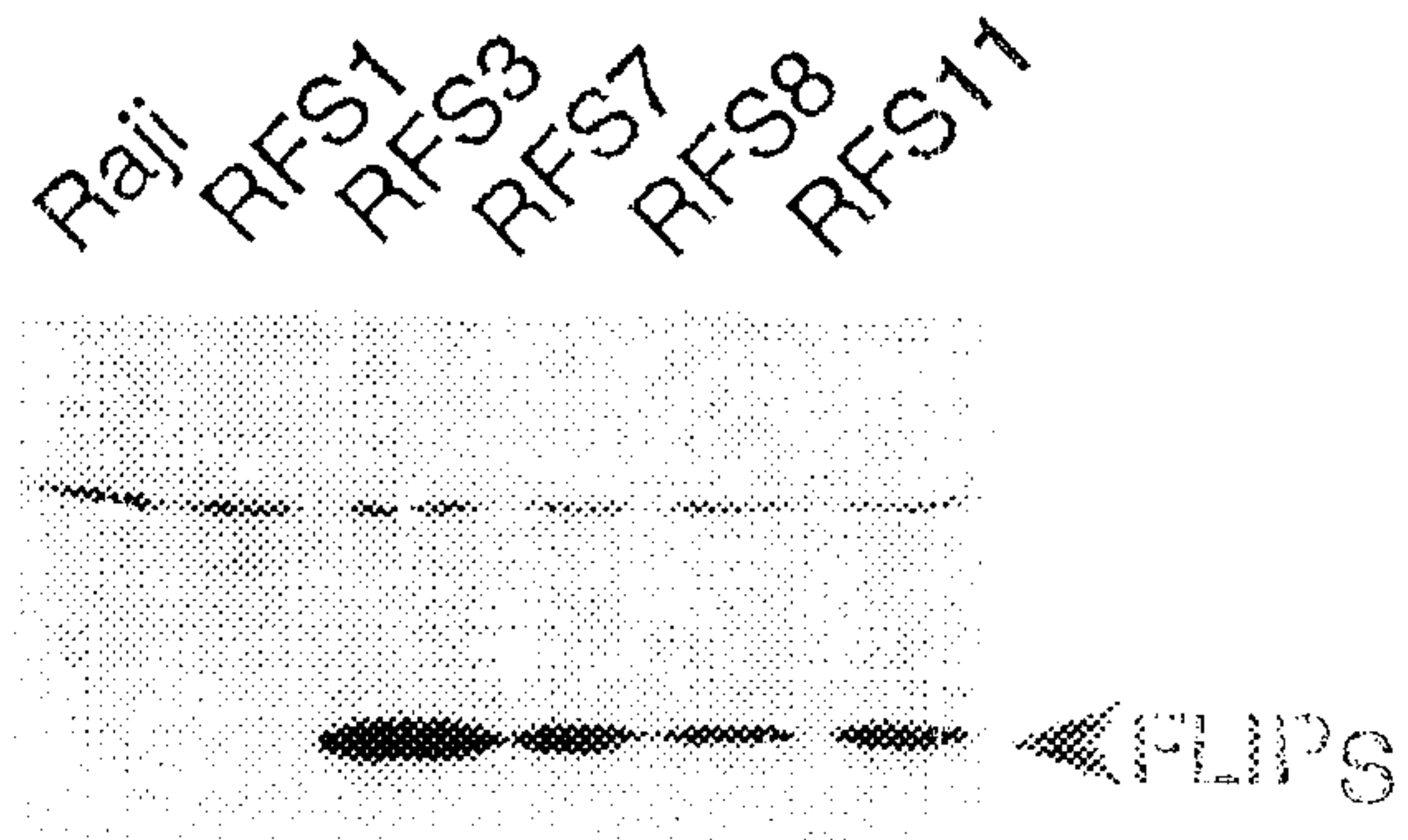
Fig. 14a



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Fig. 4b

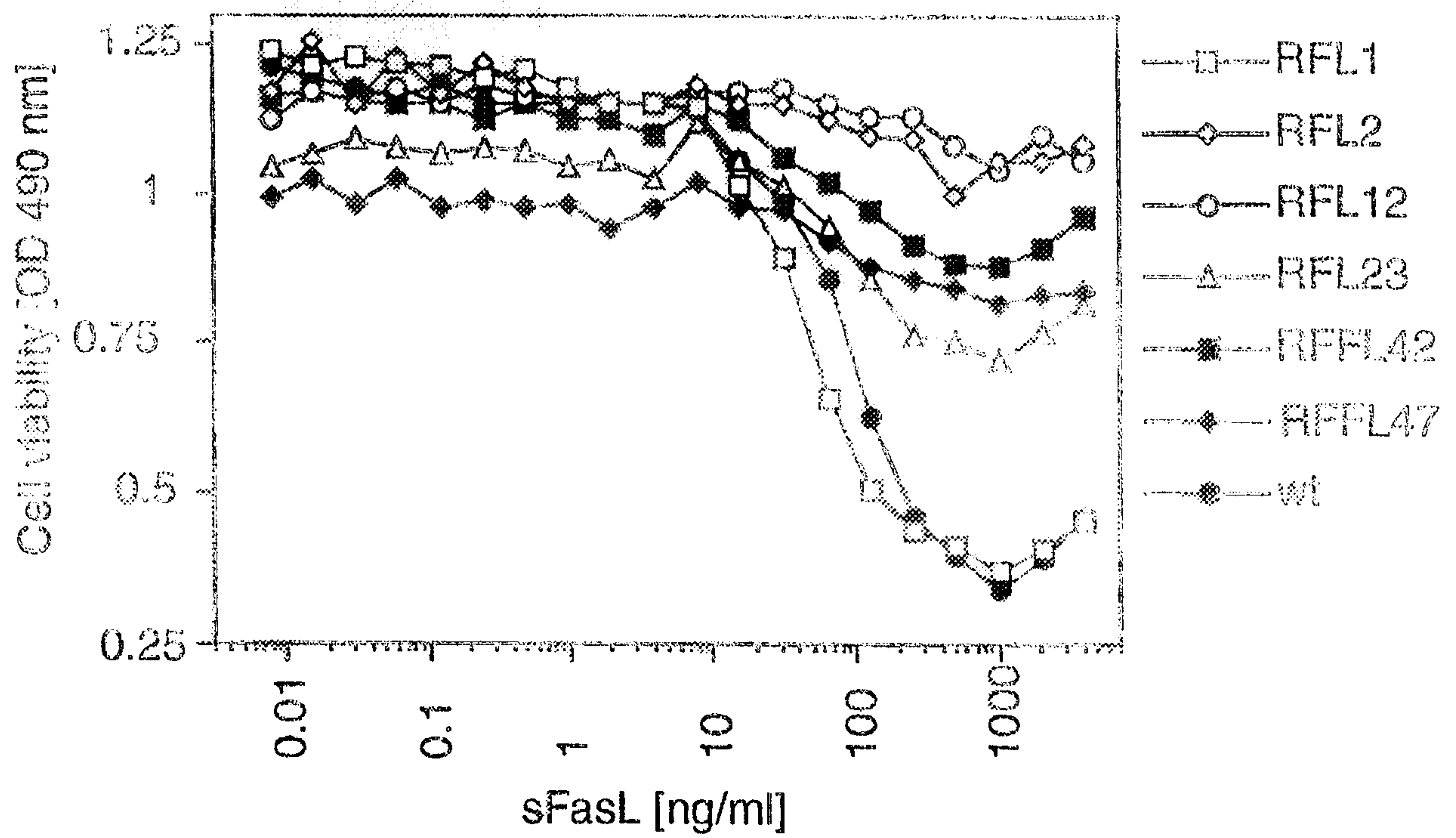
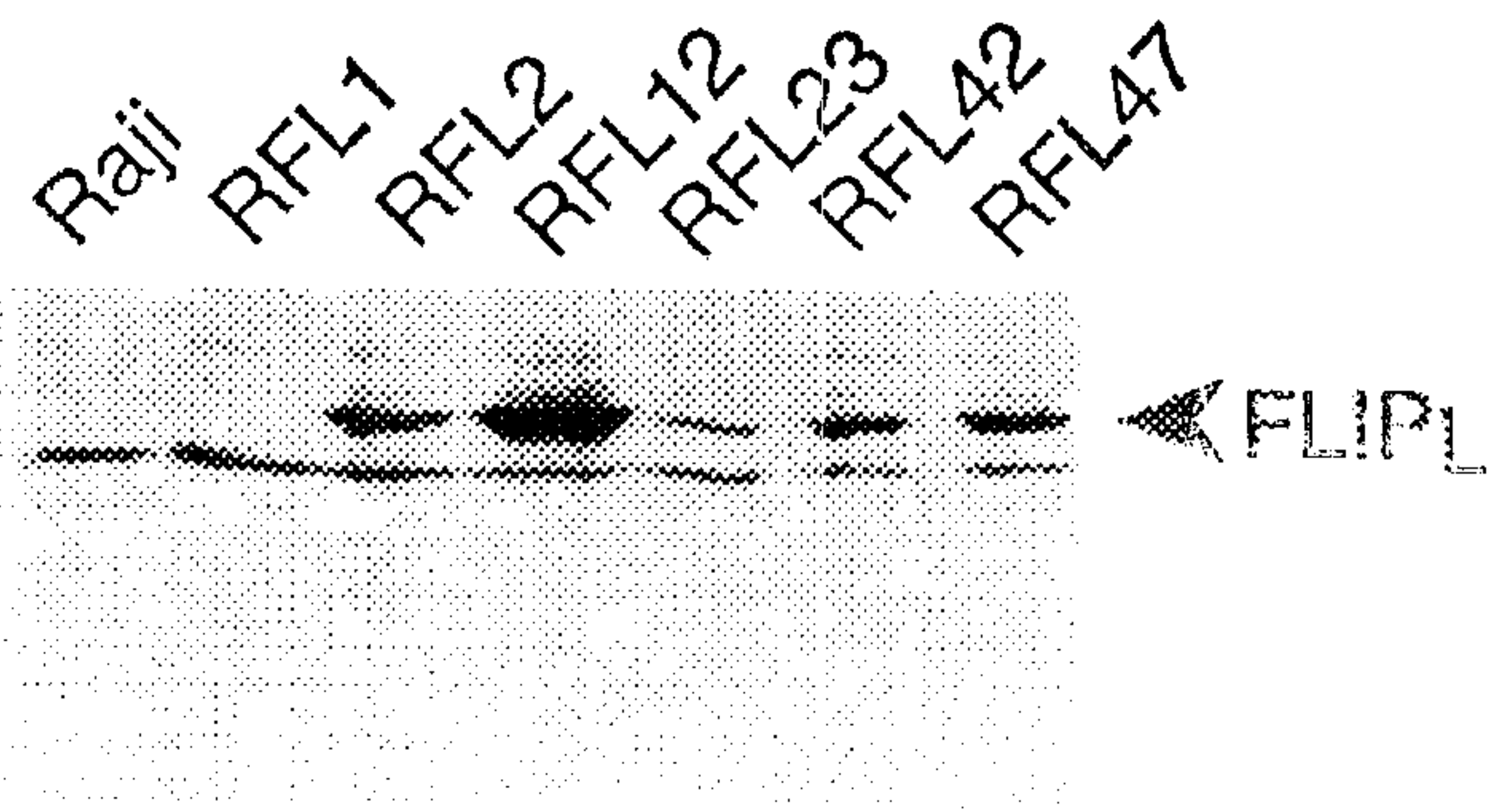
b



c

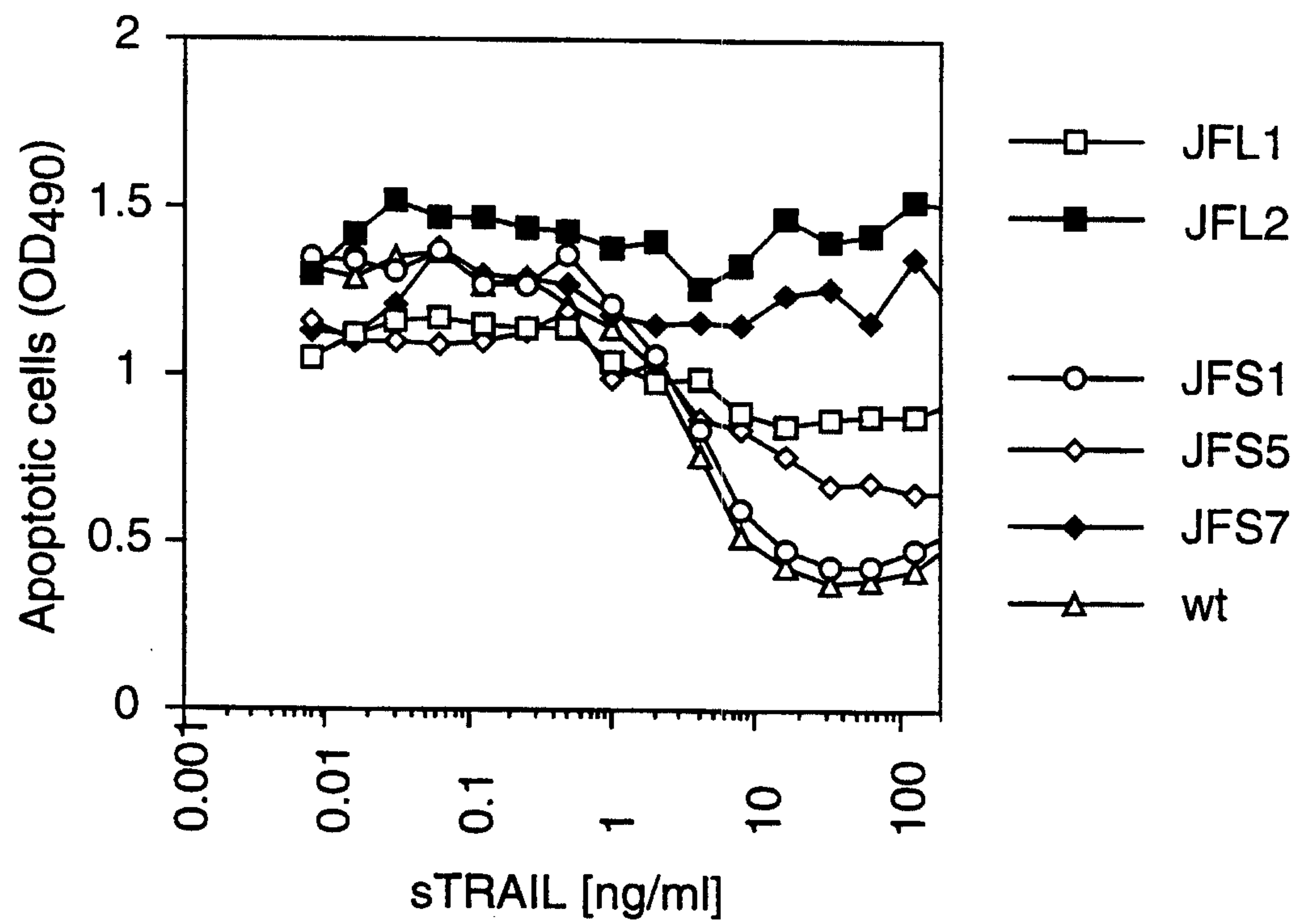
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Fig. 4b



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Fig. 15



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ORF71 aus Human Herpesvirus 8 (HHV8)

MATYEVLC EV ARKLGTD DRE VVLFLLNVEI PQPTLAQLIG ALRALKEEGR LTFPLLAEC L
FRAGR RDLLR DLLHLDPRFL ERHLAGTMSY FSPYQLTVLH VDGE LCARDI RSLIFLSKDT
IGSRSTPQTF LHWVYCMENL DLLGPTDVDA LMSMLRSLR VDLQRQVQTL MGLHLSG PSH
SQHYRHTP

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Fig. 16

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ORF aus Bovine Herpesvirus 4 (BHV 4)

MVTRDVLLAI ETHLNQNEKT FVMYFLLDPY IPKECKDFLP TLENLHSKRK IRYPILLIELM
YILQRFDLLR SIFLLDHRFV KDQITSSHVK YISPYKQLIF SIGQNIDDED LISIKFISMN
YIGKSPSNIK NYLDWVRAL E KVD MVGPDNL DLFETFFKQI HRMDIVKMIK NYRTRETLQI
TL

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Fig. 17