Caspase-8 Interacting Proteins

Field of the invention

The present invention is generally in the field of cysteine proteases. More specifically, the invention concerns proteins which interact with caspase-8 (MACH) and/or modulate its function in the cell death (or apoptotic) pathways mediated by CD95 (Fas/Apo-1) or by CD120a (p55-TNF receptor).

In particular, the present invention concerns proteins which interact with caspase-8/MACH directly or indirectly. The invention also relates to the preparation and use of the caspase-8/MACH interacting proteins.

Background of the Invention

Tumor Necrosis Factor (TNF-alpha) and Lymphotoxin (TNF-beta) (hereinafter, TNF, refers to both TNF-alpha and TNF-beta) are multifunctional pro-inflammatory cytokines formed mainly by mononuclear phagocytes, which have many effects on cells (Wallach, D. (1986) In: Interferon 7 (Ion Gresser, ed.), pp. 83-122, Academic Press, London; and Beutler and Cerami (1987)). Both TNF-alpha and TNF-beta initiate their effects by binding to specific cell surface receptors. Some of the effects are likely to be beneficial to the organism: they may destroy, for example, tumor cells or virus infected cells and augment antibacterial activities of granulocytes. In this way, TNF contributes to the defense of the organism against tumors and infectious agents and contributes to the recovery from injury. Thus, TNF can be used as an anti-tumor agent in which application it binds to its receptors on the surface of tumor cells and thereby initiates the events leading to the death of the tumor cells. TNF can also be used as an anti-infectious agent.

However, both TNF-alpha and TNF-beta also have deleterious effects. There is evidence that overproduction of TNF-alpha may play a major pathogenic role in several diseases. For example, effects of TNF-alpha, primarily on the vasculature, are known to be a major cause for symptoms of septic shock (Tracey et al,, 1986). In some diseases, TNF may cause

excessive loss of weight (cachexia) by suppressing activities of adipocytes and by causing anorexia, and TNF-alpha was thus called cachectin. It was also described as a mediator of the damage to tissues in rheumatic diseases (Beutler and Cerami, 1987) and as a major mediator of the damage observed in graft-versus-host reactions (Piquet et al., 1987). In addition, TNF is known to be involved in the process of inflammation and in many other diseases.

Two distinct, independently expressed, receptors, the p55 (CD120a) and the p75 (CD120b) TNF-Rs, which bind both TNF-alpha and TNF-beta specifically, initiate and/or mediate the above noted biological effects of TNF. These two receptors have structurally dissimilar intracellular domains suggesting that they signal differently (See Hohmann et al., 1989; Engelmann et al., 1990; Brockhaus et al., 1990; Loetscher et al., 1990; Schall et al., 1990; Nophar et al., 1990; Smith et al., 1990; and Holler et al., 1990). However, the cellular mechanisms, for example, the various proteins and possibly other factors, which are involved in the intracellular signaling of the CD120a and CD120b have yet to be elucidated. It is intracellular signaling, which occurs usually after the binding of the ligand, i.e., TNF (alpha or beta), to the receptor, that is responsible for the commencement of the cascade of reactions that ultimately result in the observed response of the cell to TNF.

As regards the above-mentioned cytocidal effect of TNF, in most cells studied so far, this effect is triggered mainly by CD120a. Antibodies against the extracellular domain (ligand binding domain) of CD120a can themselves trigger the cytocidal effect (see EP 412486) which correlates with the effectiveness of receptor cross-linking by the antibodies, believed to be the first step in the generation of the intracellular signaling process. Further, mutational studies (Brakebusch et al., 1992; Tartaglia et al,, 1993) have shown that the biological function of CD120a depends on the integrity of its intracellular domain, and accordingly it has been suggested that the initiation of intracellular signaling leading to the cytocidal effect of TNF occurs as a consequence of the association of two or more intracellular domains of CD120a. Moreover, TNF (alpha and beta) occurs as a homotrimer,

and as such, has been suggested to induce intracellular signaling via CD120a by way of its ability to bind to and to cross-link the receptor molecules, i.e., cause receptor aggregation.

Another member of the TNF/NGF superfamily of receptors is the FAS/APO1 receptor (CD95), which has also been called the FAS antigen, a cell-surface protein expressed in various tissues and sharing homology with a number of cell-surface receptors including TNF-R and NGF-R. CD95 mediates cell death in the form of apoptosis (Itoh et al., 1991), and appears to serve as a negative selector of autoreactive T cells, i.e., during maturation of T cells, CD95 mediates the apoptotic death of T cells recognizing self-antigens. It has also been found that mutations in the CD95 gene (lpr) cause a lymphoproliferation disorder in mice that resembles the human autoimmune disease systemic lupus erythematosus (SLE) (Watanabe-Fukunaga et al., 1992). The ligand for CD95 appears to be a cell-surface associated molecule carried by, amongst others, killer T cells (or cytotoxic T lymphocytes - CTLs), and hence when such CTLs contact cells carrying CD95, they are capable of inducing apoptotic cell death of the CD95-carrying cells. Further, a monoclonal antibody has been prepared that is specific for CD95, this monoclonal antibody being capable of inducing apoptotic cell death in cells carrying CD95 including mouse cells transformed by cDNA encoding human CD95 (Itoh et al., 1991).

While some of the cytotoxic effects of lymphocytes are mediated by interaction of a lymphocyte-produced ligand with the widely occurring cell surface receptor CD95, which has the ability to trigger cell death, it has also been found that various other normal cells, besides T lymphocytes, express CD95 on their surface and can be killed by the triggering of this receptor. Uncontrolled induction of such a killing process is suspected to contribute to tissue damage in certain diseases, for example, the destruction of liver cells in acute hepatitis. Accordingly finding ways to restrain the cytotoxic activity of CD95 may have therapeutic potential.

Conversely, since it has also been found that certain malignant cells and HIV-infected cells carry CD95 on their surface, antibodies against CD95, or the CD95 ligand, may be used to

trigger the CD95 mediated cytotoxic effects in these cells and thereby provide a means for combating such malignant cells or HIV-infected cells (see Itoh et al., 1991). Finding yet other ways for enhancing the cytotoxic activity of CD95 may therefore also have therapeutic potential.

It has been a long felt need to provide a way for modulating the cellular response to TNF (alpha or beta) and CD95 ligand. For example, in the pathological situations mentioned above, where TNF or CD95 ligand is overexpressed, it is desirable to inhibit the TNF- or CD95 ligand-induced cytocidal effects, while in other situations, e.g., wound healing applications, it is desirable to enhance the TNF effect, or in the case of CD95, in tumor cells or HIV-infected cells, it is desirable to enhance the CD95 mediated effect.

A number of approaches have been made by the applicants (see, for example, European Application Nos. EP 186833. EP 308378, EP 398327 and EP 412486) to regulate the deleterious effects of TNF by inhibiting the binding of TNF to its receptors using anti-TNF antibodies or by using soluble TNF receptors (being essentially the soluble extracellular domains of the receptors) to compete with the binding of TNF to the cell surface-bound TNF-Rs. Further, on the basis that TNF-binding to its receptors is required for the TNF-induced cellular effects, approaches by applicants (sec for example EP 568,925) have been made to modulate the TNF effect by modulating the activity of the TNF-Rs.

For example, EP 568925 relates to a method of modulating signal transduction and/or cleavage in TNF-Rs whereby peptides or other molecules may interact either with the receptor itself or with effector proteins interacting with the receptor, thus modulating the normal function of the TNF-Rs. In EP 568925, there is described the construction and characterization of various mutant forms of CD120a, having mutations in its extracellular, transmembrane, and intracellular domains. In this way, regions within the above domains of CD120a were identified as being essential to the functioning of the receptor, i.e., the binding of the ligand (TNF) and the subsequent signal transduction and intracellular signaling which ultimately results in the observed TNF-effect on the cells. Further, there is

also described a number of approaches to isolate and identify proteins, peptides or other factors which are capable of binding to the various regions in the above domains of CD120a, which proteins, peptides and other factors may be involved in regulating or modulating the activity of TNF-Rs. A number of approaches for isolating and cloning the DNA sequences encoding such proteins and peptides; for constructing expression vectors for the production of these proteins and peptides; and for the preparation of antibodies or fragments thereof which interact with CD120a or with the above proteins and peptides that bind various regions of CD120a are also set forth in EPO 368925. However, EP 568925 does not specify the actual proteins and peptides which bind to the intracellular domains of the TNF-Rs (e.g., CD95), nor does it describe the yeast two-hybrid approach to isolate and identify such proteins or peptides which bind to the intracellular domains of TNF-Rs. Similarly, in EP 568925 there is no disclosure of proteins or peptides capable of binding the intracellular domain of CD95.

Thus, when it is desired to inhibit the effect of TNF, or of the CD95 ligand, it would be desirable to decrease the amount or the activity of TNF-Rs or CD95 at the cell surface, while an increase in the amount or the activity of TNF-Rs or CD95 would be desired when an enhanced TNF or CD95 ligand effect is sought. To this end the promoters of both the CD120a and the CD120b have been sequenced, analyzed and a number of key sequence motifs have been found that are specific to various transcription regulating factors, and as such the expression of these TNF-Rs can be controlled at their promoter level, i.e., inhibition of transcription from the promoters for a decrease in the number of receptors, and an enhancement of transcription from the promoters for an increase in the number of receptors (EP 606869 and WO 9531206).

While it is known that the tumor necrosis factor (TNF) receptors, and the structurally-related receptor CD95, trigger in cells, upon stimulation by leukocyte-produced ligands, destructive activities that lead to their own demise, the mechanisms of this triggering are still little understood. Mutational studies indicate that in CD95 and CD120a signaling for cytotoxicity involve distinct regions within their intracellular domains

(Brakebusch et al., 1992; Tartaglia et al., 1993., Itoh and Nagata, 1993). These regions (the 'death domains') have sequence similarity. The 'death domains' of both CD95 and CD120a tend to self-associate. Their self-association apparently promotes the receptor aggregation which is necessary for initiation of signaling (see Song et al., 1994; Wallach et al., 1994; Boldin et al., 1995), and at high levels of receptor expression can result in triggering of ligand-independent signaling (Boldin et al., 1995).

Some of the cytotoxic effects of lymphocytes are mediated by interaction of a lymphocyte-produced ligand with CD95, a widely occurring cell surface receptor which has the ability to trigger cell death (see also Nagata and Goldstein, 1995); and that cell killing by mononuclear phagocytes involves a ligand-receptor couple, TNF and its receptor CD120a that is structurally related to CD95 and its ligand (see also Vandenabeele et al,. 1995). Like other receptor-induced effects, cell death induction by the TNF receptors and CD95 occurs via a series of protein-protein interactions, leading from ligand-receptor binding to the eventual activation of enzymatic effector functions, which in the case studies have elucidated non- enzymatic protein-protein interactions that initiate signaling for cell death: binding of trimeric TNF or the CD95 ligand molecules to the receptors, the resulting interactions of their intracellular domains (Brakebusch et al., 1992; Tartaglia et al., 1993; Itoh and Nagata, 1993) augmented by a propensity of the death-domain motifs to self-associate (Boldin et al., 1995a), and induced binding of two cytoplasmic proteins (which can also bind to each other) to the receptors' intracellular domains - MORT-1 (or FADD) to CD95 (Boldin et al., 1995b; Chinnaiyan et al., 1995; Kischkel et al., 1995) and TRADD to CD120a (Hsu et al., 1995; Hsu et al., 1996). Three proteins that bind to the intracellular domain of CD95 and CD120a at the 'death domain' region involved in cell-death induction by the receptors through hetero-association of homologous regions and that independently are also capable of triggering cell death were identified by the yeast two-hybrid screening procedure. One of these is the protein, MORT-1 (Boldin et al. 1995b), also known as FADD (Chinnaiyan et al., 1995) that binds specifically to CD95. The second one, TRADD (see also Hsu et al., 1995, 1996). binds to CD120a, and the third, RIP (see also Stanger et al., 1995), binds to both CD95 and CD120a. Besides their binding

to CD95 and CD 120a, these proteins are also capable of binding to each other, which provides for a functional "cross-talk" between CD95 and CD120a. These bindings occur through a conserved sequence motif, the 'death domain module' common to the receptors and their associated proteins. Furthermore, although in the yeast two-hybrid test MORT-1 was shown to bind spontaneously to CD95, in mammalian cells, this binding takes place only after stimulation of the receptor, suggesting that MORT-1 participates in the initiating events of CD95 signaling. MORT- 1 does not contain any sequence motif characteristic of enzymatic activity, and therefore, its ability to trigger cell death seems not to involve an intrinsic activity of MORT- 1 itself, but rather, activation of some other protein(s) that bind MORT-1 and act further downstream in the signaling cascade. Cellular expression of MORT-1 mutants lacking the N-terminal part of the molecule have been shown to block cytotoxicity induction by CD95 or CD120a (Hsu et al., 1996; Chinnaiyan et al., 1996), indicating that this N-terminal region transmits the signaling for the cytocidal effect of both receptors through protein-protein interactions.

Recent studies have implicated a group of cytoplasmic thiol proteases which are structurally related to the *Caenorhabditis elegans* protease CED3 and to the mammalian interleukin-1 beta converting enzyme (ICE) in the onset of various physiological cell death processes (reviewed in Kumar, 1995 and Henkart, 1996). There have also been some indications that protease(s) of this family may take part in the cell-cytotoxicity induced by CD95 and TNF-Rs. Specific peptide inhibitors of the proteases and two virus-encoded proteins that block their function, the cowpox protein crmA and the Baculovirus p35 protein, were found to provide protection to cells against this cell-cytotoxicity (Enari et al., 1995; Los et al., 1995; Tewari et al., 1995; Xue et al., 1995; Beidler et al., 1995). Rapid cleavage of certain specific cellular proteins, apparently mediated by protease(s) of the CED3/ICE family, could be demonstrated in cells shortly after stimulation of CD95 or TNF-Rs.

One such protease and various isoforms thereof (including inhibitory ones), is known as MACH (now caspase-8) which is a MORT-1 binding protein and which serves to modulate

the activity of MORT-1 and hence, of CD95 and CD120a, and which may also act independently of MORT-1, has been recently isolated, cloned, characterized, and its possible uses also described, as is set forth in detail and incorporated herein in their entirety by reference, in co-owned, copending Israel Patent Application Nos. IL 114615, 114986, 115319, 116588 and 117932, as well as their corresponding PCT counterpart No. PCT/US96/10521, and in a recent publication of the present inventors (Boldin et al., 1996). Another such protease and various isoforms thereof (including inhibitory ones), designated Mch4 (also called caspase-10) has also recently been isolated and characterized by the present inventors (unpublished) and others (Fernandes-Alnemri et al., 1996; Srinivasula et al., 1996). Caspase-10 is also a MORT-1 binding protein which serves to modulate the activity of MORT-1 and hence likely also of CD95 and CD120a, and which may also act independently of MORT-1. Thus, details concerning all aspects, features, characteristics and uses of caspase-10 are set forth in the above noted publications, all of which are incorporated herein in their entirety by reference.

It should also be noted that the caspases, caspase-8 and caspase-10, which have similar prodomains (see Boldin et al., 1996; Muzio et al., 1996; Fernandes-Alnemri et al., 1996; Vincent and Dixit, 1997) interact through their prodomains with MORT-1, this interaction being via the 'death domain motif' or 'death effector domain', DED, present in the N-terminal part of MORT-1 and present in duplicate in caspase-8 and caspase-10 (see Boldin et al., 1995b; Chinnalyan et al., 1995).

Such proteases, now known as caspases (cysteine aspartate-specific proteinases), are a growing family of cysteine proteases that share several common features. Most of the caspases have been found to participate in the initiation and execution of programmed cell death or apoptosis, while the others appear to be involved in the production of proinflammatory cytokines (Nicholson DW et al. 1997, Salvesen GS et al.1997, Cohen GM 1997) They are synthesized as catalytically inactive precursors and are generally activated by cleavage after specific internal aspartate residues present in interdomain linkers. The cleavage sites of caspases are defined by tetrapeptide sequences (X-X-X-D) and cleavage

always occurs downstream of the aspartic acid. As a result certain mature active caspases can process and activate their own as well as other inactive precursors (Fernandes-Alnemri T et al. 1996, Srinivasula SM et al. 1996).

Activation of the programmed cell death process is generally specific and involves sequential processing of downstream caspases named "execution" caspases by upstream caspases named "initiator" caspases. The functional characteristics of the two classes of caspases are also reflected by their structure. In fact the "initiator caspases" contain longer prodomain regions as compared to the "executioner" caspases (Salvesen GS et al. 1997, Cohen GM 1997). The long prodomain allows the initiator or "'apical" caspases to be activated by triggering of the death receptors of the TNF receptor family. Upon ligand-induced trimerization of the death receptors, the initiator caspases are recruited through their long N-terminal prodomain to interact with specific adapter molecules to form the death inducing signaling complex (Cohen GM 1997, Kischkel FC et al., 1995). For example, caspase-8/MACH and probably caspase-10, which contain two Death Effector Domains (DED) or FADD domains, are recruited to the receptor complex by the adapter molecules FADD/MORT-1, whereas caspase-2 is recruited by CRADD/RAIDD and RIP (Nagata S et al. 1997, MacFarlane M et al. 1997, Ahmad M et al. 1997, Duan H et al. 1997). Due to the trimeric nature of the activated receptor complex at least two caspase molecules are thought to be brought in close proximity to each other thus leading to their activation by autocatalytic processing (Yang et al. 1998, Muzio et al. 1998).

Caspases are synthesized as proenzymes consisting of three major subunits, the N-terminal prodomain, and two subunits, which are sometimes separated by a linker peptide. The two subunits have been termed "long" or subunit 1 containing the active enzymatic site, and "short" or subunit 2. For full activation of the enzyme, the prodomain and the two subdomains are cleaved. The two cleaved subunits form a heterodimer, whereby the long domain is derived from the N-terminus, and the short subunit is derived from the C-terminal region of the caspase precursor. Based on the deduced three dimensional structure of caspase-3, it appears that the C-terminal end of the long domain as well as the

N-terminus of the short subdomain have to be freed and the C-terminus of the short subunit has to be brought into close proximity with the N-terminus of the long subunit in order to yield a correctly folded and active enzyme (Rotonda et al 1996, Mittl et al. 1997, Srinivasula et al. 1998).

N-acetylglucosamine-6-phosphate deacetylase is an intracellular enzyme known to be involved in the intracellular metabolism of glucosamine. A genomic DNA fragment containing N-acetylglucosamine-6-phosphate deacetylase was cloned from the chitinase-producing bacterium *Vibrio cholerae* (Yamano et al., Biosci Biotechnol Biochem. 61, p. 1349-53, 1997).

The nagA gene encoding E. coli N-acetylglucosamine-6-phosphate deacetylase is also known [see, e.g., Peri et al., January 1990, 68(1), pp. 123-137]. Human N-acetylglucosamine-6-phosphate deacetylase has so far not yet been reported as cloned and sequenced.

SUMMARY OF THE INVENTION

The invention provides a caspase-8 interacting protein, or an isoform, allelic variant, fragment, functional analog, mutant or derivative thereof, which is capable of interacting with subunit 1 and/or subunit 2 of caspase-8.

The invention further provides human N-acetylglucosamine-6-phosphate deacetylase, or an isoform, allelic variant, fragment, functional analog, mutant or derivative thereof.

Still further, the invention provides a protein which comprises the amino acid sequence of Fig. 2, 3, 5B or 6.

The invention also provides a protein which comprises the amino acid sequence of the Tip-60 protein excluding amino acids 94 to 145.

Further encompassed in the scope of the invention is a protein which comprises the amino acid sequence encoded by the clones P27, P70, P79, L7, L12, M26, B4, B17, J40, B13, B37, B33 or P74, and its splice variants P16 and P43, as described hereinbelow.

The invention also provides a protein as defined above, which is cleaved in vitro or in vivo by caspase-8.

The invention also provides an isolated DNA sequence coding for a protein of the invention. Comprised within that scope is the DNA sequence of Fig. 2 and Fig. 3. Also comprised within the scope of the invention is an isolated DNA capable of hybridizing to said DNA sequence under moderately stringent conditions.

The invention also provides a vector comprising a DNA sequence as defined above.

The invention further provides an eukaryotic or prokaryotic host cell containing a vector of the invention.

The invention further provides a method of producing a protein, isoform, allelic variant, fragment, functional analog, mutant or derivative of a caspase-8 interacting protein of the invention, comprising growing a host cell of the invention under conditions that allow production of said protein, affecting post-translational modifications as necessary for obtaining said protein, isoform, allelic variant, fragment, functional analog, or mutant, or derivative, and isolating said protein, isoform, allelic variant, fragment, functional analog, or mutant, or derivative.

The invention also provides said method wherein the cell is a prokaryotic cell.

The invention further provides said method wherein the cell is an eukaryotic cell.

The invention also provides said method wherein the cell is a mammalian, insect, or yeast cell.

The invention also provides said method wherein the cell is a HeLa or 293 T HEK cell.

The invention further provides said method wherein as a promoter, the human CMV promoter is employed.

Still further, the invention provides a caspase-8 interacting peptide comprising at least 4 consecutive amino acids of a protein of the invention.

A derivative of said peptide is encompassed within the scope of the invention. Further encompassed within the scope of the invention is said peptide derivative, capable of forming a covalent bond with caspase-8 upon contacting said caspase-8.

The invention also provides a ribozyme specific for a nucleotide sequence corresponding to a DNA sequence of the invention.

The invention further provides an antisense oligonucleotide comprising at least 9 nucleotides of a sequence corresponding to a DNA sequence of the invention.

The invention also provides an antibody directed at an epitope of a protein of the invention.

The invention also provides an immunoassay for the detection of a caspase-8 interacting protein, comprising the antibody of the invention.

The invention further provides an immunoassay for the detection of caspase-8, comprising a peptide of the invention.

The invention also provides an immunoassay for the detection of caspase-8, comprising a protein of the invention.

The invention further provides a method of identifying caspase-8 interacting proteins, comprising the steps of

a) providing a yeast cell having a reporter gene linked to a promoter comprising a DNA sequence motif;

a) expressing in said yeast cell a p20 subunit of said caspase-8;

b) expressing in said yeast cell a fusion protein of a DNA binding domain and the p10 and/or p20 subunit of said caspase-8, wherein said DNA binding domain is capable of binding to said DNA sequence motif;

c) optionally, expressing in said yeast cell an unfused p10 or p20 subunit of said caspase-8;

d) transforming a culture of said yeast cell with a library consisting of an expression vector driving expression of a fusion protein consisting of a cDNA library and a transcriptional activator;

e) screening the culture of transformed yeast cells for yeast cells wherein the reporter gene is activated, and

f) isolating a yeast cell from step e) and further isolating the caspase-8 interacting protein, which is expressed in its prey vector.

The invention also provides said caspase-8 interacting protein, isoform, allelic variant, fragment, functional analog, mutant, or derivative, said ribozyme, antisense oligonucleotide or antibody, for use in modulating caspase-8 activity.

The invention further provides said caspase-8 interacting protein, isoform, allelic variant, fragment, functional analog, mutant, or derivative, said ribozyme, antisense oligonucleotide or antibody, for use in modulating TNF-receptor or Fas-mediated effects.

Still further, the invention provides said caspase-8 interacting protein, isoform, allelic variant, fragment, functional analog, mutant, or derivative, said ribozyme, antisense oligonucleotide or antibody, for use in modulating apoptosis.

The invention also provides said caspase-8 interacting protein, isoform, allelic variant, fragment, functional analog, mutant, or derivative, said ribozyme, antisense oligonucleotide or antibody, for use as a medicament.

The invention further provides said caspase-8 interacting protein, isoform, allelic variant, fragment, functional analog, mutant, or derivative, said ribozyme, antisense oligonucleotide or antibody, for use as a medicament in the treatment of multiple sclerosis with primary oligodendrogliopathy, autoimmune uveoretinitis, diabetes, lupus, autoimmune myocarditis I, HCV mediated chronic hepatitis, chronic gastritis e.g., type A gastritis, mixed connective tissue disease, (MCTD), Crohn's disease, or ulcerative colitis.

The invention also provides a triplex-forming oligonucleotide capable of binding to a purine-rich sequence in the promoter region of a caspase-8 interacting gene. The triplex-forming oligonucleotide may contain chemical modifications, such as internucleoside phosphate linkages modified with the cation N,N-diethyl-ethylenediamine, Uridine, benzo[g]-quinazoline-2,4-dione-(1H,3H)-dione or benzo[f]quinazoline-2,4-dione-(1H,3H)-dione residues in place of Thymidine residues. The triplex-forming oligonucleotide may further be covalently linked to chemicals having affinity for DNA, preferably such agents as may intercalate into DNA, such as acridine and psoralene.

The term "interacting" in the context of this description, relating to interaction of a protein of the invention with caspase-8, is meant to include direct forms of interaction, such as binding, cleavage, and indirect interaction, e.g. through adapter proteins. The interaction may optionally result in modulation of the caspase-8 mediated signal.

The term "binding" in the context of this application, when referring to binding of caspase-8 interaction proteins, is meant to relate to physical association of the caspase-8 interacting protein with caspase-8. This physical association may be measured in

immunoassays, such as ELISA or RIA assays, in the two-hybrid test, in immunoprecipitation assays, or in size separation-based assays, such as non-denaturing acrylamide gel electrophoresis or size exclusion gel chromatography of a mixture of caspase-8, or a subunit thereof, and a protein of the invention. When using the two-hybrid test, it is understood that the caspase-8 or subunit thereof is expressed as a DNA activation domain fusion, and the caspase-8 interacting protein is expressed as a DNA binding domain fusion, or vice versa.

The term "two-hybrid test" relates to the two-hybrid assay wherein protein-protein interactions can be measured by introducing into yeast cells a first expression vector coding for a fusion of a first protein and a DNA binding domain, and a second expression vector coding for a fusion of a second protein with a DNA activation domain. The yeast cells must contain at least one reporter gene driven by a promoter that contains a DNA sequence motif which is recognized by said DNA binding domain. Usually, two reporter genes, e.g., Histidine synthetase and beta-Galactosidase, are used. This enables the researcher to avoid false-positives that may result from mutation. This technique has been modified and refined by the present applicants for use in screening, isolating, and testing proteins that mediate TNF-R and Fas signals, see e.g., WO 97/03998 and references therein. The two-hybrid test relies on localization of both fusion proteins to the cell nucleus.

The term "two hybrid test" as used herein further comprises a modification of the two-hybrid technique wherein cell growth signaling proteins, such as ras and sos are used (Broder et al., Curr Biol 1998, 8, p. 1121-4, Aronheim et al., Nucleic Acids Res 1997 25, p. 3373-4, and references therein). In order to be functional, these proteins require relocalization to the cell membrane. This is achieved by expressing a cell growth signaling protein as a fusion with a first protein, and expressing a cell membrane localization signal, such as a myristilation signal sequence, as a fusion with a second protein. An interaction between the first and second proteins will re-localize the cell growth signaling protein to the cell membrane and thus trigger cell growth. Aggressively growing cells are then

selected for further study. This system is different from the above-described two-hybrid system, because it does not rely on localization of both hybrids to the cell nucleus.

The term "bait" refers to the protein in the above-mentioned two-hybrid test, which is expressed as a fusion protein with a DNA binding domain.

The term "prey" refers to the protein in the above-mentioned two-hybrid test that is expressed as s fusion with a DNA activating domain.

Brief description of the figures:

Figure 1 shows the schematic representation of the single chain construct of caspase-8, used as bait in the two-hybrid screen described in Example 1 B.

Figure 2 is a preliminary partial nucleotide (SEQ ID NO:1) and deduced amino acid sequence (SEQ ID NO:2) of clone 32 encoding a caspase-8 interacting protein as obtained from a cDNA clone.

Figure 3 shows the putative full length sequence of the human nucleotide (SEQ ID NO:3) and deduced amino acid sequence (SEQ ID NO:4) N-acetylglucosamine-6-phosphate deacetylase composed of the 5' extension of clone J2, the EST clone AA460869 and exon trap clone L48741.

Figure 4A shows the functional activity of clone J2 expressed as the percentage of apoptotic cells, following transfection of HEK-293T cells either with the p55 TNF receptor alone (marked p55 TNFR) or with the p55TNF receptor together with p35, a baculovirus inhibitor of caspases (p55+p35) or with clone J2 (p55+J2), or with the p55 TNF receptor together with a non cleavable mutant of J2 (denoted J*) containing an Asp to Glu

substitution at position 346 of figure 3 (p55+J2*). This mutant can be cleaved neither *in vitro* nor *in vivo*.

Figure 4B shows the percentage of apoptotic HeLa cells alone, or following transfection with p35 or J2 or J2* treated with TNF and cycloheximide.

Figure 5A shows the 1725 coding base pairs at the 5' end of clone P74 (SEQ ID NO:5).

Figure 5B shows the 574 amino acid sequence deduced from the sequence of clone P74 of figure 5A (SEQ ID NO:6).

Figure 6 shows 1428 amino acids (SEQ ID NO:7) of an open reading frame derived from a deduced amino acid sequence of PAC clone accession number RPCI5-1057I20.

Figure 7 shows the alignment of the 574 amino acids of the open reading frame derived from the deduced amino acid sequence of clone p74 (denoted cloned) compared to the 1428 amino acids of the open reading frame derived from the deduced amino acid sequence of PAC clone RPCI5-1057I20 (denoted deduced).

Figure 8 shows the alignment of the open reading frame of the deduced amino acid sequence of PAC clone RPCI5-1057I20 (top sequence) (SEQ ID NO:8) with the sequence of histone deacetylase A (lower sequence, Genebank accession number NP-006028.1) (SEQ ID NO:9).

Figure 9A shows an autoradiography of the Bid protein and the proteins encoded by the cDNA clones J2, or P16, or P43, or P70, or P74, or P79, produced in a reticulocyte lysate in the presence of ^{35}S Methionine, separated on an SDS PAGE gel.

Figure 9B shows an autoradiography of the Bid protein and the proteins encoded by the cDNA clones J2, or P16, or P43, or P70, or P74, or P79 produced as in figure 9A analysed for binding to caspase-8 expressed in bacteria as a fusion protein of its two subunits, fused to GST. The position of the molecular weight markers is shown on the left of the gel.

Figure 10 shows the results of cleavage of the protein encoded by the partial P43 cDNA clone by Caspase-8 or by Caspase-10 or by Caspase-3 or by Caspase-9 or by mutants of Caspase 3 or of Caspase-9 or of Caspase-10. The volumes of the total bacterial lysate of the recombinant caspases expressed in E. Coli that were used, are indicated in relative units (RU). The position of the molecular weight markers is shown on the left of the gel. The proteins of interest are marked by an asterisk: the full head arrows show the full size P43 protein the open head arrows show the cleavage products.

Figure 11 shows the functional activity of the proteins encoded by cDNA clones identified by two hybrid screening expressed as the percentage of cells undergoing apoptosis, following cotransfection of HEK-293T cells with the p55 TNF receptor and with the Green Fluoresence Protein (denoted PC) without or with the cDNA inserts of clones J2 or P16 or P27 or P43 or P79 or P74 or P70.

Figure 12 shows the cell death inhibiting activity of wild type and of the noncleavable mutant of Tip60 in HEK-293T cells cotransfected with the p55 TNF receptor and with the Green Fluorescence Protein as well as the effect of the Δ32-Tip60 lacking the first 32 N-terminal amino acids. Control cells were transfected with the pCGN vector alone.

Detailed description of the invention

A number of methods of the art of molecular biology are not detailed herein, as they are well known to the person of skill in the art. Such methods include site-directed

mutagenesis, PCR cloning, phage library screening using oligonucleotide or cDNA probes, expression of cDNAs, analysis of the recombinant proteins, transformation of bacterial and yeast cells, transfection of mammalian cells, and the like. Textbooks describing such methods are e.g., Sambrook et al., Molecular Cloning A Laboratory Manual, Cold Spring Harbor Laboratory; ISBN: 0879693096, 1989, Current Protocols in Molecular Biology ,by F. M. Ausubel, ISBN: 047150338X, 1988, and Short Protocols in Molecular Biology, by F. M. Ausubel et al. (eds.) 3rd ed. John Wiley & Sons; ISBN: 0471137812, 1995. These publications are incorporated herein in their entirety by reference

In order to identify caspase-8 interacting proteins and potential substrates, by two hybrid screening method, the two hybrid or three-hybrid system may be used.

The two-hybrid system is used in the method of the invention essentially as described by Fields and Song (Nature 340, p. 245, 1989). Preferably, individual vectors, yeast strains, and libraries may be obtained from Clontech (Palo Alto, USA), as components of the Matchmaker two-hybrid system (#PT1265-1).

The preferred embodiment of the yeast two-hybrid system as used in the method of the invention has been described by Boldin et al., Cell. 85, p. 803-15, 1996. The yeast two-hybrid system has further been described in US Patent 5,580,736, Brent et al. These publications are therefore incorporated herein in their entirety by reference.

The three-hybrid system is used essentially as described by Tirode et al., J. Biol. Chem. 272, p. 22995-9, 1997. For detecting caspase-8 interacting proteins according to the invention, it is required that the two subunits of caspase-8 be expressed independently. For that purpose, the two caspase-8 subunits are preferably expressed separately under the control of different promoters. In a preferred embodiment, the caspase-8 p10 subunit (Serine 375 to Aspartic acid 479) is expressed under control of a weak promoter operable in yeast, in-frame with a DNA binding domain. Preferably, the weak promoter is the yeast ADH promoter and the DNA binding protein is the DNA binding domain of the yeast

transcriptional activator Gal-4 or of the bacterial LexA protein. The ADH promoter and the Gal4 DNA binding domain are present e.g., in the pGBD commercially available by Clontech, Palo alto, USA. However, it will be apparent to a person skilled in the art that other promoters may be used, so long as the promoter is effective in yeast cells. By the same token, other DNA binding domains may be used, as long as these DNA binding domains do not have transcriptional activator function.

The long, active, caspase-8 p20 subunit (Serine 217 to Aspartic acid 374) is expressed as unfused protein under control of an inducible promoter operable in yeast cells. Preferably, the Met25 methionine repressible promoter as described in the above Tirode et al. may be used. The use of an inducible promoter simplifies the detection of the p10-p20 complex by immunoprecipitation and polyacrylamide gel electrophoresis. The inducible promoter has further advantages in that it enables the expression in yeast of protein that may be toxic to the yeast cells, because of the possibility to limit the period wherein the potentially toxic protein is expressed.

The proteins to be screened with the method of the invention are preferably provided in the form of a cDNA library. However, also genomic libraries or combinatorial libraries may be used. The library is cloned at the C-terminal end of a transcriptional activation domain operable in yeast. Preferably, the transcriptional activation domain of the yeast Gal-4 protein is used, however, a large number of other transcriptional activators may be used. Preferably, the pGAD GH vector available from Clontech is used for cloning of the library.

The yeast strain used for screening must contain a selection marker such as histidine synthetase, under the control of a promoter that comprises a DNA sequence to which the above-mentioned DNA binding domain binds specifically. Preferably, the yeast cell also contains a reporter gene under the control of a promoter that comprises a DNA sequence to which the above-mentioned DNA binding domain binds specifically. The yeast strain HF7c, available from Clontech, may be used for screening with Gal-4 binding domain hybrids; the strain L40 may be used when lexA is the DNA binding domain used.

After transformation, the yeast cells are placed in conditions selective for active plated onto media lacking certain amino acids, as required for the stability of the plasmids introduced thereinto.

The medium is selective for yeast cells in which the gene for the above-mentioned selection marker is activated. Preferably, the selection marker is the histidine synthetase gene. Yeast cells expressing this gene may be selected for by culturing in medium lacking histidine. Am advantage of this system is the possibility of adding the histidine synthetase inhibitor 3-aminotriazole to the growth medium. It is thus possible to inhibit growth of yeast cells in which a small amount of histidine synthetase is expressed, caused by leaking of the promoter containing the sequence to which the above-mentioned DNA binding domain specifically binds. In some clones, a weak, non-specific interaction between the caspase-8 p10 and/or p20 subunit and the said clone may cause spurious activation of said promoter. Thus, by raising the concentration of said inhibitor in the medium used for selection of interacting clones, it is possible to select only clones that interact with a certain minimal strength. The concentration of 3-aminotriazole is preferably 7.5 mM.

Clones identified by their ability to grow in medium lacking histidine are further analyzed by quantification of their reporter gene activity. Preferably, the lacZ gene is used a reporter gene. Quantification of lacZ activity is done preferably in liquid culture, as described in Boldin et al., J. Biol. Chem. 270, 7795-8, 1995.

The above-described screening method may be carried out similarly using the two-hybrid test. The essential difference is that the two subunits of the caspase-8 are expressed as a single peptide chain, which is a fusion protein with the above-noted DNA binding domain. The p20 subunit is mutated in its active site (cysteine 360-Serine 360) as described below.

Preferably, the p10 subunit is separated from the p20 subunit by a linker, which is preferably between 10 and 50 amino acids in length. Said linker comprises preferably