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(57) Abstract

The present invention provides an isolated nucleic acid encoding an endoplasmic reticulum associated amyloid–beta peptide binding (ERAB) polypeptide. The ERAB polypeptide may comprise human ERAB polypeptide. The present invention provides a purified ERAB polypeptide, as well as a method for treating a neurodegenerative condition in a subject which comprises administering to the subject an agent in amount effective to inhibit ERAB polypeptide binding to amyloid–beta peptide so as to thereby treat the neurodegenerative condition.

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INTRACELLULAR AMYLOID-BETA BINDING (ERAB) POLYPEPTIDE

This application is a continuation in part of U.S. Application Serial No. 08/815,225, filed March 12, 1997, the contents of which are hereby incorporated by reference into the present application.

The invention disclosed herein was made with Government support under NIH (Aging Institute) Grant No. AG 006902, from the Department of Health and Human Services. Accordingly, the U.S. Government has certain rights in this invention.

Throughout this application, various references are referred to by numbers within parentheses. Disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the claims, in numerical order corresponding to the numbers within parentheses.

Background of the Invention

Processing of the beta-amyloid precursor protein (APP) leads to a range of proteolyzed forms (1-6), some of which assemble into beta-amyloid fibrils and are cytotoxic. ß-amyloid moieties, such as amyloid-beta peptide (Aβ), are closely associated with neuronal dysfunction and death in Alzheimer's disease (AD). Increased expression of amyloid-beta peptide is linked to mutations in APP (6-10) and in presenilins (11-13), both of which occur in familial AD. The mechanisms underlying the cellular stress phenotype brought about in cells by amyloid-beta peptide-derived peptides are likely related to the neurotoxicity leading to dementia. Most attention has been focussed on mechanisms by which extracellular amyloid-beta peptide exerts its effects

on cells, since the most visible accumulations of amyloid-beta peptide occur extracellularly in plaques. Amyloid-beta peptide aggregates, especially those that assemble into fibrils, can be cytotoxic by nonspecifically disturbing the integrity of cell membranes, and by elaborating reactive oxygen intermediates (14-15), thereby resulting in elevation of cytosolic calcium eventually followed by cell death (15-16). Cell surface receptors for amyloid-beta peptide (17-19) could also activate signal transduction mechanisms. The receptor RAGE, an immunoglobulin superfamily molecule, is one such neuronal cell surface docking site which binds amyloid-beta peptide and facilitates amyloid-beta peptide-mediated cellular oxidant stress (19).

Summary of the Invention

The present invention provides an isolated nucleic acid encoding an endoplasmic reticulum associated amyloid-beta peptide binding (ERAB) polypeptide. The ERAB polypeptide may comprise human ERAB polypeptide. The present invention provides a purified ERAB polypeptide, as well as a method for treating a neurodegenerative condition in a subject which comprises administering to the subject an agent in amount effective to inhibit ERAB polypeptide binding to amyloid-beta peptide so as to thereby treat the neurodegenerative condition.

Description of the Figures

Figures 1A, 1B, 1C, 1D, 1E-1 and 1E-2. Identification and cloning of a cell-associated binding protein for amyloid-beta peptide. Fig. 1A. Schematic depiction of fragments of human amyloid precursor protein (APP) cloned into the yeast expression vector pGBT9. The line at the top designates the full-length APP cDNA (numbering is according to the Genbank sequence accession #X06989) and thickened portions indicate the region encoding A4 (APP amino acids 653 to 694). Open boxes show APP fragments encoded by the indicated plasmid fusion proteins. ATG, start codon; TAG, stop codon; E, EcoRI; S, StyI; and B, BamHI. **Fig. 1B.** Visualization of ß-galactosidase reaction product when yeast express the two indicated constructs. The left panel of Fig. 1B demonstrates the presence of yeast on the agar and the right panel of Fig. 1B shows the same area stained for ß-galactosidase reaction product. Each experiment is shown in triplicate. pAβ002 is one of three ERAB-containing clones derived from the HeLa cell Matchmaker™ cDNA library. pVA3 indicates murine p53 in pGBT9, pTD1 indicates SV40 large T-antigen in pGAD3F, and the combination of these two plasmids is used as a positive control. **Fig. 1C.** Quantitative analysis of ß-galactosidase gene product from an experiment using the same constructs as in Fig. 1B. **Fig. 1D.** cDNA sequence and deduced amino acid sequence of the endoplasmic reticulum binding polypeptide (ERAB polypeptide) identified in the yeast two-hybrid system (Seq. Id. Nos. 1 and 2). **Figs. 1E1-1E2.** Alignment of ERAB polypeptide deduced amino acid sequence (Sequence Id. No. 3) with that for 20-ß-hydroxysteroid dehydrogenase (BHD) (Seq. Id. No. 4). The boxed areas correspond to amino acid domains involved in binding of nicotinamide adenine dinucleotide (NAD). The boxed area with bold letters corresponds to the putative active center of the steroid binding domain. One dot and two dots indicated similar and identical amino acid residues, respectively. Methods: Fragments of human amyloid

precursor protein (APP) were cloned into the yeast expression vector pGBT9 (a GAL4 DNA-binding domain hybrid cloning vector; Clontech). The 3'-end of the EcoRI fragment from the APP cDNA was cloned into pGBT9 to generate pGBT/APP-23, which encodes a fusion protein comprising the C-terminus of APP (amino acids 655-751). A stop codon (TGA) was introduced after the sequence of A4 by site-directed mutagenesis and the resulting EcoRI fragment was cloned into pGBT9 to generate pBA-6, which encodes a fusion protein containing A4 peptide sequence (amino acids 3-42). A BamHI site was introduced at the beginning of A4 by PCR amplification and the resulting fragment was subcloned into the unique BamHI site of pGBT9 to generate pGBT/AB-13, the latter encoding a fusion protein with the A4 peptide sequence (amino acids 1-42). The plasmid constructs pBA-6 and pGBT/AB-13 were used as bait to screen human brain and HeLa Matchmaker libraries according to the manufacturer's instructions. Quantitative assay of ß-galactosidase activity was performed using o-nitrophenyl ß-D-galactopyranoside (ONPG) as substrate.

Figures 2A, 2B, 2C, 2D, 2E, 2F, and 2G. Expression of ERAB fusion protein: amyloid-beta peptide binding properties. Fig. 2A. SDS-PAGE (12%; reduced) followed by immunoblotting of TrcHis-ERAB polypeptide (Fig. 2A, lane 1; 2 μg) and TrcHis-chloramphenicol acetyltransferase (CAT; Fig. 2A, lane 2; 2 μg) fusion proteins using anti-ERAB polypeptide IgG (3 μg/ml). Figure 2A lane 3 has the same sample as in Figure 2A lane 1, except that free peptide used as immunogen was present at an ≈25-fold molar excess over the antibody. **Figs. 2B and 2C.** The indicated concentrations of TrcHis-ERAB polypeptide (Fig. 2B; or in Fig. 2C, 5 μg/well) were incubated in microtiter wells, excess sites on the plastic surface were blocked, and a binding assay was performed by adding ^{125}I-amyloid-beta peptide (100 nM; 1-40) alone or in the presence of an 100-fold molar excess of unlabelled amyloid-beta peptide (1-40; Fig. 2B). In Fig. 2C, anti-ERAB polypeptide IgG (a-ERAB polypeptide; 10 μg/ml) or nonimmune

(NI) IgG (10 μg/ml) was added. Specific binding (that observed in wells with ^{125}I-amyloid-beta peptide alone minus binding observed in wells with ^{125}I-amyloid-beta peptide + unlabelled amyloid-beta peptide), the mean ± SEM of quadruplicate determinations, is shown. **Fig. 2D.** TrcHis-ERAB polypeptide (5 μg/well) was incubated in microtiter wells, and the binding assay was performed using the indicated concentrations of ^{125}I-amyloid-beta peptide (1-40) alone or in the presence of excess unlabelled amyloid-beta peptide. Binding was analyzed by nonlinear least squares analysis using the method of Klotz and Hunston (60). **Fig. 2E.** Competitive binding study: TrcHis-ERAB polypeptide (5 μg/well) was incubated in microtiter wells, and the binding assay employed freshly prepared ^{125}I-amyloid-beta peptide (100 nM;1-40). Where indicated one of the following unlabelled competitors was added: freshly prepared synthetic amyloid-beta peptide(1-20), amyloid-beta peptide(1-40), amyloid-beta peptide(25-35), scrambled amyloid-beta peptide(25-35), amyloid-beta peptide purified from AD brain (61), Arg-Gly-Asp-Ser (Seq. ID. No. 5), or amyloid-beta peptide(1-40) incubated for 3 days at 37°C under conditions to promote aggregation (6) (in each case the competitor was added at 10 μM). **Fig. 2F and 2G.** Binding of ^{125}I-TrcHis-ERAB polypeptide to amyloid-beta peptide(1-42) adsorbed to microtiter wells. In Fig. 2F, the indicated concentration of amyloid-beta peptide(1-42) was incubated in wells, excess sites on the plate were blocked, and the binding assay was performed by adding ^{125}I-TrcHis-ERAB polypeptide alone or in the presence of an 100-fold molar excess of unlabelled TrcHis-ERAB polypeptide. In Fig. 2G, the binding assay was performed in the presence of the indicated concentration of ^{125}I-TrcHis-ERAB polypeptide (alone or in the presence of an 100-fold molar excess of unlabelled TrcHis-ERAB polypeptide) in wells with adsorbed amyloid-beta peptide (1-42; 5 μg/well was added). 0 indicates wells coated with albumin alone. The mean ± SEM of specific binding of quadruplicates is shown. * indicates $p<0.01$. Methods: A fusion protein construct was prepared by

subcloning an approximately 1116 bp EcoRI fragment (including 318 bp of 3'-untranslated sequence) into the unique EcoRI site of pTrcHis C vector (Invitrogen) to express TrcHis-ERAB polypeptide fusion protein. Following transformation of E. coli with this latter construct, the TrcHis-ERAB polypeptide fusion protein was purified as described by the manufacturer (Invitrogen). Control TrcHis-CAT construct (Invitrogen) was also used to transform E. coli and the fusion protein was purified as above. Antibody to ERAB polypeptide was prepared by immunizing rabbits with ERAB polypeptide-derived peptides, corresponding to residues 100-116 and 133-147, conjugated to keyhole limpet hemocyanin. These antibodies were demonstrated to be specific for ERAB polypeptide (see Figs. 2A-2G and description), and were used for immunoblotting, immunohistochemistry and functional studies. For immunoblotting shown in Fig. 2A, proteins were subject to reduced SDS-PAGE (12%), electrophoretic transfer to nitrocellulose, and reaction with anti-ERAB polypeptide antibody; sites of anti-ERAB polypeptide IgG binding were detected using the alkaline phosphatase method (Sigma). ^{125}I-amyloid-beta peptide(1-40) was radiolabelled as described (19) and ^{125}I-TrcHis-ERAB polypeptide polypeptide was labeled by the lactoperoxidase method (62). The latter tracer demonstrated a specific radioactivity of 2,000-2,500 cpm/ng, was >95% precipitable in trichloroacetic acid, and migrated as a single band, M_r ≈29 kDa, on SDS-PAGE. The binding assay to demonstrate direct interaction of ERAB polypeptide and amyloid-beta peptide utilized a modification of a previously described method (19). In brief, the indicated protein, ERAB polypeptide or amyloid-beta peptide, was diluted in carbonate buffer and incubated for 2 hrs at 37°C (for ERAB polypeptide) or overnight at 4°C (for amyloid-beta peptide) in Nunc MaxiSorb plates, excess sites in wells were blocked by exposure to albumin-containing solution (1% for 2 hr at 37°C), and then the radioiodinated tracer alone or in the presence of excess unlabelled material was added in minimal essential medium with bovine

serum albumin (1%; Sigma; fraction V, fatty acid-free) at 37°C. Incubation time was 3 hrs, when ^{125}I-TrcHis-ERAB polypeptide and ^{125}I-amyloid-beta peptide were the tracers, respectively. Unbound radioactivity was removed by washing wells five times with ice cold phosphate-buffered saline containing Tween 20 (0.05%), and bound radioactivity was eluted by incubation with Nonidet P-40 (1%).

Figures 3A, 3B, 3C, 3D, 3E, 3F-1 and 3F-2. Expression of ERAB polypeptide in human tissue. Figs. 3A, 3B, and 3C. Northern analysis of total RNA harvested from the indicated normal organs (Fig. 3A) or brain subregions (Figs. 3B and 3C; Clontech) hybridized with full-length ^{32}P-labelled human cDNA encoding for ERAB polypeptide. Controls were performed by hybridizing the same blots with ^{32}P-labelled cDNA for ß-actin. **Fig. 3D.** Immunoblotting of brain extracts with anti-ERAB polypeptide IgG. Extracts of human brain temporal lobe were prepared as described (19) and subjected to SDS-PAGE (12%; reduced; 100 μg protein/lane) and immunoblotting. Blots were reacted with rabbit anti-ERAB polypeptide IgG (3 μg/ml) and sites of IgG binding were detected by the chemiluminescence method (Amersham®). In Fig. 3D, lanes 1-2 (designated AD) and Fig. 3D, lanes 3-4 (designated C), extracts were prepared from temporal lobes of two different AD and age-matched normal brains, respectively. Figure 3D lanes 5-6 show the same samples as in Fig. 3D lanes 1-2, but excess TrcHis-ERAB polypeptide fusion protein was present (20 μg/ml). **Figs. 3E, 3F-1 and 3F-2.** Immunostaining for ERAB polypeptide in age-matched normal (Fig. 3E) and AD (Fig. 3F) brain. Brains, obtained within 8 hrs of expiration of the patients, were fixed in paraformaldehyde (4%), paraffin-embedded, sectioned and stained with anti-ERAB polypeptide IgG (30 μg/ml). Fig. 3F-2 shows double staining displaying amyloid-beta peptide (black) (19) and ERAB polypeptide (red), and was performed as described (19). Scale bar: Figs. 3E and 3F-1 = 31 μm; Fig. 3F-2 = 25 μm.

Figures 4A, 4B, 4C, 4D, 4E, 4F, 4G, 4H, and 4I. Expression of ERAB polypeptide in cultured cells: localization to the endoplasmic reticulum and change in distribution following addition of amyloid-beta peptide. Fig. 4A. Immunoblotting of ERAB polypeptide in human neuroblastoma SK-N-SH (Figure 4A, lane 1) and HeLa (Figure 4A, lane 2) cells. Cell extracts (50 μg protein/lane) were subjected to immunoblotting using anti-ERAB polypeptide IgG (3 μg/ml). **Figs. 4B, 4C, and 4D.** Confocal microscopy demonstrating immuno-fluorescence staining for ERAB polypeptide alone (Fig. 4B; red), protein disulfide isomerase alone (Fig. 4C; green), or simultaneous colocalization of these two antigens (Fig. 4D). Scale bar= 25 μm. **Fig. 4E.** Subcellular fractionation of ERAB nucleic acid-transfected human neuroblastoma cells. Transfected cells ($5x10^8$) were pelletted and fractionated in a series of sucrose steps (38%, 30% and 20%) by ultracentrifugation (100,000 for 3 hrs). Layered (Fig. 4E lanes 1-4) and pelletted fractions (Fig. 4E lane 5) were subjected to Western blotting using either anti-ERAB polypeptide IgG (upper panel) or anti-GRP78 IgG (lower panel). Lanes correspond to cytosol (Fig. 4E lane 1), plasma membrane (Fig. 4E lane 2), Golgi apparatus (Fig. 4E lane 3), and endoplasmic reticulum (Fig. 4E lanes 4-5). **Figs. 4F and 4G.** Effect of exogenous amyloid-beta peptide(1-42) on ERAB polypeptide distribution. Neuroblastoma cells were incubated in buffer alone or in the presence of amyloid-beta peptide(1-42; 1 μM), and the distribution of ERAB polypeptide was determined by immunofluorescence with anti-ERAB polypeptide IgG. The antigen becomes localized in packet apparently apposed to plasma membrane. Scale bars E= 31 μm and F= 34 μm. **Figs. 4H and 4I.** Co-immunoprecipitation of ERAB polypeptide and amyloid-beta peptide. Neuroblastoma cells ($2x10^7$) were incubated with ^{125}I-amyloid-beta peptide (100 nM) for 6 hrs at 37°C, unbound tracer was removed by extensive washing followed by dissolution of cells in lysis buffer (19). Immunoprecipitation was performed with anti-ERAB polypeptide IgG (10 μg/ml; lane 2) or nonimmune IgG (10 μg/ml; lane 1),

and samples were subjected to tris-tricine gel electrophoresis (Fig. 4I). Alternatively, neuroblastoma cells ($2x10^7$) were incubated with ^{125}I-amyloid-beta peptide (100 nM) for 6 hrs at 37°C, disuccinimidyl suberate (DSS; 0.2 mM; Pierce) was added for 30 min at 25°C, cells were washed extensively to remove free ^{125}I-amyloid-beta peptide and nonreacted DSS, followed by dissolution of cells in lysis buffer and processing of samples as above (samples were run on nonreduced SDS-PAGE, 10%). In lane 1, immunoprecipitation was performed with nonimmune IgG and in lane 2 anti-ERAB polypeptide IgG was employed. Methods: For indirect immunofluorescence, cells were plated on coverslips, fixed in formaldehyde (3.7%) for 15 min, and permeabilized in Triton X-100 (0.5%) for 5 min. For co-staining, coverslips were sequentially incubated for periods of 1-1.5 hrs at room temperature with monoclonal anti-protein disulfide isomerase (StressGen), anti-mouse FITC (Pierce), rabbit anti-ERAB polypeptide IgG and anti-rabbit TRITC IgG (Pierce). Samples were visualized on a Leica TCS confocal microscope. For subcellular fractionation studies, human neuroblastoma cells were transfected with pcDNA3-ERAB polypeptide encoding vector using the lipofectamine method (19), and fractionated as described following freeze thawing, nitrogen cavitation, homogenization and ultracentrifugation (63).

Figures 5A, 5B, 5C, 5D, 5E, and 5F. ERAB polypeptide contributes to amyloid-beta peptide-induced cellular toxicity in ERAB nucleic acid-transfected COS cells. Fig. 5A. Immunoblotting of mock-transfected (Fig. 54 lane 1) or ERAB nucleic acid-transfected COS cells (Fig. 5A lane 2; in each case 50 μg protein/lane was loaded) using anti-ERAB polypeptide IgG (3 μg/ml). Protein extracts were subjected to SDS-PAGE (12%; reduced) followed by immunoblotting as described above. **Fig. 5B.** ERAB nucleic acid-transfected COS cells displayed enhanced suppression of MTT reduction in the presence of amyloid-beta peptide. COS cells were transiently transfected with the pcDNA3-ERAB vector or

pcDNA3 alone, and, 24 hrs later (at which time ERAB polypeptide is expressed at high levels), amyloid-beta peptide (2 μM) was added for 24 hrs at 37°C. Then, MTT reduction was determined (36); the mean ± SEM of six replicate determinations is shown. * indicates $p<0.05$. **Figs. 5C, 5D, 5E, and 5F.** COS cells were transfected with either vector alone (Fig. 5C), pcDNA3-ERAB vector (Fig. 5D) or pcDNA3-amyloid-beta peptide(1-42; Fig. 5E), or were co-transfected with pcDNA3-ERAB vector and pcDNA3-amyloid-beta peptide(1-42) vectors (Fig. 5F). Overexpression of ERAB polypeptide and/or amyloid-beta peptide was observed in each case, and cultures were photographed at that time. Marker bar= 138 μm. Methods: Transfection was achieved using pcDNA3 vectors bearing either the cDNA for ERAB polypeptide and/or amyloid-beta peptide (A4 corresponding to residues 1-42) using lipofectamine according to the manufacturer's instructions. Maximal cellular expression of ERAB polypeptide and amyloid-beta peptide was observed 24-72 hrs later. MTT reduction was performed and photomicrographs obtained as described (19,36).

Figures 6A, 6B, 6C, 6D, 6E, 6F, and 6G. ERAB polypeptide contributes to amyloid-beta peptide-induced cytotoxicity in neuroblastoma cells. A-B. Introduction of anti-ERAB polypeptide and nonimmune $F(ab')_2$ into neuroblastoma cells (SK-N-SH). $F(ab')_2$ fragments derived from either anti-ERAB polypeptide IgG (Fig. 6A; prepared according to the manufacturer's instructions; Pierce) or nonimmune IgG (Fig. 6B; 13 μg in each case) were introduced into neuroblastoma cells using lipofectamine as described (65). Immunostaining demonstrates cellular association of the $F(ab')_2$ fragments in each case. Scale bar = 18 μm. **Fig. 6C.** Effect of anti-ERAB polypeptide $F(ab')_2$ on amyloid-beta peptide-induced suppression of MTT reduction. Anti-ERAB polypeptide or nonimmune $F(ab')_2$ was introduced into SK-N-SH cells as above, and, 24 hrs later, cultures were incubated with the indicated concentrations of amyloid-beta peptide for 20 hrs at 37°C. The mean ± SEM of six replicates is shown. *

indicates $p<0.05$. **Figs. 6D, 6E, 6F, and 6G.** Effect of anti-ERAB polypeptide $F(ab')_2$ on amyloid-beta peptide-induced morphologic changes. Anti-ERAB polypeptide or nonimmune $F(ab')_2$ was introduced into SK-N-SH cells, cultures were incubated with amyloid-beta peptide (1-42; 1 μM), and were then photographed: Fig. 6D, untreated cultures; Fig. 6E, cultures exposed to amyloid-beta peptide; Fig. 6F, cultures into which anti-ERAB polypeptide $F(ab')_2$ was introduced using liposomes then exposed to amyloid-beta peptide; and Fig. 6G, cultures into which nonimmune $F(ab')_2$ was introduced using liposomes then exposed to amyloid-beta peptide. Scale bar= 138 μm.

Figure 7. Overexpression of a mutant form of beta-amyloid precursor protein (βAPP) which causes increased expression of amyloid-beta protein (βA), and ERAB causes increased toxicity (DNA fragmentation) to COS cells.

Detailed Description

As used herein, a polypeptide is an amino acid polymer of amino acids linked together by peptide bonds; a nucleic acid is a deoxyribonucleotide or ribonucleotide polymer of nucleotides linked together by phosphodiester bonds; an antisense nucleic acid is a nucleic acid that is the reverse complement of another nucleic acid which may be capable of inhibiting transcription or translation of the other nucleic acid; a vector is a nucleic acid that can self replicate; a host cell is a cell containing a vector; an antibody is a protein with a specific affinity for an antigen; an antibody binding fragment (F(ab)) is a portion of an antibody which binds to the antigen; and a transgenic animal is an animal whose cells comprise a foreign nucleic acid or naturally occurring nucleic acid in unnatural quantities or frequency (84).

As used herein, the term "ERAB" is an abbreviation for "endoplasmic reticulum associated amyloid-beta peptide binding." The term "ERAB polypeptide" refers to an endoplasmic reticulum associated amyloid-beta binding polypeptide. ERAB polypeptide has a biological activity characterized by the capability of binding to amyloid-beta peptide. ERAB polypeptide is also characterized by hydroxysteroid dehydrogenase activity. The term "ERAB nucleic acid" refers to a nucleic acid encoding ERAB polypeptide. The term "ERAB antisense nucleic acid" refers to the noncoding nucleic acid corresponding to an ERAB nucleic acid. The term "ERAB antibody" refers to an antibody to the antigen "ERAB polypeptide." The term "ERAB antibody F(ab)" refers to the antigen binding fragment of an ERAB antibody.

This invention provides an ERAB polypeptide. In an embodiment, the ERAB polypeptide has a theoretical weight of about 27,000 daltons. In another embodiment, the ERAB polypeptide has an SDS-PAGE-determined weight of about

29,000 daltons. One embodiment of an ERAB polypeptide is human ERAB polypeptide or a murine ERAB polypeptide. In a specific embodiment a human ERAB polypeptide has the amino acid sequence shown in Figure 1D (Seq. Id. No. 2). Another embodiment may be a portion of the ERAB polypeptide amino acid sequence shown in Figure 1D (Seq. Id. No. 2).

The present invention includes variants of ERAB polypeptide which encompass proteins and peptides with amino acid sequences having seventy percent or eighty percent homology with the amino acid sequence shown in Figure 1D (Seq. Id. No. 2) or a naturally occurring ERAB polypeptide. More preferably the sequence homology is at least ninety percent, or at least ninety-five percent.

Variants in amino acid sequence of ERAB polypeptide are produced when one or more amino acids in naturally occurring ERAB polypeptide is substituted with a different natural amino acid, an amino acid derivative, a synthetic amino acid, an amino acid analog or a non-native amino acid. Particularly preferred variants include homologous ERAB polypeptide of humans or of different species of animals. Variants of an ERAB polypeptide may include biologically active fragments of naturally occurring ERAB polypeptide, wherein sequences of the variant differ from the wild type ERAB polypeptide sequence by one or more conservative amino acid substitutions. Such substitutions typically would have minimal influence on the secondary structure and hydrophobic nature of the ERAB polypeptide. Variants may also have sequences which differ by one or more non-conservative amino acid substitutions, deletions or insertions which do not abolish the biological activity associated with ERAB polypeptide. Conservative substitutions (substituents) typically include the substitution of one amino acid for another with similar characteristics such as substitutions within the following groups: valine, glycine; glycine, alanine; valine, isoleucine; aspartic acid, glutamic acid; asparagine,

glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine. The non-polar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid.

Other conservative substitutions are exemplified Table 1, and yet others are described by Dayhoff in the Atlas of Protein Sequence and Structure (1988) (86).

Table 1: Conservative Amino Acid Replacements

For Amino Acid	Code	Replace with any of
Alanine	A	D-Ala, Gly,beta-ALa, L-Cys,D-Cys
Arginine	R	D-Arg, Lys,homo-Arg, D-homo-Arg, Met,D-Met, Ile, D-Ile, Orn, D-Orn
Asparagine	N	D-Asn,Asp,D-Asp,Glu,D-Glu, Gln,D-Gln
Aspartic Acid	D	D-Asp,D-Asn,Asn, Glu,D-Glu, Gln, D-Gln
Cysteine	C	D-Cys, S-Me-Cys,Met,D-Met,Thr, D-Thr
Glutamine	Q	D-Gln,Asn, D-Asn,Glu,D-Glu,Asp, D-Asp
Glutamic Acid	E	D-Glu,D-Asp,Asp, Asn, D-Asn, Gln, D-Gln
Glycine	G	Ala, D-Ala,Pro, D-Pro, Beta-Ala, Acp
Isoleucine	I	D-Ile, Val, D-Val, Leu, D-Leu, Met, D-Met

Leucine	L	D-Leu, Val, D-Val, Met, D-Met
Lysine	K	D-Lys,Arg, D-Arg, homo-Arg, D-homo-Arg, Met, D-Met, Ile, D-Ile, Orn, D-Orn
Methionine	M	D-Met, S-Me-Cys, Ile, D-Ile, Leu, D-Leu, Val, D-Val, Norleu
Phenylalanine	F	D-Phe,Tyr, D-Thr,L-Dopa,His,D-His, Trp, D-Trp, Trans 3,4 or 5-phenylproline, cis 3,4 or 5 phenylproline
Proline	P	D-Pro, L-I-thioazolidine-4-carboxylic acid, D- or L-1-oxazolidine-4-carboxylic acid
Serine	S	D-Ser, Thr, D-Thr, allo-Thr, Met, D-Met, Met(O), D-Met(O), Val, D-Val
Threonine	T	D-Thr, Ser, D-Ser, allo-Thr, Met, D-Met, Met(O) D-Met(O), Val, D-Val
Tyrosine	Y	D-Tyr,Phe, D-Phe, L-Dopa, His, D-His
Valine	V	D-Val, Leu,D-Leu,Ile,D-Ile, Met, D-Met

Other variants within the invention are those with modifications which increase peptide stability. Such variants may contain, for example, one or more non-peptide bonds (which replace the peptide bonds) in the peptide sequence. Also included are: variants that include residues other than naturally occurring L-amino acids, such as D-amino acids or non-naturally occurring or synthetic amino acids such as beta or gamma amino acids. Incorporation of D- instead of L-amino acids into the polypeptide may increase its resistance to proteases. See, e.g., U.S. Patent 5,219,990 (87).

An ERAB polypeptide variant of this invention includes an ERAB polypeptide varied by changes such as insertions, deletions and substitutions, either conservative or nonconservative where such changes might provide for certain advantages in their use such as increased potency, bioavailability, stability or decreased toxicity or degredation under physiological conditions.

In other embodiments, variants with amino acid substitutions which are less conservative may also result in desired derivatives of ERAB polypeptide, e.g., by causing desireable changes in charge, conformation and other biological properties. Such substitutions would include for example, substitution of hydrophilic residue for a hydrophobic residue, substitution of a cysteine or proline for another residue, substitution of a residue having a small side chain for a residue having a bulky side chain or substitution of a residue having a net positive charge for a residue having a net negative charge. When the result of a given substitution cannot be predicted with certainty, the derivatives may be readily assayed according to the methods disclosed herein to determine the presence or absence of the desired characteristics.

Just as it is possible to replace substituents of the scaffold (i.e., amino acids which make up the ERAB polypeptide), it is also possible to substitute functional groups which decorate the scaffold with groups characterized by similar features (i.e., R-groups which are part of each amino acid). These substitutions will initially be conservative, i.e., the replacement group will have approximately the same size, shape, hydrophobicity and charge as the original group. Non-sequence modifications may include, for example, in vivo or in vitro chemical derivatization of portions of naturally occurring ERAB polypeptide, as well as changes in acetylation, methylation, phosphorylation, carboxylation or glycosylation.

In a further embodiment the ERAB polypeptide is modified by chemical modifications in which activity is preserved. For example, the ERAB polypeptide may be amidated, sulfated, singly or multiply halogenated, alkylated, carboxylated, or phosphorylated. The ERAB polypeptide may also be singly or multiply acylated, such as with an acetyl group, with a farnesyl moiety, or with a fatty acid, which may be saturated, monounsaturated or polyunsaturated. The fatty acid may also be singly or multiply fluorinated. The invention also includes methionine analogs of ERAB polypeptide, for example the methionine sulfone and methionine sulfoxide analogs. The invention also includes salts of ERAB polypeptide, such as ammonium salts, including alkyl or aryl ammonium salts, sulfate, hydrogen sulfate, phosphate, hydrogen phosphate, dihydrogen phosphate, thiosulfate, carbonate, bicarbonate, benzoate, sulfonate, thiosulfonate, mesylate, ethyl sulfonate and benzensulfonate salts.

Variants of ERAB polypeptide may also include peptidomimetic compounds of ERAB polypeptide. Such compounds are well known to those of skill in the art and are produced through the substitution of certain R groups or amino acids in the protein with non-natural replacements. Such substitutions may increase the stability, bioavailability, or activity of such ERAB polypeptide compound.

The present invention provides an ERAB nucleic acid. One embodiment of an ERAB nucleic acid is a recombinant ERAB nucleic acid. A further embodiment of an ERAB nucleic acid is human ERAB nucleic acid. An embodiment of an ERAB nucleic acid is an ERAB deoxyribonucleic acid (DNA), a recombinant nucleic acid, or an ERAB ribonucleic acid. A specific embodiment of human ERAB nucleic acid is a cDNA sequence which corresponds to the sequence shown in Figure 1D from nucleotide 19 to nucleotide 801 or a portion thereof. One embodiment of the present invention is murine ERAB nucleic acid.

The invention also provides variants of the ERAB nucleic acid. Variants of ERAB nucleic acid may include nucleic acid sequences having seventy percent or eighty percent homology with the ERAB nucleic acid sequence shown in Figure 1D. More preferably the sequence homology is at least ninety percent, or at least ninety-five percent. Variants of ERAB nucleic acid may include alleles of ERAB nucleic acid, truncated ERAB nucleic acid, alternatively spliced ERAB nucleic acid, ERAB nucleic acid with silent mutations or conservative mutations. A variant of ERAB nucleic acid may also include portions of naturally occuring ERAB nucleic acid or ERAB nucleic acid with deletions of some regions of the nucleic acid.

The present invention provides an ERAB antisense nucleic acid. In one embodiment, the ERAB antisense nucleic acid is human ERAB antisense nucleic acid or a portion of human ERAB antisense nucleic acid. In a further embodiment, the human ERAB antisense nucleic acid sequence in the 3′ to 5′ direction corresponds to the nucleic acid sequence shown in Figure 1D in the 5′ to 3′ direction.

The antisense nucleic acid may be capable of inhibiting translation or transcription of an ERAB nucleic acid or an mRNA derived from an ERAB nucleic acid. The antisense sequence to ERAB nucleic acid may be linked to a replicable vector. The ERAB antisense nucleic acid may be administered to a subject in order to treat a neurodegenerative condition. The ERAB antisense nucleic acid may inhibit the binding of an ERAB polypeptide in a cell to amyloid-beta peptide.

The present invention provides for a replicable vector containing an ERAB nucleic acid or a variant thereof. In one embodiment the vector is a prokaryotic expression vector, a yeast expression vector, a baculovirus expression, a mammalian expression vector, an episomal mammalian expression vector, pKK233-2, pEUK-C1, pREP4, pBlueBacHis A,

pYES2, pSE280, or pEBVHis. Methods for the using these replicable vectors may be found in Sambrook et al., 1989, (79) or Kriegler, 1990, (88).

The present invention provides for an host cells comprising an ERAB nucleic acid or variant thereof. In one embodiment the host cell is a eukaryotic cell, a somatic cell, a germ cell, a neuronal cell, a myocyte, a prokaryotic cell, a virus packaging cell, or a stem cell.

This invention provides the production of biologically active ERAB polypeptide in a prokaryotic expression system, a eukaryotic expression system, a mammalian expression system, a baculovirus expression system, an insect expression system or a yeast expression system. This production may provide for the post-translational modifications which exist in naturally occurring ERAB polypeptide. For protocols describing bacterial expression of mammalian proteins, see Sambrook et al, 1989 (79).

The present invention provides a transgenic animal whose cells express ERAB nucleic acid or a variant thereof. In one embodiment, the cells of the transgenic animal contain human ERAB nucleic acid. In another embodiment the transgenic animal is a non-human mammal. In another embodiment, the non-human mammal is a mouse, monkey, a dog, a swine, a fowl or a rat. In a specific embodiment, the transgenic animal may be a non-human mammal whose germ and somatic cells contain a human ERAB nucleic acid, the nucleic acid having been stably introduced into the non-human mammal at the single cell stage or an embryonic stage, and wherein the nucleic acid is linked to a promoter and integrated into the genome of the non-human mammal. The transgenic animal may be a descendant of an animal containing cells which have been transfected with ERAB nucleic acid or a variant thereof.

The present invention provides an antibody which is an antibody or a portion thereof which specifically binds to an ERAB polypeptide or variant thereof. In one embodiment the antibody is a polyclonal antibody, a monoclonal antibody or an antigen binding fragment (F(ab)) of antibody. In another embodiment, the antibody comprises an antibody which binds to human ERAB polypeptide or to murine ERAB polypeptide or to a portion of the polypeptide shown in Figure 1D.

One of ordinary skill in the art would know how to make and use such an antibody. The present invention provides for a fragment of an antibody to ERAB polypeptide, namely [$F(ab')_2$] which is capable of blocking the binding activity of ERAB polypeptide to amyloid-beta peptide. Such antibodies may be introduced into cells and may be capable of suppressing the toxicity or cell stress due to amyloid-beta peptide. The antibody may be a small synthetic peptide. The antibody may block the binding of ERAB polypeptide to amyloid-beta peptide by binding to a binding site on an ERAB polypeptide or on an amyloid-beta peptide.

The present invention provides a method to determine the ability of an agent to inhibit binding of ERAB polypeptide to amyloid-beta peptide which comprises: (a) incubating ERAB polypeptide and the agent with amyloid-beta peptide; (b) determining the amount of amyloid-beta peptide bound to ERAB polypeptide; and (c) comparing the determined amount with an amount determined when the agent is absent, thereby determining the ability of the agent to inhibit the binding of ERAB polypeptide to amyloid-beta peptide. The agent may be a peptide, a peptidomimetic compound, a nucleic acid, or a small molecule. In one embodiment, the agent may be marked with a reporter molecule. In another embodiment, the ERAB polypeptide or amyloid-beta peptide may be marked with a reporter molecule.

A "reporter molecule", as defined herein, is a molecule or atom which, by its chemical nature, provides an identifiable

signal allowing detection of the level of production of a particular protein, mRNA or transcript. Detection can be either qualitative or quantitative. The present invention provides for the use of any commonly used reporter molecule including radionucleotides, enzymes, biotins, psoralens, fluorophores, chelated heavy metals, and luciferase. The most commonly used reporter molecules are either enzymes, fluorophores, or radionucleotides linked to the nucleotides which are used in transcription. Commonly used enzymes include horseradish peroxidase, alkaline phosphatase, glucose oxidase and α-galactosidase, β-glucuronidase among others. The substrates to be used with the specific enzymes are generally chosen because a detectably colored product is formed by the enzyme acting upon the substrate. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for horseradish peroxidase, 1.2-phenylenediamine, 5-aminosalicylic acid or toluidine are commonly used. The probes so generated have utility in the detection of a specific ERAB nucleic acid target in, for example, Southern analysis, Northern analysis, *in situ* hybridization to tissue sections or chromosomal squashes and other analytical and diagnostic procedures. The methods of using such hybridization probes are well known and some examples of such methodology are provided by Sambrook et al, 1989 (79). The reporter molecule may be linked to a probe for an ERAB nucleic acid or an ERAB antibody and may be used for prognosis determination in a subject for neurodegenerative conditions.

An example of a model agent which would inhibit the binding of ERAB polypeptide to amyloid-beta peptide includes an $F(ab')_2$ prepared from anti-ERAB polypeptide-derived IgG, IgM, IgE or any other immunoglobulin molecule. The $F(ab')_2$ fragment of the antibody may be used instead of the whole IgG molecule since it is to be introduced into a cell and the whole antibody would likely have nonspecific effects due to its Fc region. The agent described herein may work when it is introduced into cells. Such introduction may be

achieved via liposomes, Lipofectin, calcium precipation and other methods. Such methods of introducing compounds, protein, nucleic acid and polypeptides into cells are well known to one of ordinary skill in the art.

The present invention also provides assays for the determination or identification of compounds capable of inhibiting the binding of ERAB polypeptide to amyloid-beta peptide. Such an assay may include the binding of radiolabelled ^{125}I-amyloid-beta peptide or labelled amyloid-beta peptide of any form to ERAB polypeptide. The amyloid-beta peptide or the ERAB polypeptide may be bound to a solid support. The amyloid-beta peptide or the ERAB polypeptide may be a fusion protein. The assay may also detect the binding of amyloid-beta peptide to immobilized ERAB polypeptide. Compounds may be added to the reaction mixture and detection of binding or the inhibition of binding may be determined. Such assays are useful for the identification of compounds which could be used as agents capable of inhibiting the binding of the amyloid-beta peptide with ERAB polypeptide. Such an agent may be used as a drug or a pharmaceutical composition or a preventative drug to ameliorate, prevent, treat or improve neurodegenerative conditions in subjects. The subjects may be humans, animals, mice, dogs, cats or other animals. Such animals may be useful human model systems.

The present invention provides a method to determine the prognosis of a neurodegenerative condition in a subject which comprises: (a) obtaining a sample from the subject; (b) contacting the sample with a reagent capable of binding to an element in the sample, the element comprising ERAB nucleic acid or ERAB polypeptide or variant thereof, under conditions such that the reagent binds only if the element is present in the sample; and (c) detecting the presence of the reagent bound to the element and thereby determining the prognosis of the neurodegenerative condition of the subject.

In an embodiment of this invention the subject is a mammal. Examples of suitable mammalian subjects include, but are not limited to, murine animals such as mice and rats, hamsters, rabbits, goats, pigs, sheep, cats, dogs, cows, monkeys and humans.

The present invention further provides a method to treat or cure a neurodegenerative condition in a subject which comprises administering to the subject a pharmaceutically acceptable composition, such composition comprising an agent capable of inhibiting the interaction or binding of ERAB polypeptide with amyloid-beta peptide in the subject, in an amount effective to treat or cure the neurodegenerative condition in the subject.

The agent or compound determined or identified by the above assays or methods may be used as a drug or in a pharmaceutical composition. Such drug or pharmaceutical composition may be administered to subjects suffering from a neurodegenerative condition. The administration times, concentrations, and duration would be known by one of ordinary skill in the art and would depend on a number of discernable factors including the body weight of the subject, the type of the neurodegenerative condition, the stage of the condition, the age of the subject, the health of the subject, among other factors.

The present invention provides a pharmaceutical composition which comprises an agent capable of inhibiting binding of amyloid-beta peptide to ERAB polypeptide and a pharmaceutically acceptable carrier thereof. The carrier may be a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

In the practice of any of the methods of the invention or preparation of any of the pharmaceutical compositions, a "therapeutically effective amount" is an amount which is effective to alter the binding of ERAB polypeptide to

amyloid-beta peptide and thus treat the neurodegenerative condition of the subject. Accordingly, the effective amount will vary with the subject being treated, as well as the condition to be treated. For the purposes of this invention, the methods of administration are to include, but are not limited to, administration cutaneously, subcutaneously, intravenously, parenterally, orally, topically, or by aerosol.

As used herein, the term "suitable pharmaceutically acceptable carrier" encompasses any of the standard pharmaceutically accepted carriers, such as phosphate buffered saline solution, water, emulsions such as an oil/water emulsion or a triglyceride emulsion, various types of wetting agents, tablets, coated tablets and capsules. An example of an acceptable triglyceride emulsion useful in intravenous and intraperitoneal administration of the compounds is the triglyceride emulsion commercially known as Intralipid®.

Typically such carriers contain excipients such as starch, milk, sugar, certain types of clay, gelatin, stearic acid, talc, vegetable fats or oils, gums, glycols, or other known excipients. Such carriers may also include flavor and color additives or other ingredients.

By means of well-known techniques such as titration and by taking into account the observed pharmacokinetic characteristics of the agent in the individual subject, one of skill in the art can determine an appropriate dosing regimen (85).

The present invention further provides for a method for treating a neurodegenerative condition in a subject which comprises administering to the subject an amount of an agent effective to inhibit ERAB binding to amyloid-beta peptide thereby treating the neurodegenerative condition.

The neurodegenerative condition may comprise Alzheimer's disease, Down's syndrome, Parkinson's disease, Huntington's disease, schizophrenia, multiple sclerosis, senility, stroke, demyelinating disease, or a heritable condition.

As used herein, the term "neurotoxicity" encompasses the negative metabolic, biochemical and physiological effects on a neuronal cell which may result in a debilitation of the neuronal cellular functions. Such functions may include memory, learning, perception, neuronal electrophysiology (ie. action potentials, polarizations and synapses), synapse formation, both chemical and electrical, channel functions, neurotransmitter release and detection and neuromotor functions. Neurotoxicity may include neuronal cytotoxicity.

As used herein, the term "neuronal degeneration" encompasses a decline in normal functioning of a neuronal cell. Such a decline may include a decline in memory, learning, perception, neuronal electrophysiology (ie. action potentials, polarizations and synapses), synapse formation, both chemical and electrical, channel functions, neurotransmitter release and detection and neuromotor functions. In the present invention, the subject may be a mammal or a human subject. The administration may be intralesional, intraperitoneal, intramuscular or intravenous injection; infusion; liposome-mediated delivery; gene bombardment; topical, nasal, oral, anal, ocular or otic delivery.

The present invention provides for a pharmaceutical composition which comprises an antibody specific for ERAB polypeptide and a pharmaceutically acceptable carrier thereof. The present invention also provides for a pharmaceutical composition which comprises an antisense nucleic acid corresponding to ERAB nucleic acid or a variant thereof and a pharmaceutically acceptable carrier thereof. The present invention provides for a pharmaceutical composition which comprises a portion of an ERAB polypeptide

sufficient to inhibit binding of ERAB polypeptide to amyloid-beta peptide in a cell and a pharmaceutically acceptable carrier.

Also provided by the invention are pharmaceutical compositions comprising therapeutically effective amounts of products of the invention together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvants and/or carriers. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. [One example would be a sufficient amount to treat or ameliorate a neurodegenerative condition in a subject, such as Alzheimer's Disease.] Such compositions are liquids or lyophilized or otherwise dried formulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (e.g., Tween 20, Tween 80, Pluronic F68, bile acid salts), solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polyglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erythrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of *in vivo* release, and rate of *in vivo* clearance. The choice of compositions will depend on the physical and chemical properties of the exact protein or compound having the activity of inhibiting the neurdegenerative condition. For example, a product derived from a membrane-bound form of a protein may require a formulation containing detergent.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g., poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal, oral, injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial or intralesional.

Another embodiment of this invention is a method for evaluating the ability of an agent to inhibit binding of an amyloid-beta peptide with an ERAB polypeptide in a cell which includes: a) contacting the cell with the agent; b) determining the amount of amyloid-beta peptide bound to ERAB polypeptide in the cell; and c) comparing the amount of bound amyloid-beta peptide to ERAB polypeptide determined in step b) with the amount determined in the absence of the agent, thus evaluating the ability of the agent to inhibit the binding of amyloid-beta peptide to ERAB polypeptide in the cell.

The agent may be capable of specifically binding to the amyloid-beta peptide or to ERAB polypeptide. The agent may bind to amyloid-beta peptide at the site where ERAB polypeptide interacts. The agent may be a soluble portion of an ERAB polypeptide which is not associated with a cell membrane. The agent may be bound to a solid support. The agent may be expressed on the surface of a cell or may be produced inside of the cell.

Portions of the compound of the invention may be "labeled" by association with a detectable marker substance (e.g.,

radiolabeled with ^{125}I or biotinylated) to provide reagents useful in detecting and quantifying the presence of ERAB polypeptide or ERAB nucleic acid or their derivatives in solid tissue and fluid samples such as blood, cerebral spinal fluid or urine.

When administered, agents are often cleared rapidly from the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent injections of relatively large doses of bioactive agents may by required to sustain therapeutic efficacy. Agents modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified agents (80, 82, 83). Such modifications may also increase the agent's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the agent, and greatly reduce the immunogenicity and reactivity of the agent. As a result, the desired *in vivo* biological activity may be achieved by the administration of such polymer-agent adducts less frequently or in lower doses than with the unmodified agent.

Attachment of polyethylene glycol (PEG) to agents or compounds is particularly useful because PEG has very low toxicity in mammals (81). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treatment of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicity and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species without the risk of triggering a severe immune response. The agent may be delivered in a microencapsulation device so

as to reduce or prevent an host immune response against the agent or against cells which may produce the agent. The agent of the present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Polymers such as PEG may be conveniently attached to one or more reactive amino acid residues in a protein such as the alpha-amino group of the amino terminal amino acid, the epsilon amino groups of lysine side chains, the sulfhydryl groups of cysteine side chains, the carboxyl groups of aspartyl and glutamyl side chains, the alpha-carboxyl group of the carboxy-terminal amino acid, tyrosine side chains, or to activated derivatives of glycosyl chains attached to certain asparagine, serine or threonine residues.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N-hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonate. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

The present invention incorporates U.S. Patent Nos. 5,446,128, 5,422,426 and 5,440,013 in their entireties as references which disclose the synthesis of peptidomimetic compounds and methods related thereto. The compounds of the present invention may be synthesized using these methods.

In addition to the agents disclosed herein having naturally-occurring amino acids with peptide or unnatural linkages, the present invention also provides for other structurally similar compounds or agents such as polypeptide analogs with

unnatural amino acids in the compound. Such compounds may be readily synthesized on a peptide synthesizer available from vendors such as Applied Biosystems, Dupont and Millipore.

The present invention provides for a method for treating a subject suffering from a neurodegenerative condition which comprises administering to the subject a compound capable of inhibiting binding of ERAB polypeptide to amyloid-beta peptide in the subject and thereby treating the subject suffering from a neurodegenerative condition. This present invention encompasses gene therapy, especially for patients with acute neurodegenerative conditions.

There are several protocols for human gene therapy which have been approved for use by the Recombinant DNA Advisory Committee (RAC) which conform to a general protocol of target cell infection and administration of transfected cells (66, 69, 70). In addition, U.S. Patent No. 5,399,346 (Anderson, W. F. et al., issued March 21, 1995) describes procedures for retroviral gene transfer (68). The contents of these support references are incorporated in their entirety into the subject application. Retroviral-mediated gene transfer requires target cells which are undergoing cell division in order to achieve stable integration hence, cells are collected from a subject often by removing blood or bone marrow. It may also be possible to remove cerebrospinal fluid or brain tissue. It may be necessary to select for a particular subpopulation of the originally harvested cells for use in the infection protocol. For example, white blood cells may be separated from red blood cells using an apheresis procedure. The white cells may then be placed in culture with mitogens, such as IL-2 or OKT3 (which can stimulate T-cell proliferation) for approximately 18 hrs. Then, a retroviral vector containing the gene(s) of interest would be mixed into the culture medium. The vector binds to the surface of the subject's cells, enters the cells and inserts the gene of interest

randomly into a chromosome. The gene of interest is now stably integrated and will remain in place and be passed to all of the daughter cells as the cells grow in number. The cells may be expanded in culture for a total of 9-10 days before reinfusion (69, 70). As the length of time the target cells are left in culture increases, the possibility of contamination also increases, therefore a shorter protocol would be more beneficial.

One skilled in the art would recognize that retroviruses, adenoviruses, parvoviruses, and herpes viruses have been used to transfer genes (77).

The present invention also includes a compound which is capable of inhibiting the enzymatic activity of ERAB polypeptide. The compound may inhibit or alter the synergistic effect of amyloid-beta peptide on ERAB polypeptide. The compound may prevent the interaction of ERAB polypeptide with amyloid-beta peptide thereby altering the enzymatic activity of ERAB polypeptide and thus improving a neurodegenerative condition.

Another embodiment of the present invention is a method for alleviating symptoms of a neurodegenerative condition in a subject which includes administering to the subject a compound described hereinabove, the compound being present in an amount effective to inhibit neuronal cell death and thus alleviate the symptoms of the neurodegenerative condition in the subject.

The neurodegenerative condition may be associated with aging, Alzheimer's disease, dentatorubral and pallidolyusian atrophy, Huntington's disease, Machoado-Joseph disease, multiple sclerosis, muscular dystrophy, Parkinson's disease, senility, spinocerebellar ataxia type I, spinobulbar muscular atrophy, stroke, trauma. The subject may be a mammal. The mammal may be a human. The administration may include aerosol delivery; intralesional, intraperitoneal,

intramuscular or intravenous injection; infusion; liposome-mediated delivery; anal, nasal, oral, ocular, otic or topical delivery of the pharmaceutical composition.

In one embodiment, the condition may be associated with degeneration of a neuronal cell in the subject,with formation of an amyloid-beta peptide fibril, with aggregation of amyloid-beta peptide, with infiltration of a microglial cell into a senile plaque, or with activation of a microglial cell by an amyloid-beta peptide, wherein the activation comprises production of cytokines by the microglial cell.

This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

Experimental Details

Example 1: ERAB Polypeptide - A Novel Intracellular amyloid-beta peptide Binding Polypeptide Which Mediates Neurotoxicity In Alzheimer's Disease

Amyloid-beta peptide is neurotoxic and is implicated in the pathogenesis of Alzheimer's disease(1-6). Using the yeast two-hybrid system, a novel endoplasmic reticulum-associated amyloid-beta binding polypeptide (ERAB polypeptide), putatively identified as a hydroxysteroid dehydrogenase, was shown to be expressed in normal tissues and, at higher levels, in affected neurons in the brain in Alzheimer's disease. ERAB polypeptide, constitutively produced by neuroblastoma cells was co-immunoprecipitated with amyloid-beta peptide; following exposure of cultures to exogenous amyloid-beta peptide, ERAB polypeptide was rapidly redistributed to the inner aspect of the plasma membrane. Toxicity of amyloid-beta peptide to neuroblastoma cells was prevented by liposome-mediated introduction of anti-ERAB polypeptide $F(ab')_2$ antibody into the cell and was enhanced by overexpression of ERAB polypeptide in COS cells. These data implicate a novel intracellular binding protein, ERAB polypeptide, as an integral participant in amyloid-beta-cellular interactions, and suggest a mechanism whereby amyloid-beta peptide could gain access to cellular compartments containing ERAB polypeptide. There may be other cell surface molecules capable of tethering amyloid-beta peptide, as indicated by the incomplete blockade of amyloid-beta peptide-cell surface interaction by preventing access to RAGE, and the characterization of other molecules which also bind amyloid-beta peptide, such as the type I macrophage scavenger receptor present on microglia (20-21).

Another potentially important mechanism of cytotoxicity of amyloid-beta peptide species could result from their engagement by intracellular targets. Intracellular

accumulation of amyloidogenic amyloid-beta peptide has been observed in several cell types(22-27); this could reflect aberrant cellular processing with excessive amounts of amyloid-beta peptide (≈4 kDa) generated within the cell, or from uptake of amyloid-beta peptide released into the medium. In addition, larger C-terminal fragments of APP might in themselves exert neurotoxic effects. These data led to consideration of the possibility that amyloid-beta peptide and amyloid-beta peptide-containing polypeptides might act at specific intracellular sites relevant to the neurotoxicity underlying AD. Using the yeast two-hybrid system(28-29), an intracellular amyloid-beta peptide binding protein in the endoplasmic reticulum which mediates, at least in part, the ability of amyloid-beta peptide to induce cellular toxicity was identified. These data provide the first direct evidence for an intracellular binding site for amyloid-beta peptide, and indicate that intracellular amyloid-beta peptide, in addition to amyloid-beta peptide in the extracellular space, can exert deleterious effects on cellular functions.

Identification and cloning of ERAB.

The yeast two-hybrid system was used to screen human brain and HeLa cell Matchmaker™ cDNA libraries for encoded proteins capable of binding to the fusion protein containing amyloid-ß-peptide. In order to screen the libraries, the portion of human amyloid precursor protein sequence encoding amyloid-beta peptide was subcloned into the yeast expression vector pGBT9 containing the GAL4 DNA-binding domain (**Fig. 1A**). Out of $3x10^6$ clones screened from each library, one positive clone was identified from human brain and three positive clones from HeLa cells. By DNA sequencing, all four clones (designated as ERAB, see below) gave the same cDNA sequence, except for minor variations at the 5'-end cloning site. Strong ß-galactosidase activity was observed when constructs containing nucleic acid encoding amyloid-beta peptide(1-42 and 3-42) and ERAB polypeptide were co-

expressed (**Figs. 1B and 1C**). The specificity of ERAB polypeptide interaction with amyloid-beta peptide in this system was further demonstrated by lack of ß-galactosidase activity when the amyloid-beta peptide-related construct or the ERAB nucleic acid construct was replaced with vector-only constructs (Figs. 1B and 1C). The construct pGBT/APP-23, which contained ß-amyloid precursor protein (APP)-derived amino acids 655-751 (encoding a peptide starting at residue 3 in amyloid-beta peptide and extending for 95 amino acids towards the C-terminus), showed no ß-galactosidase activity, indicating that the C-terminus of APP prevents amyloid-beta peptide interaction with ERAB polypeptide, suggesting that normally expressed APP is not a ligand for ERAB polypeptide. Using the 5'-rapid amplification of cDNA ends (RACE), the 5'-end of a human ERAB nucleic acid was cloned; the sequence was found to extend about 18 bp upstream of the first AUG and the start codon of the open reading frame was consistent with Kozak consensus sequences(30). The full-length cDNA sequence coded for a predicted 262 amino acid polypeptide (**Fig. 1D**, Seq. Id. Nos. 1 and 2). Comparison with the SWISS-PROT and Protein Data Bank databases, using the FASTA algorithm(31-32), showed amino acid sequences of ERAB polypeptide to most closely resemble the family of short-chain alcohol dehydrogenases, including hydroxysteroid dehydrogenases, Ke6, and acetoacetyl-CoA reductases(33-34). In both cases, ERAB polypeptide exhibited an identity score of 32% and a similarity score of 65%. Alignment of ERAB polypeptide with the 255 amino acids of the nicotinamide adenine dinucleotide (NAD) binding enzyme 3a,20ß-hydroxysteroid dehydrogenase (EC1.1.1.53) of *Streptomyces hydrogenans*, for which the X-ray structure at 2.6Å resolution is available(34), revealed a series of common structural motifs (**Figs. 1E-1 and 1E-2**, ERAB as Seq. Id. No. 3 and 2 BHD as Seq. Id. No. 4); both share the NAD(H) binding domains, as well as the highly conserved sequence YGASK in the bacterial enzyme (residues 152-165), which in the human ERAB polypeptide corresponds to

residues 168-172 (YSASK), putatively assigned as part of the active center of the enzyme.

To study the ability of ERAB polypeptide to bind amyloid-beta peptide, TrcHis-ERAB fusion protein was expressed in E. coli and purified. Immunoblotting with polyclonal antibodies prepared by immunizing an animal with ERAB polypeptide-derived peptides, to produce an anti-ERAB IgG antibody, indicated the presence of the target ERAB polypeptide sequence in the fusion protein, but not in the control fusion protein, TrcHis-chloramphenicol acetyltransferase (CAT)(**Fig. 2A**; lanes 1-2). The specificity of anti-ERAB polypeptide IgG antibody was shown by its recognition of the TrcHis-ERAB fusion protein when the latter was present in crude cell lysates, and disappearance of this band on addition of excess free ERAB polypeptide-derived peptide identical to that used as immunogen (**Fig. 2A**, lane 3). Purified TrcHis-ERAB adsorbed to microtiter wells bound ^{125}I-amyloid-beta peptide (synthetic 1-40) dependent on the amount of immobilized ERAB polypeptide (**Fig. 2B**), and this was blocked by antibody to ERAB polypeptide but not by nonimmune IgG (**Fig. 2C**). No specific binding of ^{125}I-amyloid-beta peptide was observed to wells coated with albumin (Fig. 2B, bar designated "0") or TrcHis-CAT. At one concentration of immobilized TrcHis-ERAB polypeptide, binding of ^{125}I-amyloid-beta peptide was saturable with K_d = 51.6 ± 15.7 nM (**Fig. 2D**). The specificity of freshly prepared ^{125}I-amyloid-beta peptide(1-40) binding to TrcHis-ERAB polypeptide was shown by competition experiments in which excess unlabelled freshly prepared synthetic amyloid-beta peptide(1-40), synthetic amyloid-beta peptide(1-40) incubated for 3 days at 37°C (under conditions promoting aggregation)(6), synthetic amyloid-beta peptide(1-20), and AD-derived amyloid-beta peptide (mainly 1-42) blocked binding, but synthetic amyloid-beta peptide(25-35), scrambled amyloid-beta peptide(25-35), and Arg-Gly-Asp-Ser (Seq. ID. No. 5) had no effect (**Fig. 2E**). Experiments in which TrcHis-ERAB

polypeptide was radioiodinated and amyloid-beta peptide(1-42) was adsorbed to microtiter wells also showed similar specific binding dependent on the concentration of amyloid-beta peptide (**Fig. 2F**). Complementary studies in which a single concentration of amyloid-beta peptide(1-42) was immobilized on wells and varying amounts of ^{125}I-TrcHis-ERAB were added showed dose-dependent binding with K_d= 88.3 ± 28.1 nM (**Fig. 2G**). In contrast, excess unlabelled TrcHis-CAT did not inhibit the binding of ^{125}I-TrcHis-ERAB to microtiter wells with adsorbed amyloid-beta peptide. These data demonstrate that ERAB polypeptide and amyloid-beta peptide interact specifically in the nanomolar range.

Tissue and cellular localization of ERAB

ERAB mRNA was expressed ubiquitously in normal human tissues and brain subregions as a single transcript of ≈1 kb in each case (**Figs. 3A, 3B, and 3C**); the highest levels of ERAB transcripts were in liver and heart, but ERAB nucleic acid was also expressed elsewhere, including normal brain. ERAB antigen, detected with anti-ERAB derived polyclonal antibody, was visualized on SDS-PAGE as a ≈29 kDa band in extracts of normal brain from two individuals (**Fig. 3D**, lanes 3-4). This apparent molecular weight is close to that predicted from analysis of the ERAB polypeptide sequence (26925.7 Daltons). Intensity of the band observed on immunoblots with AD brain from two different patients (Fig. 3D, lanes 1-2) was greater than that observed in brain extracts from age-matched controls (Fig. 3D, lanes 3-4; a total of five AD and three controls were analyzed). The immunoreactive band in AD brain was blocked by addition of excess free TrcHis-ERAB (Fig. 3D, lanes 5-6), but not by free TrcHis-CAT. Immunostaining of normal brain showed ERAB polypeptide to be predominately localized in neurons (**Fig. 3E**); controls with nonimmune IgG or with anti-ERAB IgG in the presence of excess ERAB peptide were unstained. Increased neuronal ERAB polypeptide expression was observed in AD brain (**Fig. 3F-1**), especially near deposits of

amyloid-beta peptide (**Fig. 3F-2**). These histologic results are representative of seven AD brains and five age-matched normal brains, the postmortem times of which were <8 hrs.

Histologic evidence and the apparent absence of a signal peptide or transmembrane spanning domain indicated that ERAB polypeptide was intracellular. Immunoblotting demonstrated expression of ERAB polypeptide as a single ≈29 kDa band in both human neuroblastoma (SK-N-SH; Fig. 4A, lane 1) and HeLa cells (Fig. 4A, lane 2) whose appearance was blocked by excess free TrcHis-ERAB. Confocal microscopy of HeLa cultures showed ERAB polypeptide to be distributed (**Fig. 4B**) identically with the endoplasmic reticulum marker protein disulfide isomerase(35)(**Fig. 4C**), as shown in double immunofluorescence images for both antigens (**Fig. 4D**). Similar results were obtained with neuroblastoma cells and ERAB-transfected COS cells. In neuroblastoma cells transfected to overexpress ERAB polypeptide, subcellular fractionation showed ERAB antigen in fractions containing the endoplasmic reticulum marker GRP78 (**Fig. 4E**, lanes 4-5), but not in fractions containing plasma membrane, cytosol, or Golgi (Fig. 4E, lanes 1-3). Based on immunostaining with ERAB IgG and on binding studies with labelled anti-ERAB IgG, ERAB polypeptide was not identified on the cell surface. Furthermore, in studies using markers for mitochondria, lysosomes and Golgi complex, ERAB polypeptide was not found in these compartments. These data suggested that ERAB polypeptide was only present in the endoplasmic reticulum.

These observations also posed an apparent paradox, for, how could amyloid-beta peptide, most of which is extracellular, interact with a polypeptide associated with endoplasmic reticulum? First, it was observed that the presence of amyloid-beta peptide influenced the distribution of ERAB polypeptide; addition of amyloid-beta peptide(1-42) to neuroblastoma cells resulted in a marked change in the cellular localization of ERAB polypeptide from its original intracellular sites (**Fig. 4F**) to granular-particulate

accumulations of apparently associated with the inner aspect of the cell membrane (**Fig. 4G**; no ERAB polypeptide was detected in nonpermeabilized cells). These changes were specific for amyloid-beta peptide, as neither scrambled amyloid-beta peptide nor an unrelated peptide (Arg-Gly-Asp-Ser)(Seq. ID. No. 5) similarly perturbed the distribution of ERAB polypeptide. Second, it was determined that ERAB polypeptide became associated with amyloid-beta peptide, and that amyloid-beta peptide-induced effects on ERAB polypeptide were not likely mediated by an indirect mechanism. To demonstrate this, SK-N-SH cells were incubated with ^{125}I-amyloid-beta peptide(1-40), and immunoprecipitation of cell-associated peptide was performed using antibodies to ERAB. From cell lysates, anti-ERAB IgG precipitated ^{125}I-labelled ≈4 kDa molecule visualized on tris-tricine gels which corresponded to amyloid-beta peptide (**Fig. 4H**, lane 2), whereas nonimmune IgG did not precipitate any bands (Fig. 4H, lane 1). To confirm that ERAB polypeptide and amyloid-beta peptide interacted in intact cultured cells, neuroblastoma cells were incubated for 6 hrs at 37°C with ^{125}I-amyloid-beta peptide followed by addition of the crosslinker disuccinimidyl suberate (**Fig. 4I**). Cultures were then washed extensively, to remove unbound tracer and unreacted crosslinker, followed by lysis of the cells and immunoprecipitation with anti-ERAB IgG or nonimmune IgG. Antibody to ERAB polypeptide precipitated a ^{125}I-labelled band with Mr ≈38 kDa (Fig. 4I, lane 2), most likely corresponding to a complex of ERAB polypeptide with an oligomer (dimer-trimer) of ^{125}I-amyloid-beta peptide; in contrast, no bands were precipitated with nonimmune IgG (Fig. 4I, lane 1). These data show that amyloid-beta peptide directly affects the cellular distribution of ERAB polypeptide, and that ERAB polypeptide-amyloid-beta peptide interaction occurs in intact cells.

ERAB polypeptide contributes to amyloid-beta peptide-mediated cellular toxicity.

Elucidating the role of ERAB polypeptide in amyloid-beta peptide-induced cellular stress was critical to evaluating its functional significance. Following transfection, COS-1 cells overexpressed ERAB polypeptide (**Fig. 5A**, lane 2), compared with mock-transfected cultures (Fig. 5A, lane 1). Exposure to amyloid-beta peptide of cultures overexpressing ERAB polypeptide suppressed their capacity to reduce 3-(4,5-dimethylthiazol-2-yl)-2,5, diphenyl tetrazolium bromide (MTT)(36), as compared with mock-transfected controls (**Fig. 5B**). COS cells were then transfected with vectors causing overexpression of either ERAB polypeptide, amyloid-beta peptide or both ERAB polypeptide and amyloid-beta peptide. In cells transfected with the plasmid causing overexpression of amyloid-beta peptide there were increased levels of amyloid-beta peptide, both cell-associated peptide and that released into the medium, which reached a maximum after 24 hrs. Cells transfected with pcDNA3-ERAB nucleic acid showed increased expression of ERAB polypeptide which was only cell-associated. Cytotoxicity was especially evident in COS cells cotransfected with vectors causing overexpression of both ERAB polypeptide and amyloid-beta peptide: compared with COS-1 transfected with vector alone (**Fig. 5C**), or those overexpressing only ERAB polypeptide (**Fig. 5D**) or amyloid-beta peptide (**Fig. 5E**), cells from cultures cotransfected with both ERAB nucleic acid and amyloid-beta peptide appeared shrunken and clumped (**Fig. 5F**).

The effect of ERAB nucleic acid and protein expression on sensitivity of neuroblastoma cells (which normally express ERAB polypeptide) to amyloid-beta peptide-induced stress was studied using blocking $F(ab')_2$ prepared from anti-ERAB IgG. For these studies, anti-ERAB or nonimmune $F(ab')_2$ was introduced into neuroblastoma cells using lipofectamine. In each case, the $F(ab')_2$ fragments became cell-associated (**Fig. 6A-B**). After liposome-mediated introduction of the $F(ab')_2$,

SK-N-SH cultures were incubated with a range of amyloid-beta peptide concentrations; whereas cultures exposed to nonimmune-derived $F(ab')_2$ showed marked amyloid-beta peptide-induced suppression of MTT reduction (comparable to untreated controls, anti-ERAB-derived $F(ab')_2$ protected against amyloid-beta peptide-induced suppression of MTT reduction (**Fig. 6C**). In contrast, addition of anti-ERAB $F(ab')_2$ directly to the medium (i.e., not in liposomes) had no affect on amyloid-beta peptide-induced MTT suppression, consistent with the intracellular localization of ERAB polypeptide. Parallelling the protective effect of blocking ERAB polypeptide intracellularly, introduction of anti-ERAB $F(ab')_2$ into SK-N-SH cells using liposomes suppressed amyloid-beta peptide-induced changes in cellular morphology. Control SK-N-SH cultures display typical short neurites and an extended cell body (**Fig. 6D**); but, after exposure to amyloid-beta peptide, retraction of neurites and rounding up of cell bodies was observed (**Fig. 6E**). Introduction of anti-ERAB $F(ab')_2$ into SK-N-SH cells prevented, in large part, these amyloid-beta peptide-induced morphologic changes (**Fig. 6F**), whereas $F(ab')_2$ derived from nonimmune IgG had no effect (**Fig. 6G**).

DISCUSSION

Neurotoxicity in Alzheimer's disease may result from a concatenation of factors, such as: (1) enhanced cellular processing of APP, releasing amyloidogenic amyloid-beta peptide peptides; (2) interaction of amyloid-beta peptide with cell membranes, both at specific cell binding sites and, presumably, by direct actions on the membrane itself; (3) nonenzymatic glycation of macromolecules whose turnover is delayed in AD brain, producing modified structures which can form crosslinks and generate reactive oxygen intermediates; and, (4) cellular components that comprise the protective response to oxidant stress and/or increase susceptibility to apoptotic stimuli(1-6,15,16,36-40). Mutant presenilins, associated with most familial forms of Alzheimer's disease(11-12)and present in endoplasmic

reticulum and Golgi complex(41), provide an instructive example of this multifactorial pathogenesis. Increased production of amyloid-beta peptide occurs in transgenic mice overexpressing mutant presenilins, and in the plasma and cultured fibroblasts from carriers of familial AD linked to chromosome 1(42-44). In addition, presenilin 2, which is homologous to ALG-3, a protein involved in programmed cell death, can function as a susceptibility factor for apoptosis in differentiated PC12 cells, in response to growth factor/serum withdrawal or to addition of amyloid-beta peptide(45).

ERAB polypeptide adds a new dimension to the pathogenetic interaction of amyloid-beta peptide with cellular elements. Like presenilin 2(45), ERAB polypeptide also appears to potentiate cellular perturbations due to amyloid-beta peptide. However, the true physiologic roles of both ERAB polypeptide and presenilins remain unknown. Addition of amyloid-beta peptide to COS cells overexpressing ERAB polypeptide caused more exaggerated suppression of MTT reduction, and greater retraction of the cells than that induced by the peptide alone. Similarly, in neuroblastoma cells, which constitutively express ERAB polypeptide, amyloid-beta peptide-induced impairment of MTT reduction and of cell morphology were suppressed by introduction of anti-ERAB $F(ab')_2$. Therefor it was proposed that ERAB polypeptide may contribute to AD pathology as a progression factor, enhancing cellular stress due to increased levels of amyloid-beta peptide. Although the mechanism through which ERAB polypeptide promotes amyloid-beta peptide-mediated changes in cellular functions is not clear, ERAB polypeptide and amyloid-beta peptide appear to interact in intact cells. First, addition of amyloid-beta peptide to cells expressing ERAB polypeptide changes its intracellular distribution. Second, COS cells, transfected to overexpress ERAB polypeptide and then exposed to exogenous ^{125}I-amyloid-beta peptide, formed immunoprecipitable ERAB - ^{125}I-amyloid-beta peptide complexes. Radioligand binding studies indicated

the specificity of ERAB-amyloid-beta peptide interaction, but it is not yet known whether ERAB polypeptide binds preferentially to monomer, oligomer, or fibrillar forms of amyloid-beta peptide. A cross-linking study with ^{125}I-amyloid-beta peptide-ERAB polypeptide strongly suggests that a soluble, probably dimer or small oligomer of amyloid-beta peptide(46-47)can serve as a ligand for ERAB polypeptide.

Analysis of the ERAB nucleic acid and protein sequence has suggested that ERAB polypeptide might be a part of the cellular enzymatic machinery, possibly functioning as a hydroxysteroid dehydrogenase. The interaction of amyloid-beta peptide with such an intracellular polypeptide is certainly not something that would have been predicted. However, there may be an analogy in binding of glyceraldehyde-3-phosphate dehydrogenase to the expanded polyglutamine repeats coded for by the CAG triplet(48)in mutant forms of the Huntington protein(49), and in other polypeptides associated with other neurodegenerative conditions(49-52). There may be an effect of amyloid-beta peptide on the enzymatic activity of ERAB polypeptide. But, how can amyloid-beta peptide gain access to this intracellular polypeptide which is apparently confined to the endoplasmic reticulum? After addition of amyloid-beta peptide to neuroblastoma cultures expressing ERAB polypeptide, it still cannot be detected on the cell surface. Thus, in order for ERAB polypeptide to interact with amyloid-beta peptide, the amyloidogenic peptide must gain access to the proper intracellular compartment. In this context, although some past studies have demonstrated amyloid-beta peptide aggregates and amyloid fibrils within cells(53-59). Such deposits, however, are usually localized to an endosomal-lysosomal compartment. The association of amyloid-beta peptide with intracellular neurofibrillary tangles has also been noted, suggesting that some amyloid-beta peptide gains access to the cytosolic milieu (it would be difficult to visualize the free ERAB peptide unless it

becomes associated to a morphologically identifiable structure, such as a neurofibrillary tangle or an amyloid fibril). Potentially, following endocytosis, amyloid-beta peptide could be diverted from the endosomal-lysosomal compartment, where it is also generated(1-6), at least in part, and gain access to the endoplasmic reticulum by a pathway yet to be defined. Alternatively, based on the location of cleavage sites in APP which produce amyloid-beta peptide, including one within the transmembrane spanning domain(1-6), another possible mechanism might be release of peptide to both intra- and extra-cellular spaces.

ERAB transcripts are apparently widely distributed in the body, with highest expression in normal liver and heart. It is possible that ERAB polypeptide participates in homeostatic cellular functions. By virtue of its localization in the endoplasmic reticulum, it may usually function in cellular metabolism, perhaps as a dehydrogenase in steroid biogenesis. ERAB polypeptide may modulate cellular function in normal and perturbed cells.

Example 2: ERAB is critical for ERAB potentiation of amyloid-beta cytotoxicity. The following data indicates that an inhibitor of ERAB activity could provide a therapy to protect neurons from Alzheimer's disease.

A mutant of ERAB was created in which tyrosine (169) and lysine (173) were replaced by glycine. These two residues, tyrosine and lysine, are essential components of the active site of ERAB which is an enzyme. The mutant ERAB was expressed and characterized as follows: (1) its immunoblotting showed the same molecular weight (about 27,000 Da) as the native ERAB which is expected for the small change introduced by the mutation, (2) the mutant form is devoid of enzyme activity because enzyme assays for ERAB activity as an NAD-dependent short chain alcohol dehydrogenase (octanol as the substrate) showed no activity and the mutant ERAB did showed no activity towards 17-beta-

estradiol (whereas native ERAB is an hydroxysteroid dehydrogenase) or towards S-acetoacetyl-Coenzyme A (whereas native ERAB is an hydroxyacyl Coenzyme A dehydrogenase), (3) when cells were transfected with the mutant ERAB with mutant amyloid-beta precursor protein which produces more amyloid-beta peptide, no increased toxicity to the cell was observed which contrasts with the increased apoptosis, as measured by ELISA for cytoplasmic histone-associated DNA fragments, caused by the cotransfection of wild-type ERAB with mutant amyloid-beta precursor protein.

These findings indicate that an inhibitor of ERAB could protect neurons from the toxic effects of amyloid-beta peptide.

Figure 7 shows that expression of a mutant form of beta-amyloid precursor protein (βAPP), which causes increased expression of amyloid-beta protein (Aβ) and ERAB causes increased toxicity (DNA fragmentation) to COS cells.

Experimental Procedure:

COS cells were grown in cell culture and transfected using lipofectamine procedure with the following DNA constructs:

1. Wild-type ERAB.
2. Mutant ERAB. This was made by site-directed mutagenesis resulting in replacement of tyrosine [169] and lysine [173] with glycine (at 169 and 173). Mutant ERAB results in the expression of a protein of molecular weight ≈ 27 kDa which has no enzymatic activity compared with native wild-type ERAB. Native or wild-type ERAB has three distinct enzymatic activities which were assayed: (a) short chain alcohol dehydrogenase (octanol as the substrate); (b) β-hydroxyacyl-Coenzyme A dehydrogenase (S-acetoacetyl-Coenzyme A as substrate); and (c) hydroxysteroid dehydrogenase (17-β-estradiol as the substrate).
3. Mutant βAPP. This was derived from the London mutation of βAPP in which valine at position 717 is replaced by

glycine. A reference describing a plasmid which can be used to express this molecule is Wild-Bode, C., et al. (1997) Intracellular generation and accumulation of amyloid β-peptide termination at amino acid 42. J. Biol. Chem. 272:16085-16088.

The assay for cell toxicity was measurement of DNA fragmentation by determining cytoplasmic histone-associated DNA fragments (one could use Cell Death ELISA from Boehringer-Mannheim®).

Experimental Protocol:

Cells were transfected with either wild type ERAB alone, mutant βAPP alone, mutant ERAB alone or lipofectamine alone. Lipofectamine is the lipid to make the vesicles for transfection and serves as a negative control. Cells were cotransfected with either wild type ERAB and mutant βAPP (see Figure 7; wt-ERAB/mut-APP) or mutant ERAB and mutant APP (see Figure 7; mut-ERAB/mut-APP).

Cells were allowed to incubate for 24 hours at 37°C, and then the cell death ELISA assay was performed.

Results:

Increased DNA fragmentation, consistent with the occurrence of apoptosis (programmed cell death) occurred in cells cotransfected with wild type ERAB and mutant βAPP. This did not occur when cells were transfected with either wild type ERAB or mutant βAPP alone. In contrast, cells cotransfected with mutant ERAB and mutant βAPP did NOT show increased DNA fragmentation. Expression of mutant ERAB and mutant βAPP was confirmed by Western blotting, thus the proteins are expressed but apoptosis did not occur.

Thus, the enzymatically inactivated ERAB does not promote the toxicity of Aβ in the same manner as wild type Aβ. Note that mutant βAPP produces increased amounts of Aβ and causes cytotoxicity which is enhanced by ERAB.

References

1. Haass, C., & Selkoe, D. Cell 7:1039-1042 (1994).
2. Kosik, K. J. Cell Biol. 127:1501-1504 (1994).
3. Yankner, B. et al. Science 245:417-420 (1989).
4. Scheuner, D. et al. Nature Med. 2:864-869 (1996).
5. Roses, A. Nature Med. 2:267-269 (1996).
6. Cai, X. et al. Science 259:514-516 (1993).
7. Citron, M. et al. Nature 360:672-674 (1992).
8. Hendriks, L. et al. Nature Genet. 1:218-222 (1992).
9. Mullan, M. et al. Nature Genet. 1:345-347 (1992).
10. Haass, C. et al. J. Biol. Chem. 269:17741-17748 (1994).
11. Sherrington, R. et al. Nature 375:754-760 (1993).
12. Citron, M. et al. Nature Med. 3:67-72 (1997).
13. Levy-Lahad, E. et al. Science 269:970-972 (1995).
14. Hensley, K. et al. Proc. Natl. Acad. Sci. (USA) 91:3270-3274 (1994).
15. Mattson, M. and Goodman, Y. Brain Res. 676:219-224 (1995).
16. Mattson, M. Neurobiol. Aging 16:661-674 (1995).
17. Yankner, B. et al. Science 250:279-282 (1990).
18. Boland, K. et al. J. Biol. Chem. 271:18032-18044 (1996).
19. Yan, S-D. et al. Nature 382:685-691 (1996).
20. Paresce, D. et al. Neuron 17:553-565(1996).
21. El-Khoury, J. et al. Nature 382:716-719 (1996).
22. Yoshikawa, K. et al. Nature 359:64-67 (1992).
23. Fukuchi, K. et al. Biochem. Biophys. Res. Commun. 182:165-173 (1992).
24. Martin, B. et al. J. Biol. Chem. 270:26727-26730 (1995).
25. Fuller, S. et al. Biochemistry 34:8091-8098 (1995).
26. Matsumoto, A. Biochim. Biophys. Acta 1225:304-310 (1994).
27. Frackowiak, J. et al. Brain. Res. 676:225-230 (1995).
28. Fields, S. and Song, O-K. Nature 340:245-246 (1991).
29. Chien, C-T. et al. PNAS(USA) 88:9578-9582 (1991).

30. Kozak, M. Nucleic Acids Res. 15:8125-8131 (1987).
31. Bernstein, F. et al. J. Mol. Biol. 112:535-542 (1977).
32. Devereux, J. et al. Nucleic Acids. Res. 12:387-395 (1984).
33. Ghosh, D. et al. PNAS(USA) 88:10064-10068 (1991).
34. Schembri, M. et al. J. Bacteriol. 177:4501-4507 (1995).
35. Edman, J. et al. Nature 317:267-270 (1985).
36. Behl, C. et al. Cell 77:817-827 (1994).
37. Busciglio, J. and Yankner, B. Nature 378:776-779 (1995).
38. Vitek, M. et al. PNAS(USA) 91:4766-4770 (1994).
39. Yan, S-D. et al. PNAS(USA) 91:7787-7791 (1994).
40. Ledesma, M. et al. J. Biol. Chem. 269:21614-2169 (1994).
41. Kovacs, D. et al. Nature Med. 2:224-229 (1996).
42. Duff, K. et al. Nature 383:710-713 (1996).
43. Borchelt, D. et al. Neuron 17:1005-1013 (1996).
44. Scheuner, D. et al. Nature Med. 2:864-869 (1996).
45. Wolozin, B. et al. Science 274:1710-1713 (1996).
46. Kuo, Y-M. et al. J. Biol. Chem. 271:4077-4081 (1996).
47. Roher, A. et al. J. Biol. Chem. 271:20631-20635 (1996).
48. Burke, J. R. et al. Nature Med. 2:347-350 (1996).
49. Huntington's Disease Collaborative Research Group Cell 72:971-983 (1993).
50. Koide, R. et al. Nature Genet. 6:9-13 (1994).
51. Nagafushi, S. et al. Nature Genet. 6:14-18 (1994).
52. Willems, P. Nature Genet. 8:213-215 (1994).
53. Knauer, M. et al. PNAS(USA) 89:7437-7441 (1992).
54. Perry, G. et al. Am. J. Pathol. 140:283-290 (1992).
55. Scott, R., et al. J. Biol. Chem. 271:8966-8970 (1996).
56. Fuller, S. et al. Biochem. 34:8091-8098 (1995).
57. Perry, G. et al. Am. J. Pathol. 143:1586-1593 (1993).
58. Yang, A. et al. J. Biol. Chem. 270:14786-14792 (1995).
59. Martin, B. et al. J. Biol. Chem. 270:26727-26730 (1995).

60. Klotz, I. and Hunston, D. J. Biol. Chem. 258:11442-11445 (1984).

61. Roher, A. et al. J. Biol. Chem. 268:3072-3083 (1993).

62. David, G. and Reisfeld, R. Biochem. 13:1014-1029 (1974).

63. Kuwabara, K. et al. J. Biol. Chem. 271:5025-5032 (1996).

64. Adelman, M. et al. Cell Biol. 56:191-205 (1973).

65. Yan, S-D. et al. Nature Med. 1:693-699 (1995).

66. Anderson, W.F. (1992) Human Gene Therapy. Science *256*:808-813.

67. Anderson W.F. et al. (1990) Human Gene Therapy *1(3)*:327-62.

68. Anderson, W. F. et al. Gene Therapy, U. S. Patent No. 5,399,346, March 21, 1995, filed March 30, 1994, U. S. Serial No. 220,175.

69. Culver, K.W. et al. (1991a) Lymphocyte Gene Therapy, Human Gene Therapy 2(2):107-9.

70. Culver K.W. et al. (1991b) Transplantation Proceedings (1 Pt 1):170-1.

71. Larrick J.W. and Burck K.L. (1991) Gene Therapy: Application of Molecular Biology, New York, Elsevier.

72. Miller A.D. (1990) Blood 76:(2):271-8.

73. Miller A.D. (1990) Retrovirus Packaging Cells, Human Gene Therapy 1(1):5-14.

74. Miller A.D. (1992) Human Gene Therapy Comes of Age. Nature 357:455-460.

75. Miller A.D. and Buttimore C. (1986) Redesign of retrovirus packaging cell lines to avoid recombination leading to helper virus production. Molec. Cell Biol. 6:2895-2902.

76. Miller A.D. et al. (1991) Construction and properties of retrovirus packaging cells based on Gibbon Ape Leukemia Virus. J.Virol. 65:2220-2224.

77. Mulligan, R.C. (1993). The basic science of gene therapy. Science *260:*926-932.

78. Rosenberg S.A. et al. (1990) Gene transfer into humans - immunotherapy of patients with advanced melanoma,

using tumor-infiltrating lymphocytes modified by retroviral gene transduction. New England Journal of Medicine 323:570-8.

79. Sambrook et al. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed.

80. Abuchowski et al., In: "Enzymes as Drugs", Holcenberg et al., eds. Wiley-Interscience, New York, NY, 367-383 (1981).

81. Carpenter et al., *Toxicol. Appl. Pharmacol.*, 18:35-40 (1971).

82. Katre et al., *Proc. Natl. Acad. Sci. USA* 84:1487-1491 (1987).

83. Newmark et al., *J. Appl. Biochem.* 4:185-189 (1982).

84. Stryer, L. (1988) Biochemistry.

85. Benet, et al., "Clinical Pharmacokinetics" in ch. 1 (pp. 20-32) of Goodman and Gilman's The Pharmacological Basis of Therapeutics, 8th edition, A.G. Gilman, et al. eds. (Pergamon, New York 1990).

86. Dayhoff. (1988) Atlas of Protein Sequence and Structure.

87. U.S. Patent 5,219,990.

88. Kriegler, M. (1990) Gene Transfer and Expression: A Laboratory Manual, Stockton Press, New York, New York.

What is claimed is:

1. A purified ERAB polypeptide.

2. The polypeptide of claim 1, wherein the polypeptide comprises the amino acid sequence shown in Figure 1D (Seq. Id. No. 2) or a variant thereof.

3. The polypeptide of claim 2, wherein the polypeptide comprises a portion of the amino acid sequence shown in Figure 1D (Seq. Id. No. 2) or a portion of a variant thereof.

4. The polypeptide of claim 1, wherein the polypeptide is human or murine.

5. The polypeptide of claim 1, wherein the polypeptide comprises a molecular weight of about 27,000 to 29,000 daltons.

6. An isolated nucleic acid which encodes an ERAB polypeptide.

7. The nucleic acid of claim 6, wherein the ERAB polypeptide comprises human ERAB polypeptide.

8. The nucleic acid of claim 6, wherein the nucleic acid comprises the nucleic acid sequence shown in Figure 1D (Seq. Id. No. 1) from nucleotide 19 to nucleotide 801 or a variant thereof.

9. The nucleic acid of claim 6, wherein the nucleic acid is DNA, RNA, or a recombinant nucleic acid.

10. A replicable vector comprising the nucleic acid of claim 6.

11. The replicable vector of claim 10, wherein the vector is a prokaryotic expression vector, a yeast expression vector, a baculovirus expression vector, a mammalian expression vector, an episomal mammlian expression vector, pKK233-2, pEUK-C1, pREP4, pBlueBacHis A, pYES2, pSE280, or pEBVHis.

12. A host cell comprising the vector of claim 10.

13. The host cell of claim 12, wherein the host cell is a eukaryotic cell, a somatic cell, a germ cell, a neuronal cell, a myocyte, a prokaryotic cell, a virus packaging cell, or a stem cell.

14. The nucleic acid of claim 6, wherein the nucleic acid comprises antisense oriented nucleic acid.

15. A cell comprising a foreign nucleic acid, which nucleic acid comprises at least a portion of the nucleic acid sequence shown in Figure 1D from nucleotide 19 to a nucleotide 801 or a variant thereof.

16. An antibody to the polypeptide of claim 1.

17. The antibody of claim 16, wherein the antibody is a polyclonal antibody, a fragment of an antibody, or a monoclonal antibody.

18. A transgenic non-human mammal whose germ and somatic cells contain and express a nucleic acid molecule encoding human ERAB polypeptide or a biologically active variant thereof, the nucleic acid molecule having been stably introduced into the non-human mammal at the single cell stage or an embryonic stage, and wherein the nucleic acid molecule is linked to a promoter and integrated into the genome of the non-human mammal.

19. The transgenic non-human mammal of claim 18, wherein the animal is selected from the group consisting of a mouse, a rat, a swine, a fowl, a dog, or a nonhuman primate.

20. A method for evaluating the ability of an agent to inhibit binding of ERAB polypeptide to amyloid-beta peptide which comprises:

(a) incubating ERAB polypeptide, the agent and amyloid-beta peptide under suitable binding conditions;

(b) determining the amount of amyloid-beta peptide bound to ERAB polypeptide from the incubate of (a); and

(c) comparing the amount of bound amyloid-beta peptide determined in step (b) with an amount of amyloid-beta peptide bound to ERAB polypeptide determined in the absence of the agent, thereby evaluating the ability of the agent to inhibit binding of ERAB polypeptide to amyloid-beta peptide.

21. The method of claim 21, wherein the agent comprises a peptide, a peptidomimetic compound, a nucleic acid, or a small molecule.

22. A pharmacuetical composition which comprises an agent capable of inhibiting an interaction between an amyloid-beta peptide and an ERAB polypeptide and a pharmaceutically acceptable carrier.

23. The pharmaceutical composition of claim 23, wherein the carrier is a diluent, an aerosol, a topical carrier, an aqueous solution, a nonaqueous solution or a solid carrier.

24. A method for treating a neurodegenerative condition in a subject which comprises administering to the subject an agent, capable of inhibiting binding of an ERAB polypeptide to an amyloid-beta peptide, in an amount effective to inhibit such binding and thereby treat the neurodegenerative condition in the subject.

25. The method of claim 25, wherein the neurodegenerative condition comprises Alzheimer's disease, Down's syndrome, Parkinson's disease, Huntington's disease,

schizophrenia, a demyelinating disease or multiple sclerosis.

FIG. 1A

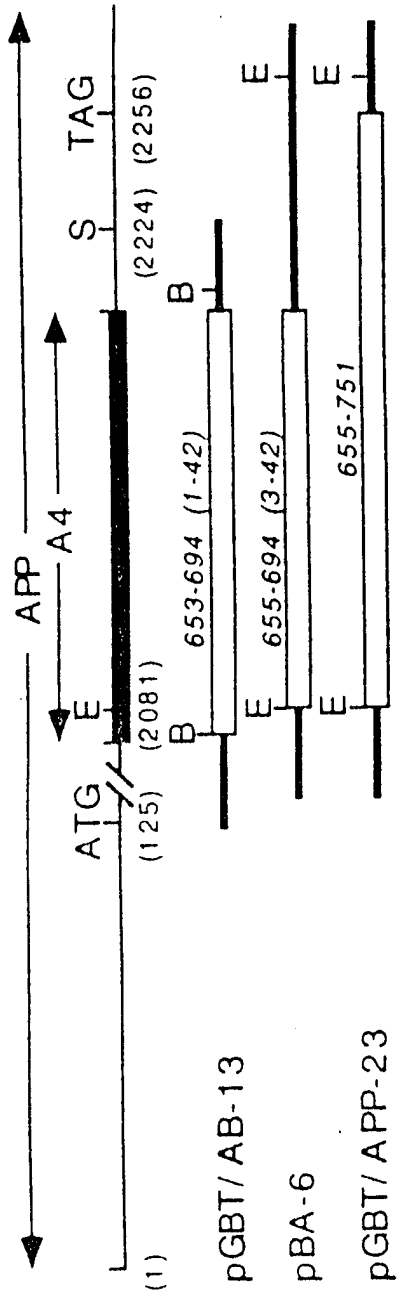

APP
A4
ATG
TAG
S
E
B
(1)
(125)
(2081)
(2224)
(2256)
653-694 (1-42)
655-694 (3-42)
655-751
pGBT/AB-13
pBA-6
pGBT/APP-23

FIG. 1B

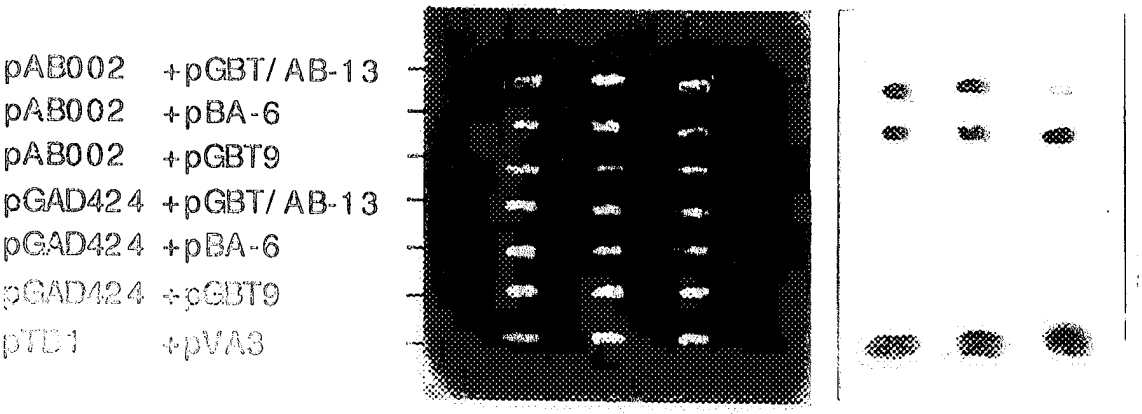

pAB002 +pGBT/AB-13
pAB002 +pBA-6
pAB002 +pGBT9
pGAD424 +pGBT/AB-13
pGAD424 +pBA-6
pGAD424 +pGBT9
pTD1 +pVA3

FIG. 1C

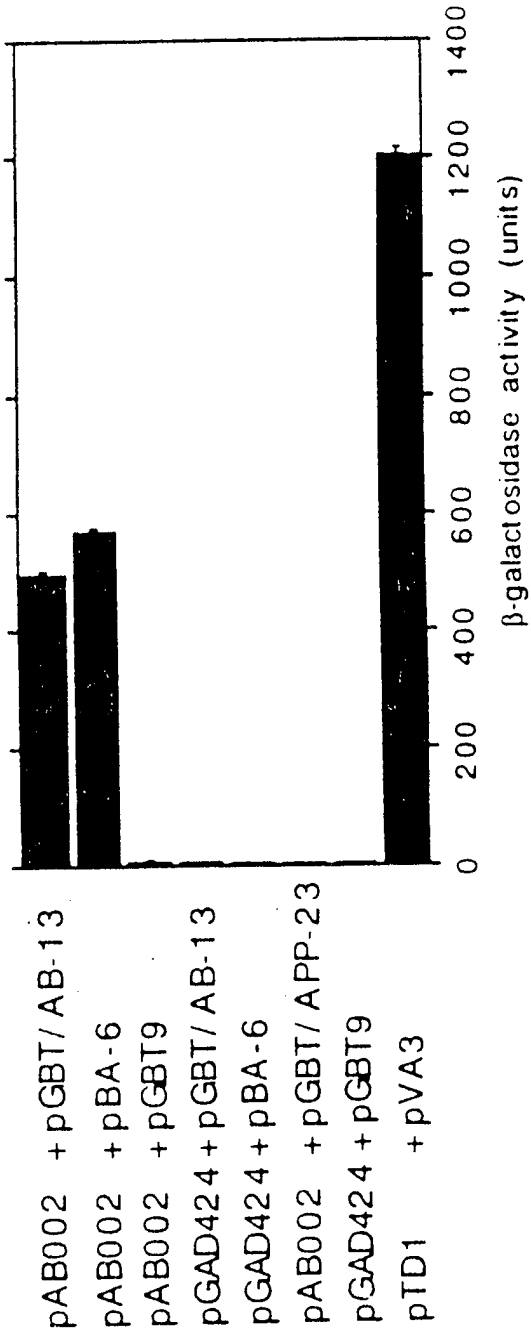

0
200
400
600
800
1000
1200
1400
β-galactosidase activity (units)
pAB002 + pGBT/AB-13
pAB002 + pBA-6
pAB002 + pGBT9
pGAD424 + pGBT/AB-13
pGAD424 + pBA-6
pAB002 + pGBT/APP-23
pGAD424 + pGBT9
pTD1 + pVA3

FIG. 1D

```
                                                        GGAGTGGCCGGCGACAAG  18

ATG GCA GCA GCG TGT CGG AGC GTG AAG GGC CTG GTG GCG GTA ATA ACC  66
M   A   A   A   C   R   S   V   K   G   L   V   A   V   I   T

GGA GGA GCC TCG GGC CTG GGC CTG GCC ACG GCG GAG CGA CTT GTG GGG  114
G   G   A   S   G   L   G   L   A   T   A   E   R   L   V   G

CAG GGA GCC TCT GCT GTG CTT CTG GAC CTG CCC AAC TCG GGT GGG GAG  162
Q   G   A   S   A   V   L   L   D   L   P   N   S   G   G   E

GCC CAA GCC AAG AAG TTA GGA AAC AAC TGC GTT TTC GCC CCA GCC GAC  210
A   Q   A   K   K   L   G   N   N   C   V   F   A   P   A   D

GTG ACC TCT GAG AAG GAT GTG CAA ACA GCT CTG GCT CTA GCA AAA GGA  258
V   T   S   E   K   D   V   Q   T   A   L   A   L   A   K   G

AAG TTT GGC CGT GTG GAT GTA GCT GTC AAC TGT GCA GGC ATC GCG GTG  306
K   F   G   R   V   D   V   A   V   N   C   A   G   I   A   V

GCT AGC AAG ACG TAC AAC TTA AAG AAG GGC CAG ACC CAT ACC TTG GAA  354
A   S   K   T   Y   N   L   K   K   G   Q   T   H   T   L   E

GAC TTC CAG CGA GTT CTT GAT GTG AAT CTC ATG GGC ACC TTC AAT GTG  402
D   F   Q   R   V   L   D   V   N   L   M   G   T   F   N   V

ATC CGC CTG GTG GCT GGT GAG ATG GGC CAG AAT GAA CCA GAC CAG GGA  450
I   R   L   V   A   G   E   M   G   Q   N   E   P   D   Q   G

GGC CAA CGT GGG GTC ATC ATC AAC ACT GCC AGT GTG GCT GCC TTC GAG  498
G   Q   R   G   V   I   I   N   T   A   S   V   A   A   F   E

GGT CAG GTT GGA CAA GCT GCA TAC TCT GCT TCC AAG GGG GGA ATA GTG  546
G   Q   V   G   Q   A   A   Y   S   A   S   K   G   G   I   V

GGC ATG ACA CTG CCC ATT GCT CGG GAT CTG GCT CCC ATA GGT ATC CGG  594
G   M   T   L   P   I   A   R   D   L   A   P   I   G   I   R

GTG ATG ACC ATT GCC CCA GGT CTG TTT GGC ACC CCA CTG CTG ACC AGC  642
V   M   T   I   A   P   G   L   F   G   T   P   L   L   T   S

CTC CCA GAG AAA GTG TGC AAC TTC TTG GCC AGC CAA GTG CCC TTC CCT  690
L   P   E   K   V   C   N   F   L   A   S   Q   V   P   F   P

AGC CGA CTG GGT GAC CCT GCT GAG TAT GCT CAC CTC GTA CAG GCC ATC  738
S   R   L   G   D   P   A   E   Y   A   H   L   V   Q   A   I

ATC GAG AAC CCA TTC CTC AAT GGA GAG GTC ATC CGG CTG GAT GGG GCC  786
I   E   N   P   F   L   N   G   E   V   I   R   L   D   G   A

ATT CGT ATG CAG CCT TGA AGGGAGAAGGCAGAGAAAACACACGCTCCTCTGCCCTTCCTT  842
I   R   M   Q   P

TCCCTGGGGTACTACTCTCCAGCTTGGGAGGAAGCCCAGTAGCCATTTTGTAACTGCCTACCAGTC  912

GCCCTCTGTGCCTAATAAAGTCTCTTTTTCTCACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  978

AAA
```

FIG. 1E-1

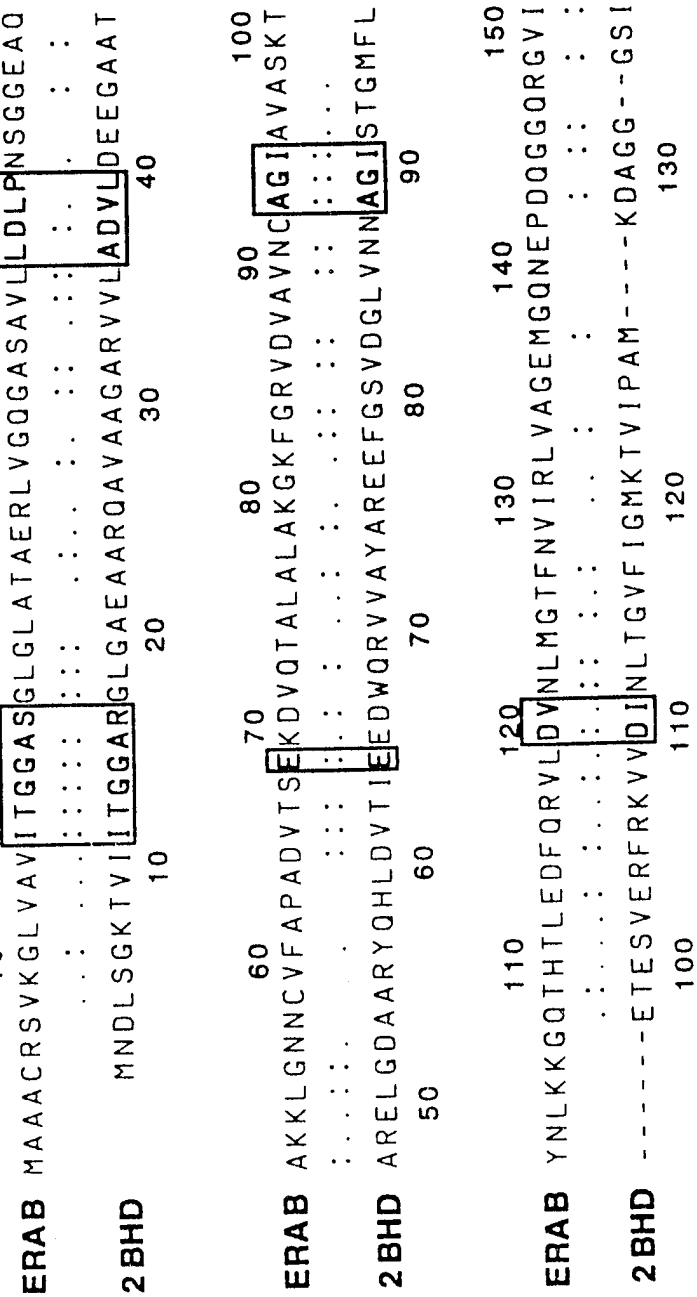

ERAB MAAACRSVKGLVAVITGGASGLGLATAERLVGQGASAVLLDLPNSGGEAQ
2BHD MNDLSGKTVITGGARGLGAEAARQAVAAGARVVLADVLDEEGAAT
ERAB AKKLGNNCVFAPADVTSEKDVQTALALAKGKFGRVDVAVNCAGIAVASKT
2BHD ARELGDAARYQHLDVTIEEDWQRVVAYAREEFGSVDGLVNNAGISTGMFL
ERAB YNLKKGQTHTLEDFQRVLDVVNLMGTFNVIRLVAGEMGQNEPDQGGQRGVII
2BHD ------ETESVERFRKVVDINLTGVFIGMKTVIPAM----KDAGG--GSI
10 20 30 40 50
60 70 80 90 100
110 120 130 140 150
10 20 30 40
50 60 70 80 90
100 110 120 130

FIG. 1E-2

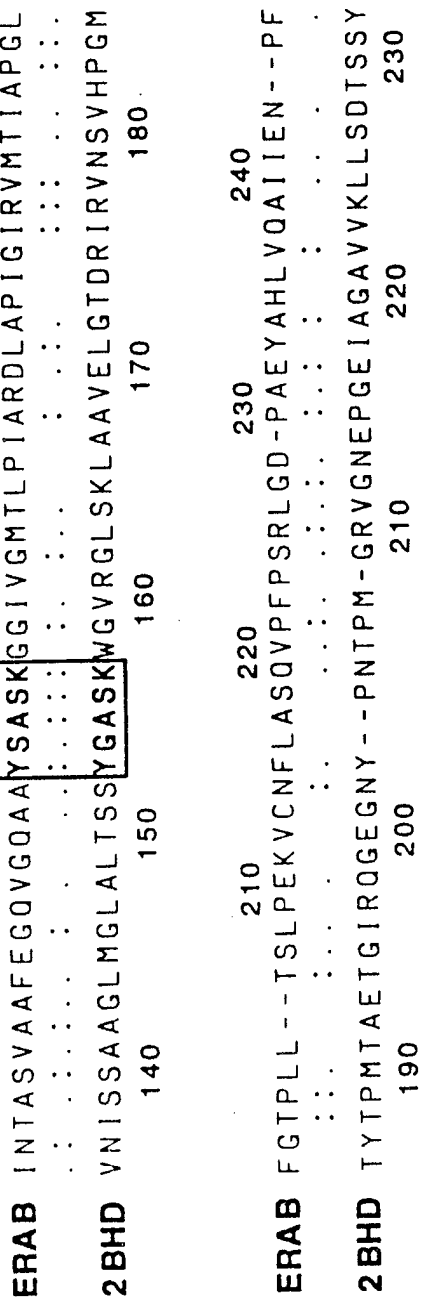

160 170 180 190 200
ERAB INTASVAAFEGQVGQAAYSASKGGIVGMTLPIARDLAPIGIRVMTIAPGL
2BHD VNISSAAGLMGLALTSSYGASKWGVRGLSKLAAVELGTDRIRVNSVHPGM
140 150 160 170 180
210 220 230 240
ERAB FGTPLL--TSLPEKVCNFLASQVPFPSRLGD-PAEYAHLVQAIIEN--PF
2BHD TYTPMTAETGIRQGEGNY--PNTPM-GRVGNEPGEIAGAVVKLLSDTSSY
190 200 210 220 230
250 260
ERAB LNGEVIRLDGAIRMQP
240 250
2BHD VTGAELAVDGGWTTGPTVKYVMGQ

FIG. 2A

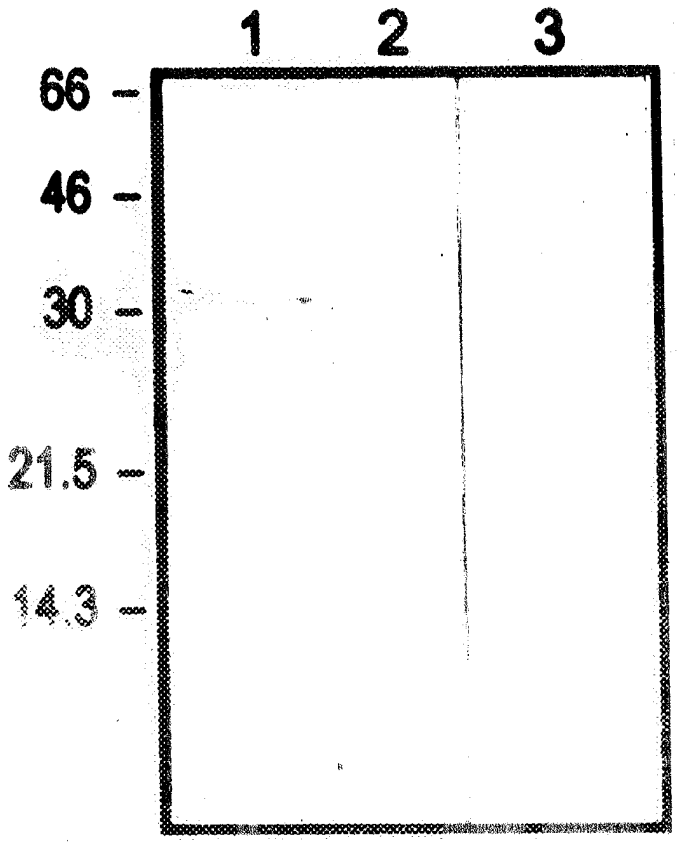


FIG. 2B

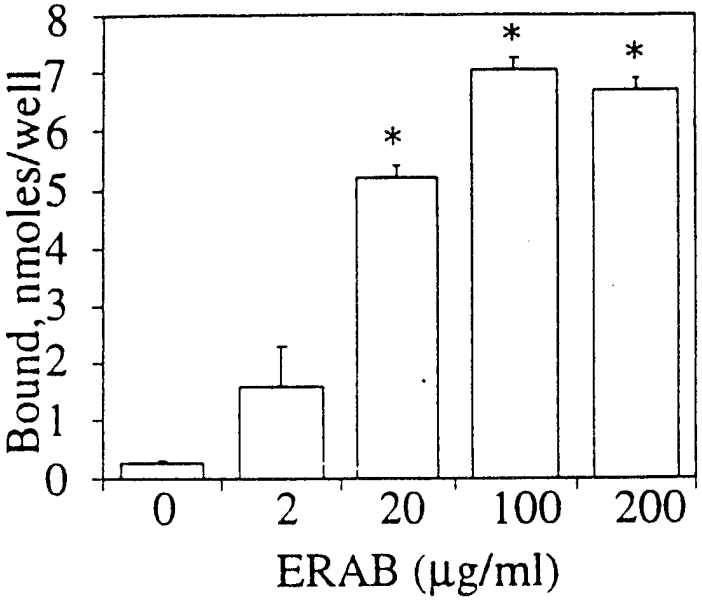

Bound, nmoles/well
0
1
2
3
4
5
6
7
8
*
*
*
0
2
20
100
200
ERAB (µg/ml)

FIG. 2C

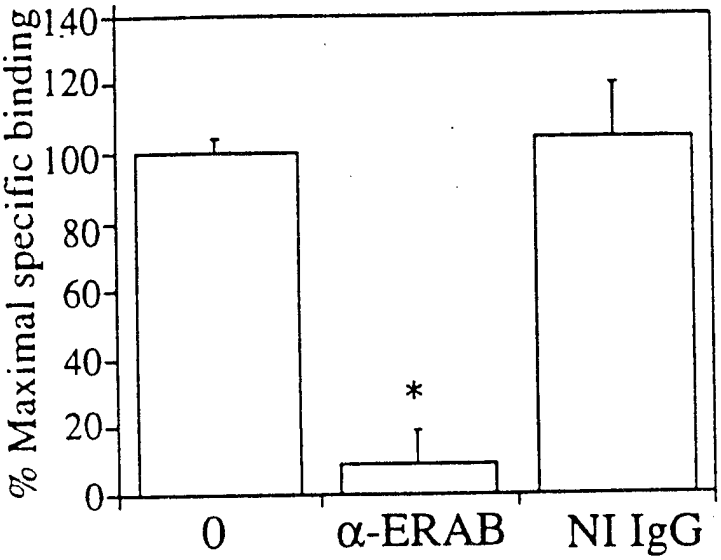

% Maximal specific binding
0
20
40
60
80
100
120
140
*
0
α-ERAB
NI IgG

FIG. 2D

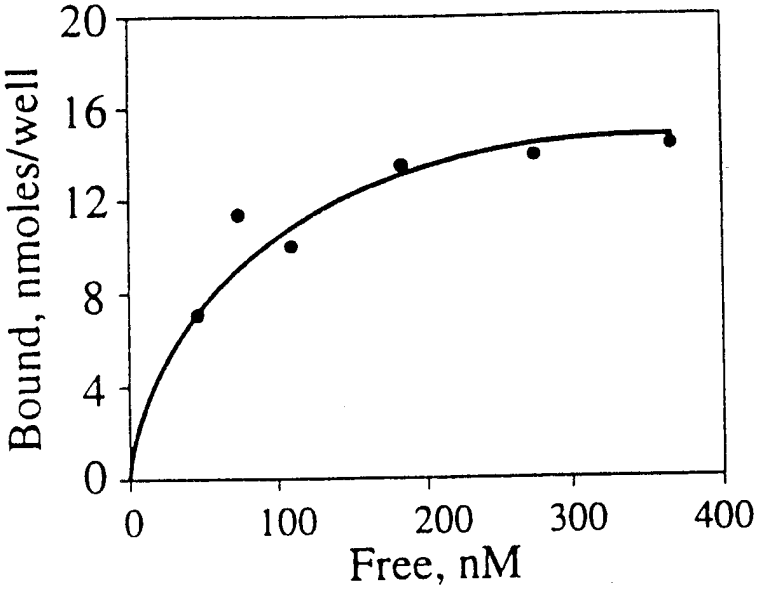

Bound, nmoles/well
20
16
12
8
4
0
0
100
200
300
400
Free, nM

FIG. 2E

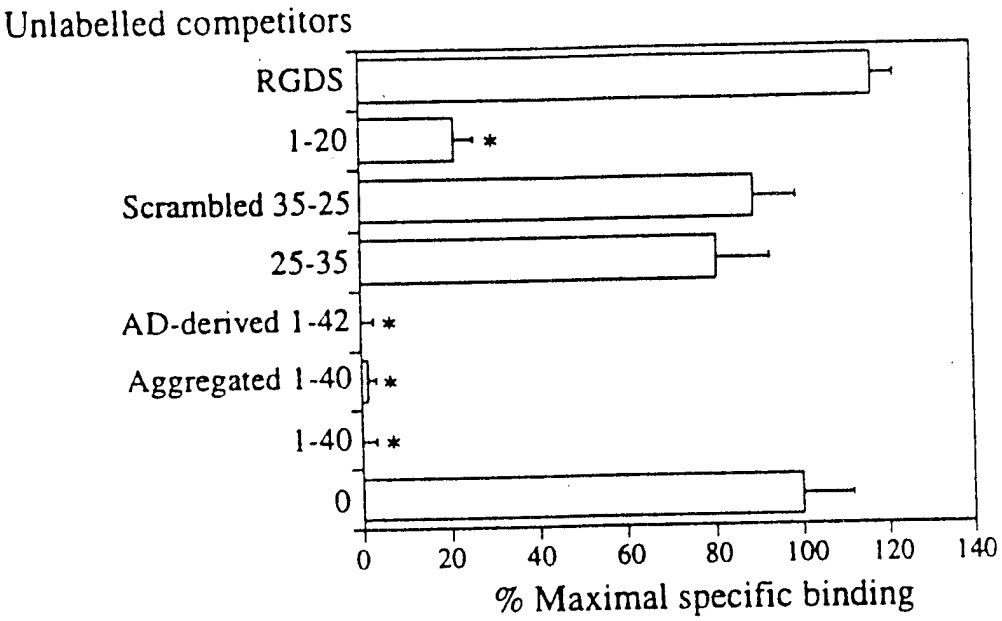

Unlabelled competitors
RGDS
1-20
Scrambled 35-25
25-35
AD-derived 1-42
Aggregated 1-40
1-40
0
*
0
20
40
60
80
100
120
140
% Maximal specific binding

FIG. 2F

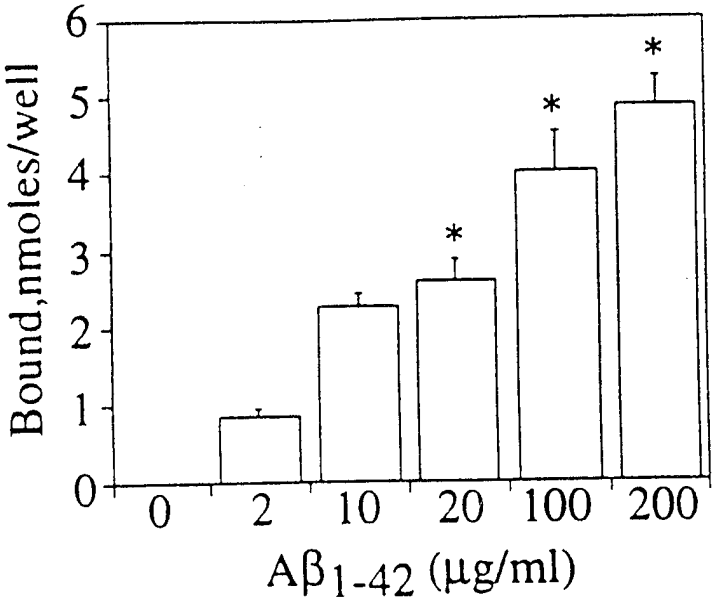

Bound,nmoles/well
0
1
2
3
4
5
6
*
*
*
0
2
10
20
100
200
Aβ1-42 (μg/ml)

FIG. 2G

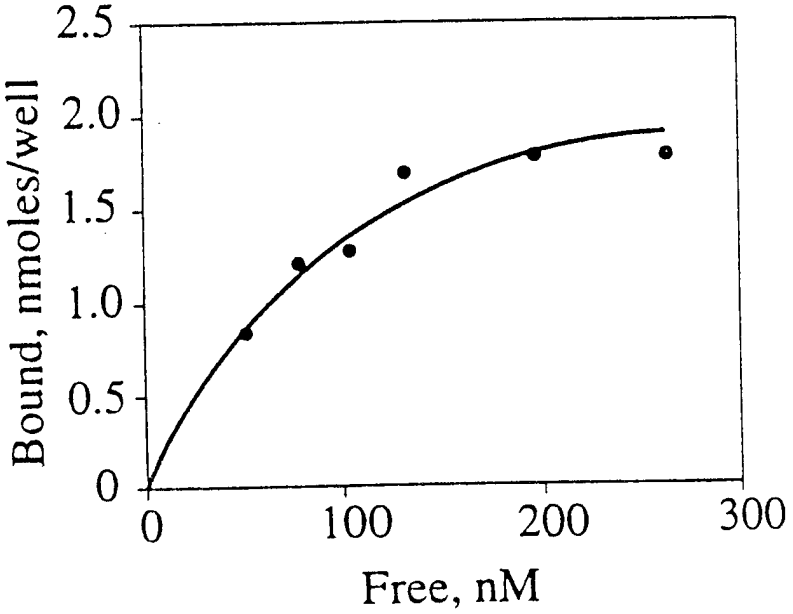

Bound, nmoles/well
0
0.5
1.0
1.5
2.0
2.5
0
100
200
300
Free, nM

FIG. 3A

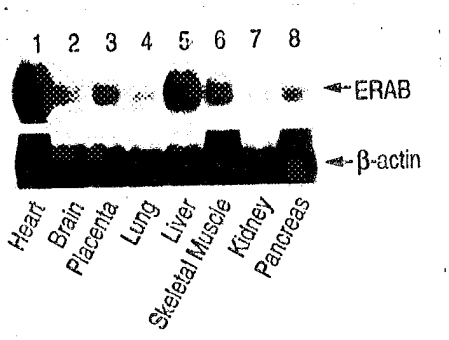


FIG. 3B

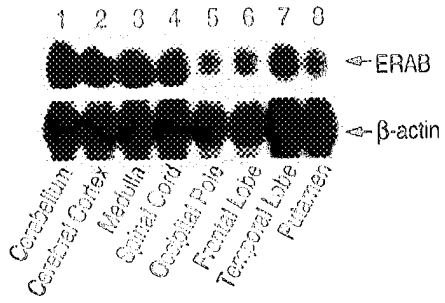


FIG. 3C

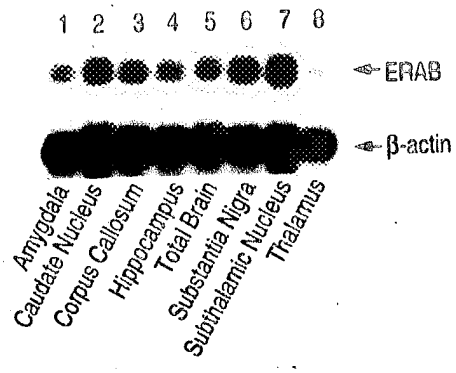


FIG. 3F-2

FIG. 3F-1

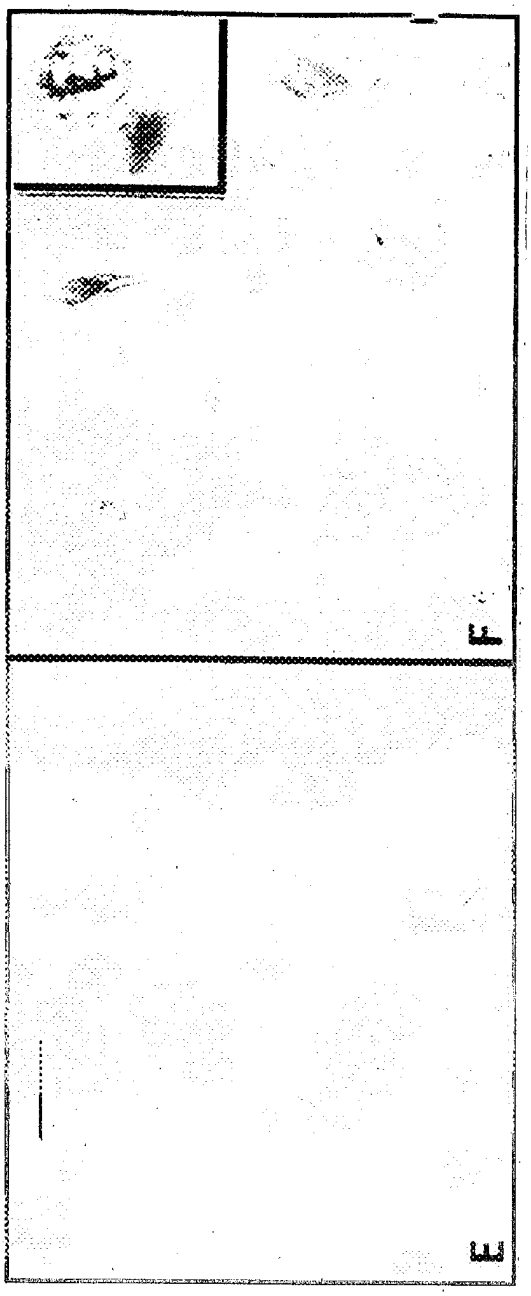
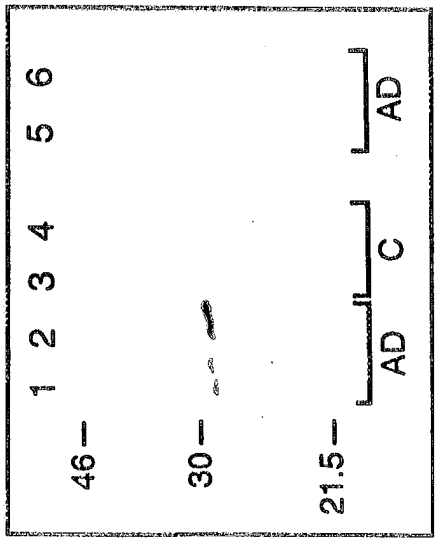


FIG. 3E

FIG. 3D



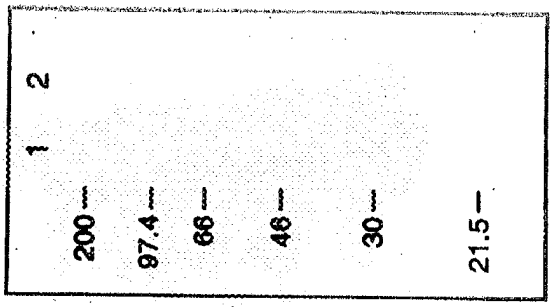


FIG. 4A

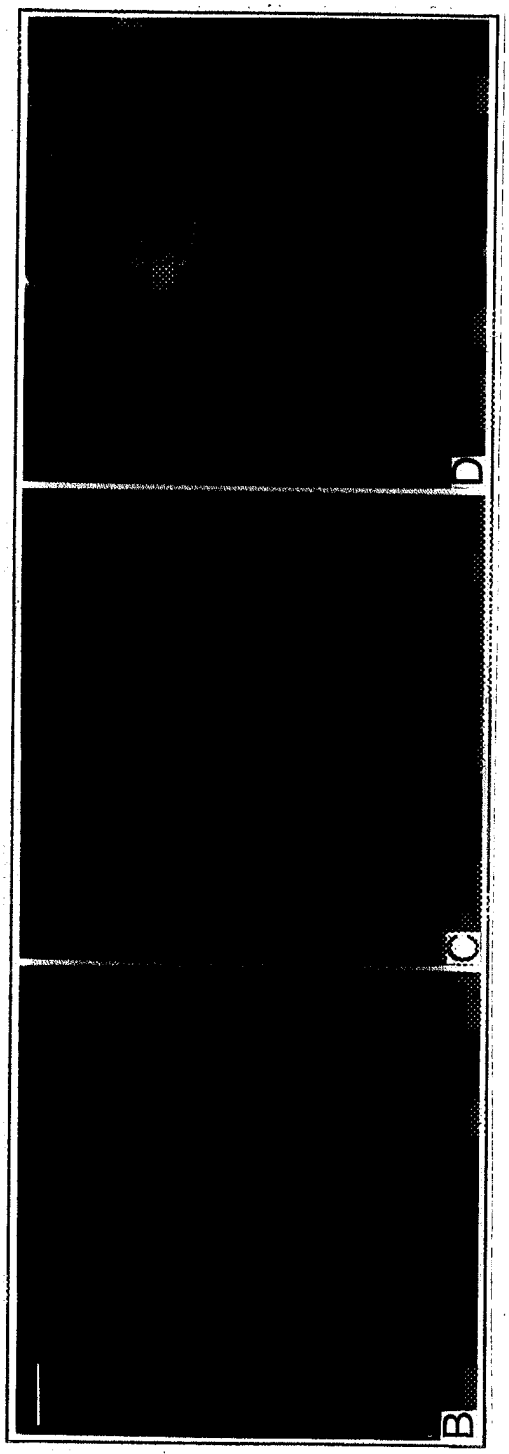


FIG. 4B

FIG. 4C

FIG. 4D

FIG. 4E

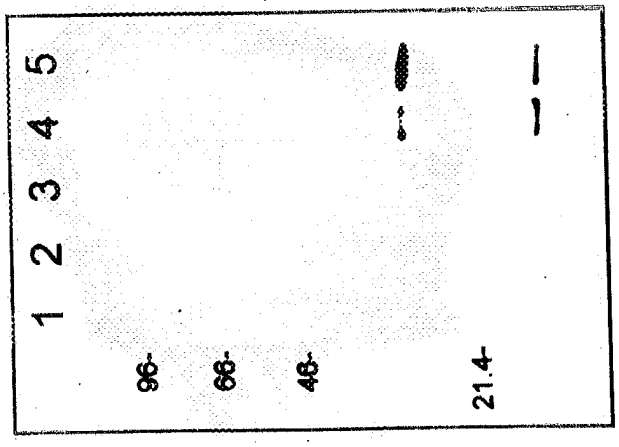


FIG. 4F

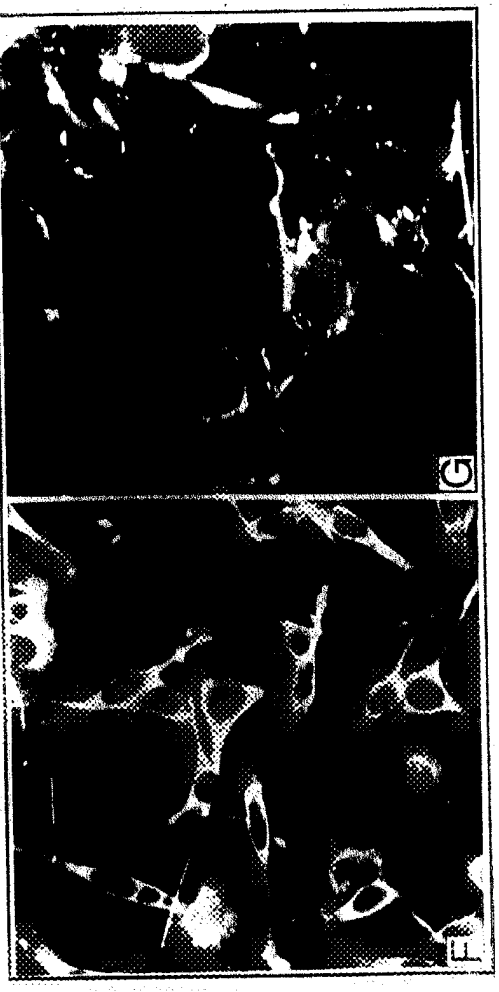


FIG. 4G

FIG. 4H

FIG. 4I

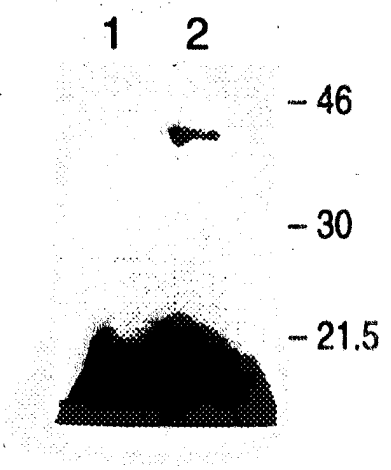


FIG. 5A

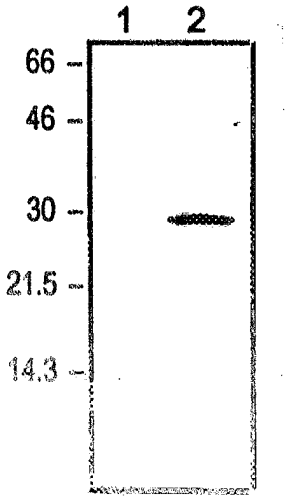


FIG. 5B

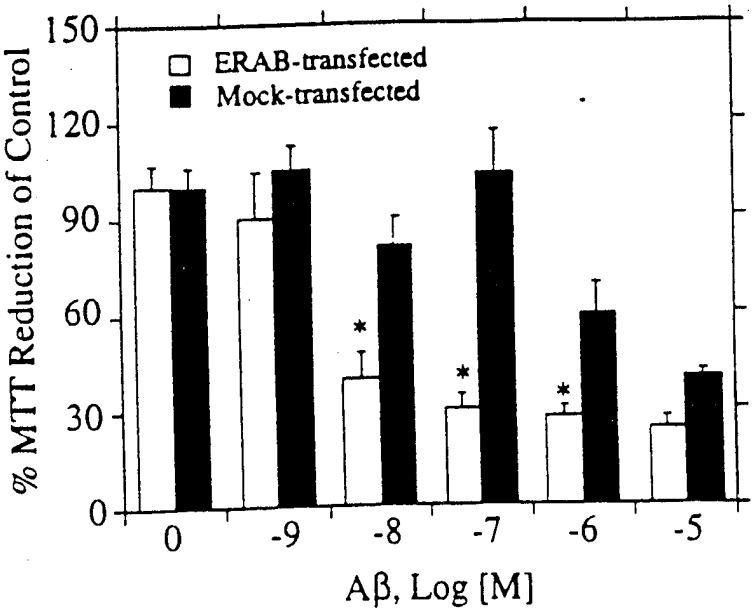

150
120
90
60
30
0
% MTT Reduction of Control
ERAB-transfected
Mock-transfected
*
*
*
0
-9
-8
-7
-6
-5
Aβ, Log [M]

FIG. 5C FIG. 5D FIG. 5E FIG. 5F

FIG. 6A FIG. 6B

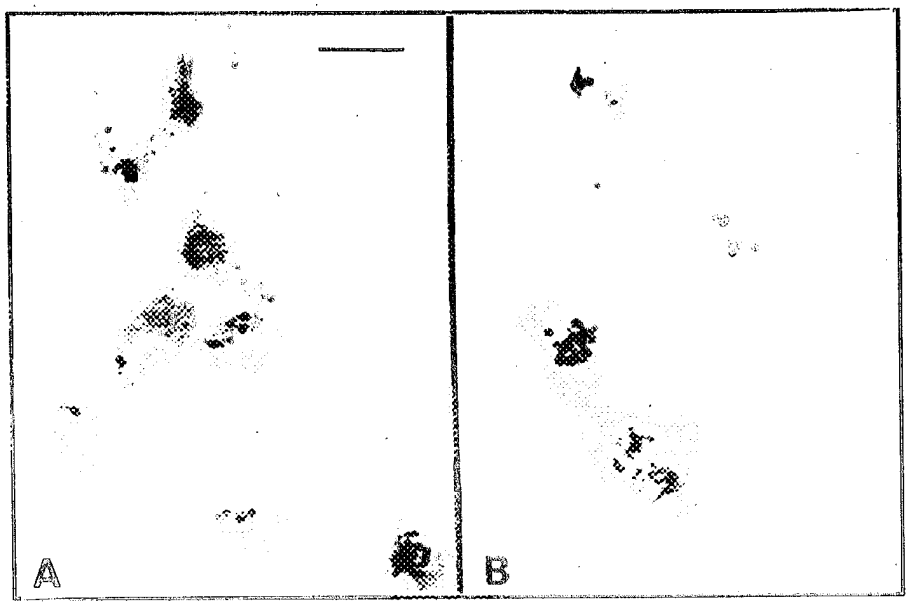


FIG. 6C

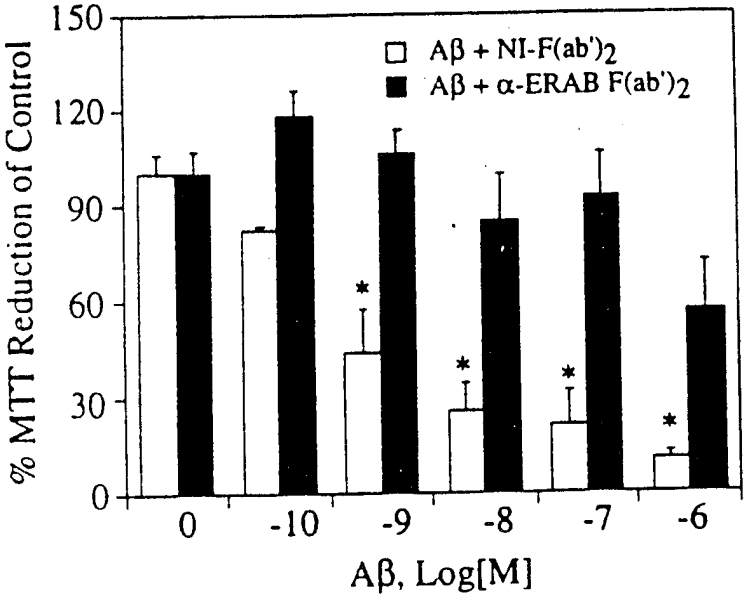
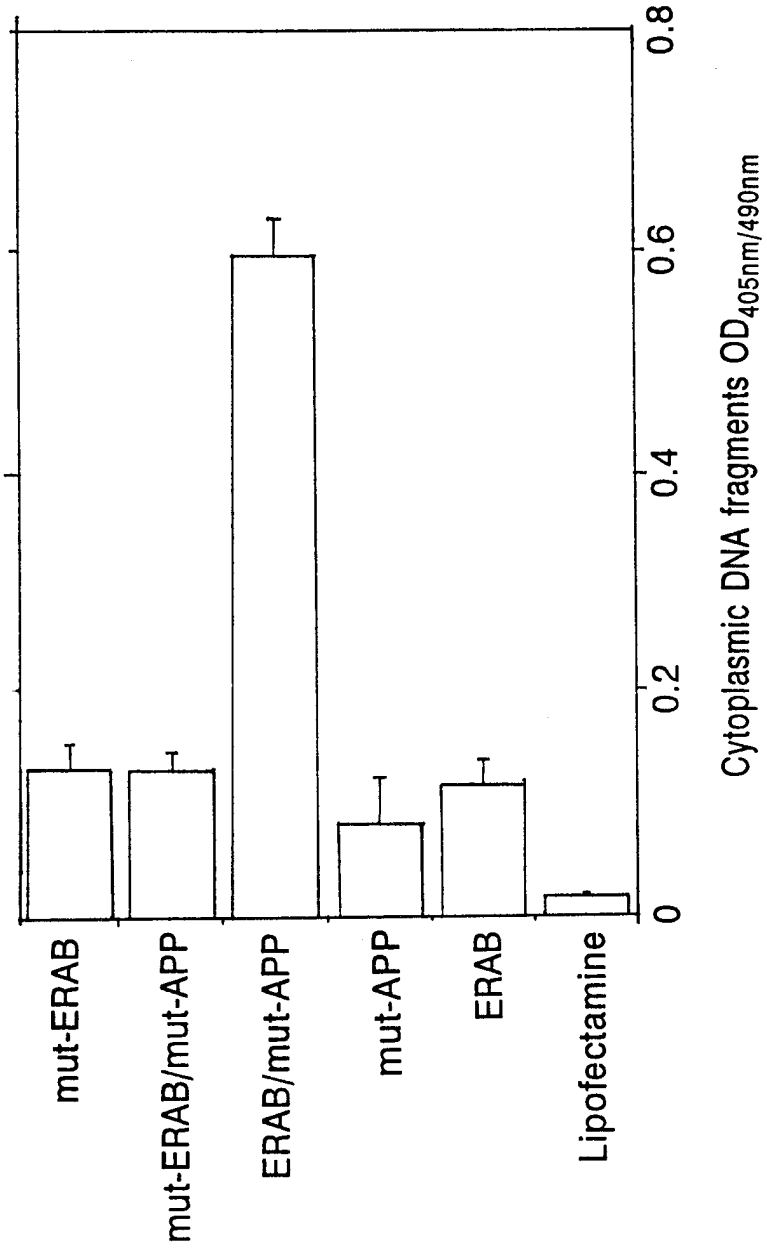

150
120
90
60
30
0
% MTT Reduction of Control
Aβ + NI-F(ab')2
Aβ + α-ERAB F(ab')2
*
*
*
*
0
-10
-9
-8
-7
-6
Aβ, Log[M]

FIG. 6D FIG. 6E FIG. 6F FIG. 6G

FIG. 7


0
0.2
0.4
0.6
0.8
Cytoplasmic DNA fragments $OD_{405nm/490nm}$
mut-ERAB
mut-ERAB/mut-APP
ERAB/mut-APP
mut-APP
ERAB
Lipofectamine

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04915

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/12, 15/63, 15/85; C07K 14/435; G01N 33/68; A61K 38/00, 39/395

US CL :Please See Extra Sheet.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 536/23.5, 24.3; 530/350; 435/7.1, 325, 252.3, 320.1; 514/2; 424/130.1

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

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

MasPar-GenBank, EMBL, Swiss-prot; Medline; Biosis; APS
search terms: ERAB, Alzheimer#, amyloid?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	FURUTA et al. Cloning and Expression of cDNA for a Newly Identified Isozyme of Bovine liver 3-Hydroxyacyl-CoA Dehydrogenase and its Import into Mitochondria. Biochimica et Biophysica Acta. 28 February 1997, Vol. 1350, pages 317-324, especially pages 318-319.	1-3, 5-15
X, P ----- Y, P	Yan et al. An Intracellular Protein that Binds Amyloid-β Peptide and Mediates Neurotoxicity in Alzheimer's Disease. Nature. 16 October 1997, Vol. 389, pages 689-695, see entire document.	1-15, 20-21 ----- 24-25

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

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

Date of the actual completion of the international search	Date of mailing of the international search report
05 JUNE 1998	20 JUL 1998

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

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

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04915

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	ADAMS et al. Initial Assessment of Human Gene Diversity and Expression Patterns Based Upon 83 Million Nucleotides of cDNA Sequence. Nature. 23 September 1995, Vol. 377, No. 6547 supplement, pages 3-17, especially page 16 concerning Accession No. AA310009 and AA306194 clones.	6-9, 14 ----- 10-13, 15
X	WO 94/03599 A1 (SAGAMI CHEMICAL RESEARCH CENTER) 17 February 1994, see pages 1 and 89-90.	1-15
X --- Y	Database GENBANK/EMBL/DDBJ, Accession No. Q99714, Zhuchenko et al, January 1997.	1-5 ----- 6-15
X --- Y	Database GENBANK/EMBL, Accession No. U73514, Zhuchenko et al, 05 October 1996.	6-9, 14 ----- 1-5, 10-13, 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04915

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-15, 20-21, 24-25

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/04915

A. CLASSIFICATION OF SUBJECT MATTER:
US CL :

536/23.5, 24.3; 530/350; 435/7.1, 325, 252.3, 320.1; 514/2; 424/130.1

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s) 1-5, drawn to purified ERAB polypeptides.
Group II, claim(s) 6-15, drawn to isolated nucleic acids that encode ERAB polypeptides, as well as vectors and host cells.
Group III, claim(s) 16-17, drawn to antibodies to ERAB proteins.
Group IV, claim(s) 18-19, drawn to non-human transgenic animals expressing human ERAB.
Group V, claim(s) 20-21, drawn to methods to identify agents that inhibit ERAB binding to amyloid-beta peptide.
Group VI, claim(s) 22-23, drawn to pharmaceutical compositions of agents that inhibit ERAB binding to amyloid-beta peptide.
Group VII, claim(s) 24-25, drawn to methods of treating neurodegenerative diseases comprising administering agents that inhibit ERAB binding to amyloid-beta peptide.

The inventions listed as Groups I-VII do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons:

Group I is directed to purified ERAB polypeptides, which is the first product. However, because Furata et al (1997) teach a protein that has sufficient homology to ERAB to be a ERAB protein, as defined on pages 14-18 of the description, no special technical feature exists for Group I as defined by PCT RULE 13.2, because it does not define a contribution over the prior art. In addition, the technical features of Groups II-IV and VI are drawn to structurally different products, which do not require each other for their practice and do not share the same or a corresponding technical feature. Groups V & VII are drawn to methods having different goals, method steps and starting materials, which do not require each other for their practice and do not share the same or a corresponding technical feature. Note that PCT Rule 13 does not provide for multiple products or methods within a single application. Because the technical feature of the Group I invention is not a special technical feature and it is not present in the Group II-VII claims, and because the technical features of the Group II-VII inventions are not present in the Group I claims, unity of invention is lacking.