Requirement of the Familial Alzheimer’s Disease Gene PS2 for Apoptosis

OPPOSING EFFECT OF ALG-3*
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ALG-3, a truncated mouse homologue of the chromosome 1 familial Alzheimer’s disease gene PS2, rescues T hybri
doma 3DO cells from T-cell receptor-induced apoptosis by inhibiting Fas ligand induction and Fas signal-
ing. Here we show that ALG-3 transfected 3DO cells express a COOH-terminal PS2 polypeptide. Overexpress-
ion of PS2 in 3DO-3 transfected 3DO cells reconstitutes sensitivity to receptor-induced cell death, suggest-
ing that the artificial PS2 polypeptide functions as a domi-
nant negative mutant of PS2. ALG-3 and antisense PS2 protect PC12 cells from glutamate-induced apoptosis
but not from death induced by hydrogen peroxide or the free radical MPP*. Thus, the PS2 gene is required for
some forms of cell death in diverse cell types, and its function is opposed by ALG-3.

Programmed cell death (PCD)† is a normal event under genetic control that regulates the life span of different cell
types in multicellular organisms. Among other physiological processes, PCD plays a pivotal role in the regu-
lation of the immune system. T-cells bearing T-cell receptors (TCR) able to recognize peptides from self-proteins are generated in the thymus, but mature T-cells do not normally react against self-
constituents and hence exhibit self-tolerance. Self-tolerance is therefore not an inherent feature of T lymphocytes but is ac-
dquired during development (negative selection or clonal dele-
tion) (1–3), and the main process through which it is estab-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EBI Data Bank with accession number(s) U49111 (ALG-3) and U57324 (mouse PS2).
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¶ The abbreviation used are: PCD, programmed cell death; TCR, T-cell
receptor; AD, Alzheimer’s disease; PAGE, polyacylamide gel
electrophoresis; β-Gal, β-galactosidase; CMV, cytomegalovirus; PBS,
phosphate-buffered saline; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-di-
phenyl tetrazolium bromide.

lished involves elimination by apoptosis of self-reactive
thymocytes and peripheral T-cells (4–7). Foreign antigens, on
the other hand, induce activation and expansion of antigen-
specific peripheral T-cells followed, if the antigen stimulates a
large fraction of T-cells and persists for a prolonged time, by
their elimination (8–11). This process, called activation-
duced cell death, is essential to protect the organism against
the deleterious effects of an uncontrolled T-cell expansion and
subsequent production of toxic levels of cytokines. Disregula-
tion of PCD in lymphoid cells can result in diseases such as
cancer, AIDS, and autoimmune disorders. The pathological
consequences of the malfunction of PCD are not limited to the
immune system but can affect a number of cell types including
neurons (12).

To better characterize the biochemical steps linking TCR
stimulation to cell death, we have used the mouse T-cell hybri-
doma 3DO which, like other T-cell hybridomas (13, 14), under-
goes PCD when stimulated with an anti-TCR antibody. Fas
ligand is induced following TCR triggering and the engagement
of Fas by Fas ligand activates the cell death program (15–17).
Therefore, 3DO is a suitable in vitro model to study the mole-
cular mechanisms of TCR-induced cell death. By means of a
functional selection strategy, we have isolated, from an expres-
sion cDNA library, genes involved in apoptosis induced by TCR
cross-linking in 3DO cells. The experimental system, called
“death trap,” selects for cDNAs able to block death by: 1) ex-
pressing in the cells adequate levels of specific antisense
RNAs or dominant-negative mutants for “apoptotic” genes and
2) producing “anti-apoptotic” proteins. One of the transcrits
identified, ALG-3 (18), was shown to be a truncated mouse
homologue of the chromosome 1 familial Alzheimer’s disease
(AD) gene PS2 (19, 20). Expression of this partial RNA, poten-
tially coding for the 103 COOH-terminal PS2 amino acids,
rescued 3DO cells from T-cell receptor-induced apoptosis by
inhibiting the induction of Fas ligand and the Fas-mediated
death signal. Whether ALG-3 induced resistance to receptor-
cross-linked mediated cell death by being translated into a polypeptide
and whether the endogenous PS2 protein was involved in PCD
remained to be determined. In the present study we show that
the Alzheimer’s disease gene PS2 is required for cell death and that
ALG-3 codes for a dominant negative mutant of PS2.

EXPERIMENTAL PROCEDURES

Cloning of Mouse PS2—A cDNA library from mouse liver (Clontech)
was screened with the ALG-3 fragment, and several positive phages
were double strand sequenced. Several shorter cDNAs corresponding to
the ~1.1 kilobases most 3’ end of the mouse PS2 were also isolated and
named PS2 short (PS2s). A detailed description of PS2s will be pub-
lished elsewhere.

Antisera Production—Rabbit polyclonal antisera were raised against
an SDS-PAGE purified GST-fusion protein containing amino acids 341–
377 of PS2 (aPS2n) and a keyhole limpet hemocyanin-linked COOH-
terminal PS2 peptide (Met-coo-He346) (aPS2m) by Spring Valley Labora-
tories. The fusion protein was produced using constructs based on the
inducible prokaryotic expression vector pGEX (Pharmacia Biotech Inc.).

In Vitro Transcription and Translation, Immunoprecipitation, and
Immunoblot Analysis—PS2, PS2s, and ALG-3 cDNAs were cloned into
cDNA3. Briefly, the cDNAs fragments coding for the mouse PS2, PS2s,
and PS1 proteins were cloned in the EcoRI site of pcDNA3 (Invitrogen)
in both orientation. ALG-3 cDNA was cloned in the EcoRV/NorI sites
of pcDNA3. 1 µg of DNA was used as a template for in vitro RNA
transcription using T7 RNA polymerase (Promega). The RNAs were
translated in vitro using rabbit reticulocyte lysate (Promega) and
[3H]leucine. For immunoprecipitation, immunocomplexes were bound
Identical residues are indicated by two dots. Open reading frame encoded by the shows that the two proteins are 95% identical. The beginning of the affinity purified nitrocellulose membranes (Gelman Science), and probed with either cated antiserum, were separated by SDS-PAGE, transferred to nitrocellulose membranes and probed with either preimmune antiserum, or the indicated antiserum, separated on SDS-PAGE, and detected by x-ray exposure. Total cell lysates, immunoprecipitated with the indicated cDNAs (1 μg) together with equal amounts of CMV β-Gal (Clontech) using DEAE-dextran (450 μg/ml; 8 h after transfection, cells (10^5/well) were cultured for 10 h in 96-well plates coated with 1 μg/ml of 2C11 to induce apoptosis. The β-Gal activity of living cells was visualized by fixing the cells for 10 min following by staining in PBS containing 20 mM each K3Fe(CN)6 and K4Fe(CN)6, 595 the following day. For in situ detection of DNA fragmentation (see Fig. 5A), the cells were fixed in 4% paraformaldehyde/PBS for 10 min, washed twice, incubated 5 min in 0.1% Triton X-100/PBS, washed three times with PBS, and then incubated with 0.1 unit/ml unmodified T7 polymerase and 0.4 M sodium-1-dATP. The color was developed using streptavidin-peroxidase and 0.125 mg/ml 3,3'-diaminobenzidine as described previously (21). The data shown represent the means ± standard deviations on at least triplicate counts of data reproduced in six independent experiments. The efficiency of transfection was determined by transfecting CMV β-Gal (Clontech). The β-galactosidase activity was visualized as described above. The number of cells present in at least four wells were counted. The percentage of reduction corresponds to the ratio of 2C11 treated versus untreated β-Gal+ cells for each individual transfection. The transfection efficiency was comparable in all experimental samples.

**RESULTS**

**ALG-3 Is Translated into a COOH-terminal PS2 Polypeptide**

In vitro metabolically labeled PS2 (A), PS1 (B), and ALG-3 (C) proteins were immunoprecipitated using either preimmune antiserum (P.I.) or the indicated antiserum, separated on SDS-PAGE, and detected by x-ray exposure. No indicates the reaction products before immunoprecipitation.

to protein A-Sepharose beads (Pharmacia), separated, and washed. Immunoprecipitates were separated on SDS-PAGE and detected by x-ray exposure. Total cell lysates, immunoprecipitated with the indicated antiserum, were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with α-ALG-2, α-P52c, or α-P52c antibodies. Western blot analysis of transfected PC12 was accomplished by fixing the cells for 10 min following by staining in PBS containing 20 mM each K3Fe(CN)6 and K4Fe(CN)6H2O and 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase for 1–3 h at 37°C. The number of blue cells present in at least four wells were counted. The percentage of reduction corresponds to the ratio of 2C11 treated versus untreated β-Gal+ cells for each individual transfection. The transfection efficiency was comparable in all experimental samples. The day after transfection, the cells were plated on 96-well dishes or Nunc chamber slides at a density of 5000 cells/well. On the second day after transfection, the cells were treated with toxic agents. Glutamate toxicity experiments were performed using undifferentiated PC12 cells grown in growth medium containing dialyzed sera (Life Technologies, Inc.); all glutamate treatments were for 24 h. MPP+ was used at 1 and 10 μM. Hydrogen peroxide was used at 2, 20, and 200 μM. The MTT assay (see Fig. 5A) was performed by incubating for 3 h in 0.5 mg/ml MTT and then adding lysis buffer (20% SDS/50% N,N-dimethylformamide/3.3 mM HCl), rocking, and measuring the A595 the following day. For in situ detection of DNA fragmentation (see Fig. 5D), the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/ml) solution for 15 min in PBS, washed twice with PBS, and then incubated with 0.1 unit/ml unmodified T7 polymerase and 0.4 M sodium-1-dATP. The color was developed using streptavidin-peroxidase and 0.125 mg/ml 3,3′-diaminobenzidine as described previously (21). The data shown represent the means ± standard deviations on at least triplicate counts of data reproduced in six independent experiments. The efficiency of transfection was determined by transfecting CMV β-Gal (Clontech). The β-galactosidase activity was visualized as described above. The number of cells present in at least four wells were counted with a phase contrast microscope.

**Fig. 3.** A COOH-terminal PS2 polypeptide is expressed in ALG-3 transfected 3DO cells. Total cell lysates prepared from 3DO or 3DO expressing the ALG-3 transgene or transfected with the empty vector were immunoprecipitated with either the αPS2c or the αALG-2 antiserum on a Western blot (W.B.). After SDS-PAGE separation, proteins were transferred to nitrocellulose membranes and probed with αPS2n, αPS2c, or αALG-2. The αALG-2 antiserum was used to normalize the amount of proteins loaded in each well. The gel was cut above 35 kDa to remove the signal derived from the heavy chain of the antiserum used for immunoprecipitation (IP). The endogenous PS2 protein (~50 kDa) and aggregated forms comigrate with the heavy chain and are therefore not detectable.

(Amersham Corp.)

**Transient Transfection in 3DO cells and β-Gal Activity—**ALG-3-3.2 cells were transiently transfected with pcDNA3 expressing the indicated cDNAs (1 μg) together with equal amounts of CMV β-Gal (Clontech) using DEAE-dextran (450 μg/ml; 8 h after transfection, cells (10^5/well) were cultured for 10 h in 96-well plates coated with 1 μg/ml of 2C11 to induce apoptosis. The β-Gal activity of living cells was visualized by fixing the cells for 10 min following by staining in PBS containing 20 mM each K3Fe(CN)6 and K4Fe(CN)6H2O and 1 mg/ml 5-bromo-4-chloro-3-indoyl-β-D-galactopyranosidase for 1–3 h at 37°C. The number of blue cells present in at least four wells were counted. The percentage of reduction corresponds to the ratio of 2C11 treated versus untreated β-Gal+ cells for each individual transfection. The transfection efficiency was comparable in all experimental samples. Glutamate toxicity experiments were performed using undifferentiated PC12 cells grown in growth medium containing dialyzed sera (Life Technologies, Inc.); all glutamate treatments were for 24 h. MPP+ was used at 1 and 10 μM. Hydrogen peroxide was used at 2, 20, and 200 μM. The MTT assay (see Fig. 5A) was performed by incubating for 3 h in 0.5 mg/ml MTT and then adding lysis buffer (20% SDS/50% N,N-dimethylformamide/3.3 mM HCl), rocking, and measuring the A595 the following day. For in situ detection of DNA fragmentation (see Fig. 5D), the cells were stained with 4′,6-diamidino-2-phenylindole (DAPI) (1 μg/ml) solution for 15 min in PBS, washed twice with PBS, and then incubated with 0.1 unit/ml unmodified T7 polymerase and 0.4 M sodium-1-dATP. The color was developed using streptavidin-peroxidase and 0.125 mg/ml 3,3′-diaminobenzidine as described previously (21). The data shown represent the means ± standard deviations on at least triplicate counts of data reproduced in six independent experiments. The efficiency of transfection was determined by transfecting CMV β-Gal (Clontech). The β-galactosidase activity was visualized as described above. The number of cells present in at least four wells were counted with a phase contrast microscope.

**RESULTS**

**ALG-3 Is Translated into a COOH-terminal PS2 Polypeptide**

**Both in Vitro and in Vivo—**ALG-3 was found to be a truncated mouse homologue of the familial Alzheimer’s disease gene PS2. However, the full-length mouse PS2 transcript revealed that the mouse protein is 95% identical to the human PS2 counterpart (Fig. 1). To verify whether ALG-3 can be translated into a truncated PS2 polypeptide, we generated two polyclonal anti-
sera specific for amino acids 341–377 (αPS2n) and 438–448 (αPS2c) of PS2. Both sequences are potentially coded by the ALG-3 cDNA. As determined by immunoprecipitation of in vitro translated proteins, the two antisera are specific for PS2 and do not cross-react with mouse PS1, the chromosome 14 Alzheimer's disease gene that is 65% identical to PS2 (22) (Fig. 2, A and B). The ALG-3 RNA is translated into a polypeptide of ~10 kDa, recognized only by the polyclonal antiserum specific for the COOH-terminal 11 amino acids of PS2 (Fig. 2C). Because the first AUG codon present in the translated frame codes for Met438 and would give rise to only an 11-amino acid peptide (Fig. 1A) and there is not in-frame AUG in the transcribed 5’ plasmid sequence, the observed ~10-kDa polypeptide must be translated from a noncanonical CUG (Lys365, Lys365, Lys365, Lys404, Lys413, Lys433, and Lys441) or ACG (Thr388) start codon (23–25).

Next, we determined whether ALG-3 could give raise to a truncated PS2 protein in vivo. To this end, we analyzed cell lysates from a 3DO T-cell hybridoma clone, named ALG-3,2, expressing the transgenic ALG-3 transcript that was protected from TCR-induced cell death (18). Immunoprecipitation followed by Western blot revealed that a truncated PS2 protein of ~10 kDa, detected only by the αPS2n antiserum, is specifically detected in the ALG-3.2 cell clone but not in 3DO cells and the mock transfected 3DO clone pc.2 (Fig. 3).

**PS2 Is Required for Apoptosis and Its Function Is Opposed by ALG-3—ALG-3 could represent a gain of function mutant of an endogenous anti-apoptotic protein, whose activity is normally downregulated during receptor-induced PCD. Alternatively, this molecule could function as a dominant negative mutant of a gene necessary to carry out the apoptotic program. If the latter hypothesis is correct, overexpression of PS2 should reconstitute sensitivity to TCR-induced PCD. To test for this, we cotransfected a plasmid carrying the β-Gal gene together with various pcDNA3 vector constructs into ALG-3.2 cells, which are resistant to TCR-induced apoptosis (18). As shown in Fig. 4, ~40% of the blue cells, transfected with both PS-2 and β-Gal, undergo PCD upon TCR triggering. This phenotype was specific to the PS2 transfected cells and was not observed with pcDNA3 expressing other cDNAs (Fig. 4).

The importance of PS2 for AD lead us to examine the role of PS2 levels in apoptosis in a different cell type. Pheochromocytoma PC12 cells, which express PS2 (Fig. 5A), were induced into apoptosis by treatment with glutamate, hydrogen peroxide, or the free radical MPP+. Cellular viability was measured using the MTT assay. Transfection with antisense PS2, which reduced the expression level of endogenous PS2 (Fig. 5A), and ALG-3 completely blocked the glutamate toxicity (Fig. 5B) but did not affect sensitivity to hydrogen peroxide or MPP+. Independent transfections performed with a β-galactosidase vector indicated a transfection efficiency of 32 ± 4.6%. Cellular apo-
ptosis was then measured by in situ DNA fragmentation assay. Transfection with antisense PS2 and ALG-3 reduced the number of apoptotic PC12 cells upon exposure for 24 h to 10 mM glutamate (Fig. 5C) but did not affect sensitivity to the other two insults (data not shown). On the contrary, overexpression of PS2 sense augmented the number of apoptotic PC12 cells (Fig. 5C). Thus, PS2 is involved in glutamate-induced cell death, and the death pathway requiring PS2 is blocked by ALG-3.

**DISCUSSION**

In this study, we have demonstrated a requirement for PS2 in some forms of PCD in both lymphoid and pheochromocytoma cells. PS2 function is opposed by ALG-3, an artificial truncated form of PS2 that encodes a COOH-terminal PS2 polypeptide. The involvement of PS2 in TCR-induced cell death suggests that this gene may play a role in negative selection and/or activation-induced cell death. In agreement with this hypothesis is the fact that PS2 is expressed in both thymus and peripheral lymphoid tissues. The generation of transgenic mice expressing ALG-3 in T-cells, in which PS2 should be functionally inactivated, and of PS2 null mice should address this question.

The involvement of PS2 in the PCD pathway emphasizes the potential importance of PCD in the pathophysiology of AD. It has been recently shown that β-amyloid, a 42-amino acid peptide that derives from the proteolytic cleavage of the β-amyloid precursor protein and accumulates in senile plaques in Alzheimer’s disease, can cause apoptosis in a cell type-dependent fashion (26, 27). Moreover, mice expressing a transgenic β-amyloid peptide in neurons present extensive neuronal degeneration followed by apoptotic death (28). In addition, in situ apoptosis has been described in Alzheimer’s disease (29, 30). Based on this evidence, a potential role for PCD in Alzheimer’s disease has been proposed (31). This hypothesis is reinforced by the evidences that PS2 also participates in cell death of neurontally differentiated PC12 cells and that a PS2 mutation associated with Alzheimer’s disease generates a molecule with constitutive apoptotic activity (32).

The PS2 protein has substantial structural and amino acid similarity with the chromosome 14 Alzheimer’s disease gene product PS1. These two proteins share a 63% overall identity and are strikingly homologous in the COOH-terminal 103 amino acids (80% sequence identity) and are both predicted to be seven transmembrane domain proteins. Therefore, it has been postulated that PS1 and PS2 are functionally related and subserve the same biochemical pathway. However, overexpression of PS1 in the ALG-3.2 cell clone does not reconstitute sensitivity to TCR-induced cell death. It is possible that the two proteins are both necessary but not sufficient to carry out the apoptotic program. Alternatively, like members of the BCL-2/ced-9 gene family, PS1 and PS2 could regulate PCD in a coordinate but opposite fashion.

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