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Gamma Secretase-Activating Protein Is a Substrate for Caspase-3: Implications for Alzheimer's Disease

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ABSTRACT

BACKGROUND: A major feature of Alzheimer's disease (AD) is the accumulation of amyloid-beta (Aβ), whose formation is regulated by the gamma-secretase complex and its activating protein (also known as GSAP). Because GSAP interacts with gamma-secretase without affecting the cleavage of Notch, it is an ideal target for a viable anti-Aβ therapy. However, despite much interest in this protein, the mechanisms involved in its neurobiology are unknown.

METHODS: Postmortem brain tissue samples from AD patients, transgenic mouse models of AD, and neuronal cells were used to investigate the molecular mechanism involved in GSAP formation and subsequent amyloidogenesis.

RESULTS: We identified a caspase-3 processing domain in the GSAP sequence and provide experimental evidence that this caspase is essential for GSAP activation and biogenesis of Aβ peptides. Furthermore, we demonstrated that caspase-3-dependent GSAP formation occurs in brains of individuals with AD and two different mouse models of AD and that the process is biologically relevant because its pharmacological blockade reduces Aβ pathology in vivo.

CONCLUSIONS: Our data, by identifying caspase-3 as the endogenous modulator of GSAP and Aβ production, establish caspase-3 as a novel, attractive and viable Aβ-lowering therapeutic target for AD.

Keywords: Alzheimer's disease, Amyloid beta, Caspase-3, Gamma secretase, Gamma secretase-activating protein, Transgenic mice

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Alzheimer's disease (AD) is the principal cause of dementia in the elderly. With increasing longevity and the absence of a cure, AD has become not only a major health problem but also a heavy economic burden worldwide. Accumulation of neurotoxic amyloid-beta (Aβ) peptides is a major characteristic of the AD brain and is responsible for its clinical manifestation. Because formation of Aβ is under the strict control of the gamma-secretase complex, its pharmacological blockade is an attractive therapeutic approach for lowering Aβ (1). However, full blockade of gamma-secretase has deleterious effects because this enzyme is also involved in the proteolytic processing of substrates other than Aβ precursor protein (APP), such as Notch-1 and cadherins (2). Recently, a study identified a gamma-secretase-activating protein (GSAP) that facilitates Aβ production by interacting directly with this secretase without affecting the cleavage of Notch (3). Therefore GSAP is potentially a relevant target for a viable therapeutic strategy aimed at interfering with pro-amyloidogenic effectors. However, while we know that this protein is derived from a C-terminal fragment (CTF) of a larger precursor called pigeon homologue protein, a protein of unknown biological function expressed in various tissues, including the brain, nothing else is known about its neurobiology (4).

In this article, we provide experimental evidence that caspase-3 is essential for GSAP formation and for the biogenesis of the amylodotic Aβ peptides. Furthermore, we demonstrate that caspase-3-dependent GSAP formation occurs in brains of individuals with AD and two different mouse models of AD. Collectively our data indicate a crucial new role for caspase-3 in AD pathogenesis and support the hypothesis that it is a viable target for the pharmacological therapy of this devastating disease.

METHODS AND MATERIALS

Cell Culture

Neuro-2 A neuroblastoma (N2A) neuronal cells stably expressing human APP carrying the K670N, M671L Swedish mutation (APPswe) were grown as previously described (5). For transfection, cells were grown to 70% confluence and transfected with 1 μg of vector (pcDNA3.1), human caspase-3 complementary (c)DNA, or caspase-7 cDNA by using Lipofectamine 2000 (Invitrogen, Carlsbad, California) according to the manufacturer's instructions. After 24 hours of transfection, supernatants were collected, and cell pellets were harvested in lytic buffer for biochemical analyses.

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Cell Treatment. N2A-APPswe cells were grown to 70% confluence and then treated with the caspase-3 inhibitor z-DEVD-fmk at 10 μmol/L, 25 μmol/L, 50 μmol/L, 100 μmol/L, and 500 μmol/L for 48 hours, after which supernatants were collected, and cell pellets were harvested in lytic buffer for biochemical analyses.

siRNA Knockdown Studies

Caspase-3 small interfering RNA (siRNA; product sc-29927) and a negative control siRNA (control siRNA-A; code sc-37007) were obtained from Santa Cruz Biotechnology (Dallas, Texas). N2Asw-APP cells were reverse transfected with 100 nmol/L siRNA using Lipofectamine 2000 transfection reagent (product no. 11668-019; Invitrogen) according to the manufacturer's instruction and as previously described (6). Cell toxicity was always monitored by measuring the amount of lactate dehydrogenase enzyme released in the supernatant at the end of the incubation time by a colorimetric assay (Cell Biolabs, San Diego, California).

Co-immunoprecipitation Studies

Cells were grown to 85%–90% confluence and then lysed in a solution containing 50 mmol/L HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 150 mmol/L NaCl, 5 mmol/L MgCl2, 5 mmol/L CaCl2, and 1% (3-[(3-Cholamidopropyl) dimethylammonio]-2-hydroxy-1-propanesulfonate) CHAPSO containing a protease inhibitor mixture. Prior to immunoprecipitation, cell lysates were diluted in lysis buffer lacking CHAPSO to give .25% final CHAPSO concentration. Cell lysates were incubated for 3 hours at room temperature with 5 μg of anticaspase-3 or anticaspase-7 antibodies. Dynabeads M-280 (50 μL; Invitrogen) sheep antirabbit immunoglobulin G were added, and samples were incubated overnight at 4°C. A control incubation of cell lysates with Dynabeads alone was also conducted. Dynabeads were collected and washed 5 times with lysis buffer containing .25% CHAPSO. Bound proteins were eluted with sodium dodecyl sulfate sample buffer containing reducing agent and subjected to Western blot analysis as described below.

Immunofluorescence Microscopy

Immunofluorescence studies were performed as previously described (7). Briefly, cells were placed on glass coverslips and on the following day fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 min at 22°C. Brain tissue sections were deparaffinized, hydrated with 3% H_2O_2 in methanol, and then retrieved antigen with citrate (10 mmol/L). After cells or sections were rinsed several times with PBS, they were incubated in a blocking solution (5% normal serum/.4% Triton X-100) for 1 hour at 22°C and then with the primary antibody separately against GSAP, caspase-3 overnight at 4°C. After being washed with PBS, samples were incubated for 1 hour with a secondary Texas Red or Alexa Fluor 488-conjugated antibody (Invitrogen). Coverslips were mounted using Vectashield mounting medium (Vector Laboratories, Burlingame, California) and analyzed with confocal laser scanning microscopy (LSM 710 model; Carl Zeiss, Gottingen, Germany). Control coverslips were processed as described above except that no primary antibody was added to the solution.

Assay of Caspase-3 Activity

For determination of caspase-3 activity, cells were rinsed with PBS once and lysed in buffer A (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L sodium chloride, 1% NP-40 (nonyl phenoxypolyethoxylethanol-40), .5% sodium deoxycholate, .1% sodium dodecyl sulfate, .02% sodium azide, and freshly added protease inhibitors [100 μg/mL phenylmethylsulfonyl fluoride and 1 μg/mL aprotinin]). Following incubation on ice for .5 hour, the samples were centrifuged at 16,000 *g* at 4°C for 15 min, and the supernatant was collected. Caspase-3 activity was measured by using a colorimetric substrate, Ac-DEVDpNA, and the production of pNA (p-nitroaniline) was monitored over 20 min by microplate reader at an optical density of 405 nm. One unit of activity was defined as the amount of enzyme required to cleave 1 pmol of pNA/min/mg of protein. The activity of caspase-3 was also measured indirectly by cleavage of PARPN (Poly [ADP-ribose] polymerase), which is a recognized substrate of caspase-3.

Mutagenesis

A putative consensus cleavage site specific for caspase-3 was identified in the full length GSAP (GSAP-FL) amino acid sequence and located in the GSAP-16kDa CTF (^{737}DLD^{739}). To obtain a caspase3-resistant GSAP mutant, the required aspartic acids (D) in positions 737 and 739 were replaced with alanine (A) and glutamic acid (E; D737A/D739E), respectively, by direct mutagenesis (Origene, Rockville, Maryland). In addition, both wild-type (WT) and mutated GSAP were DDK tagged (FLAG tag; Origene).

In Vitro Transcription/Translation of WT and Mutated GSAP and Cleavage by Caspase-3 in a Cell-Free System

WT and D737A/D739E-GSAP were transcribed and translated using 1-Step Human Coupled IVT Kit (ThermoScientific, Waltham, Massachusetts) as extensively described elsewhere (7). Briefly, 1 μg of WT and D737A/D739E-GSAP DNA was incubated with Hela lysate and reaction components for 3 hours at 30°C according to the manufacturer's instruction (ThermoScientific). Transcription/translation reaction product proteins then were purified by immunoprecipitation with anti-DDK-4C5 magnetic beads (Origene) and incubated overnight at 4°C and then competitively eluted by FLAG peptide (Sigma Chemical Co., St. Louis, Missouri) from anti-DDK-4C5 magnetic beads. To perform in vitro proteolytic analysis of caspase-3 on GSAP, purified WT and D737A/D739E-GSAP were incubated with recombinant caspase-3 (Life Science, St. Louis, Missouri) overnight at 37°C. Briefly, 8 μL of eluted WT and D737A/D739E-GSAP was incubated in 50 μL of 25 mmol/L HEPES, pH 7.5, .1% CHAPS, 5.0 mmol/L DDT with or without 50 ng of recombinant caspase-3 overnight at 37°C. Protein samples were then separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and the signals were detected by applying horseradish-peroxide-conjugated anti-DDK antibody (Origene).