Interfering with Apoptosis: Ca\(^{2+}\)-Binding Protein ALG-2 and Alzheimer’s Disease Gene ALG-3

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Two apoptosis-linked genes, named ALG-2 and ALG-3, were identified by means of a functional selection strategy. ALG-2 codes for a Ca\(^{2+}\)-binding protein required for T cell receptor-, Fas-, and glucocorticoid-induced cell death. ALG-3, a partial complementary DNA that is homologous to the familial Alzheimer’s disease gene STM2, rescues a T cell hybridoma from T cell receptor- and Fas-induced apoptosis. These findings suggest that ALG-2 may mediate Ca\(^{2+}\)-regulated signals along the death pathway and that cell death may play a role in Alzheimer’s disease.

The normal development of multicellular organisms is dependent on the removal of “unwanted” cells by a genetically controlled process termed programmed cell death (PCD) that is typically mediated by apoptosis (1). Disregulation of this process contributes to the pathogenesis of several diseases, including neurodegenerative disorders, cancer, immunodeficiency, and autoimmune diseases (2). Although the intracellular events that induce PCD are beginning to be defined, much remains to be understood. We have designed a method to select genes involved in apoptosis, using as a model PCD induced in a mouse T cell hybridoma (3DO) by T cell receptor (TCR) cross-linking (3). The selection system, which we named “death trap,” is based on the assumption that a transected complementary DNA (cDNA) library, constructed in the mammalian expression vector pLTP, should protect some recipient cells from death (4). Such inhibition may depend on inactivation of apoptotic genes by either antisense RNA or dominant negative mutants or on overexpression of proteins with anti-apoptotic activity.

Using this system, we isolated six cDNA clones, designated apoptosis-linked genes (ALG-1 to -6), that were able to inhibit TCR-induced cell death in a transient transfection assay (5). Here we describe two of them: ALG-2, a Ca\(^{2+}\)-binding protein, and ALG-3, the mouse homolog of a human gene linked to Alzheimer’s disease. ALG-2 consisted of a 435–base pair (bp) cDNA insert that identified a single 1.3-kb transcript in 3DO cells and in all adult mouse tissues analyzed; the thymus and liver showed the most expression, whereas the testis and skeletal muscles showed the least (Fig. 1A). ALG-3 consisted of an 850-bp fragment that hybridized to a transcript of about 2.4 kb that was present in the liver and, in lesser amounts, in all other adult mouse tissues analyzed (Fig. 1B), the thymus included (5). The ALG-3 probe also detected a major ~1.3-kb mRNA in the liver and a transcript of ~7 kb in the heart and skeletal muscles (Fig. 1B). The 2.4-kb mRNA is also expressed in 3DO cells and, like ALG-2 (Fig. 1A), is not regulated by TCR triggering.

DNA sequence analysis of the ALG-2 insert and of five clones isolated from a 3DO cDNA library, together with Northern (RNA) blot analysis with single-strand probes (5), revealed that the transfected ALG-2 is transcribed in the antisense orientation from the expression vector pLTP. The full-length ALG-2 cDNA is identical to a partial cDNA previously identified (6) and has an open reading frame predicted to encode a protein of 191 amino acids, containing two canonical Ca\(^{2+}\)-binding EF hand structures (7) (Fig. 2A). A rabbit antisera raised against an ALG-2–histidine tag fusion protein specifically recognized a polypeptide of the expected molecular mass (21.9 kD) in 3DO (Fig. 2B). The ALG-3 cDNA instead is expressed from the vector as a truncated sense transcript coding for a putative polypeptide that is 98% identical to the 103 COOH-terminal amino acids of STM2, the chromosome 1 familial Alzheimer’s disease gene (8) (Fig. 2C). Initial characterization of a full-length clone obtained from a mouse liver cDNA library indicates that the 2.4-kb mRNA encodes a protein that is highly homologous to STM2 (5). Thus, ALG-3 is the mouse homolog of STM2.

To confirm the data obtained in the transient transfection assay and to better characterize the steps along the death pathway that are affected by these two cDNAs, we established stable transfected 3DO cell clones. To this end, we cotransfected a plasmid carrying the neomycin resistance gene (pcDNA3) with either the ALG-2 or ALG-3 expression vectors into 3DO cells to generate G418-resistant clonal populations. Cell clones transfected with the empty plasmid or with pLTP expressing two cDNAs, called A25 and B15 (9), which scored ambiguously in the transient transfection experiment, were also produced. Thirty-six 3DO clones, expressing amounts of surface TCR comparable to those expressed by the 3DO hybridoma cells (10), were analyzed for susceptibility to TCR-induced death. Unlike clones pc.1 to -4, A25.1 to -7, and B15.1 to -6, transfected with the empty pLTP or with this same vector expressing the A25 and B15 cDNAs, 11 of the 16 ALG-2–transfected clones and all 4 ALG-3–transfected clones were resistant to TCR-induced cell death (Fig. 3A).

Because of its antisense orientation in the pLTP expression vector, the ALG-2 cDNA is predicted to produce, upon transfection, an antisense RNA that should reduce the steady-state amount of the corresponding protein required for TCR-induced cell death. To verify this hypothesis, we analyzed the amount of ALG-2 protein present in 13 of the ALG-2–transfected clones by protein immunoblot analysis (clones ALG-2.12, -15, and -16 were not analyzed). 3DO clones ALG-2.1, -2, -3, -6, -7, -10, -11, -13, and -14, which were resistant to TCR-induced death, expressed reduced amounts of ALG-2 protein (representative clones ALG-2.6, -7, and -11, which were further analyzed, are shown in Fig. 3B). The clones ALG-2.4, -5, -8, and -9, which were susceptible to receptor-trig-
gated death, expressed amounts of ALG-2 protein equivalent to those present in the mock transfectants and 3DO cells (only clones ALG-2.4 and pc.2, which were used for further analysis, are shown in Fig. 3B). This may be the consequence of either poor expression of the ALG-2 transgene or integration of pcDNA3 alone. An antibody specific for β tubulin was used to allow normalization to the amount of protein loaded on each lane (Fig. 3B). Thus, reduction of the amount of ALG-2 protein protects 3DO cells from receptor-induced cell death.

The ALG-3 cDNA was in sense orientation in the expression vector pLTP. Therefore, ALG-3-transfected 3DO clones, protected from TCR-induced PCD, should express a truncated ALG-3 transcript. Northern blot analysis with the ALG-3 cDNA probe, which detected the endogenous 2.4-kb mRNA in all tested populations, hybridizes to a ∼1-kb transgenic transcript expressed in ALG-3-transfected clones and not present in 3DO cells (only ALG-3.2, -4, and -5 clones, used for subsequent studies, are shown in Fig. 3C). Thus, expression of the truncated ALG-3 transcript correlates with resistance of 3DO cells to receptor-induced cell death.

The transfected ALG-2 and ALG-3 constructs could either specifically inter-
**Fig. 4.** (A) Fas ligand up-regulation upon TCR triggering. Protein immunoblot analysis was performed as described in Fig. 2. Fas ligand (Fas L) induction was measured on total cell lysates prepared from untreated cells or from cells stimulated for 4 hours with 2C11, with the use of an antibody to Fas ligand (Santa Cruz). The nature of the cross-reactive smaller protein is unknown. (B) Susceptibility to Fas-mediated PCD. To induce cell death, 3DO cells were incubated at room temperature for 15 min with the indicated concentrations of the antibody to Fas Jo2 (Pharmingen), then washed and plated on wells coated with an antibody to hamster immunoglobulin G (5 μg/ml) (Pharmingen).

**Fig. 5.** Analysis of apoptosis induced by other stimuli. Cells were cultured with the indicated concentrations of (A) dexamethasone (Ascent Pharmaceutical) for 18 hours, (B) staurosporine (Sigma) (9 hours), (C) actinomycin D (U.S.B.) for 20 hours, or (D) the cell-permeable synthetic ceramide C2-ceramide (BIOMOL Research Labs) for 14 hours. Cell death was measured as indicated in Fig. 3.

Fas ligand is induced after TCR stimulation in T cell hybridomas, and the engagement of Fas by Fas ligand activates the cell death program (11). Because all 3DO clones expressed equivalent amounts of Fas protein on the cell surface (10), we examined whether Fas ligand induction or Fas-mediated cell death (or both) were affected. Protein immunoblot analysis of whole-cell extracts revealed that Fas ligand up-regulation, which was detectable in the mock transfectant pc.2 and in all ALG-2–transfected clones, was completely blocked in the ALG-3–expressing cells (Fig. 4A). Stimulation of the Fas receptor with an antibody to Fas readily induced death of 3DO cells, of the mock transfectant pc.2 clone, and of the ALG-2.4 clone. In contrast, the clones resistant to TCR-mediated cell death were also protected from Fas-induced cytotoxicity (Fig. 4B). Thus, the ALG-2 protein functions downstream of Fas–Fas ligand interaction, and the ALG-3 transgenic transcript interferes with the TCR death pathway by inhibiting Fas ligand induction and, consequently, Fas-mediated autocrine suicide and by blocking the death signal transduced by the Fas molecule.

To investigate whether the effect of these two cDNAs is limited to TCR-induced PCD, we treated 3DO clones with other apoptosis-inducing agents. Dexamethasone, a synthetic corticosteroid that causes apoptosis in lymphoid cells (12), triggers a pathway that is independent of Fas–Fas ligand interaction and antagonizes TCR-induced death by inhibiting Fas ligand up-regulation (13). Ceramide has been implicated as a mediator of Fas-induced death and is a potent inducer of apoptosis (14). Staurosporine and actinomycin D induce apoptosis in many cell lines (15). Clones expressing reduced amounts of ALG-2 protein were less sensitive to steroid- and staurosporine-induced cell death.
ALG-3-transfected actinomycin 524 served has shift was binant EGTA lanes (Fig. GST, removed mutant proteins are schematically represented gate NH2-terminal the inducible (black boxes) GST, produced extracellular Ca2+-flux or Ca2+ release from intracellular storage compartments might mediate, at least in part, this functional activation. To test whether ALG-2 can bind Ca2+, we performed a 45Ca2+ ligand-blotting experiment with recombinant proteins. As shown in Fig. 6A, a GSTALG-2 fusion protein specifically binds 45Ca2+ and both Ca2+-binding EF hands are required for efficient binding. In addition, like other Ca2+-binding proteins, ALG-2 migrates faster during SDS-polyacrylamide gel electrophoresis (PAGE) in the presence of calcium (Fig. 6B). Thus, by these two criteria, ALG-2 is identified as a Ca2+-binding protein.

In this study, two cDNAs involved in PCD were described. One, ALG-2, codes for a Ca2+-binding protein and is a component of both TCR- and glucocorticoid-triggered cell death. Apoptosis induced by other agents is also affected. The involvement of Ca2+ in apoptosis was first suggested by studies assessing the biochemical requirements for DNA cleavage (17). Subsequently, it has been shown that glucocorticoid and TCR triggering stimulate sustained calcium increases in thymocytes, and that cell death is prevented by blocking of the calcium increase (18, 19). A requirement for extracellular Ca2+ in TCR-induced Fas ligand up-regulation and for intracellular Ca2+ for Fas-mediated death has been recently demonstrated (20). Moreover, calmodulin (19, 21) and the calcium-dependent cysteine protease calpain (22) have been suggested to be involved in PCD. ALG-2 is the first Ca2+-binding protein shown to be directly involved in PCD and might represent the prototype of a family of genes that mediate Ca2+-regulated signals along the death pathway. Cysteine proteases of the ICE/CED-3 family have been suggested to be involved in Fas-mediated (23) but not glucocorticoid-induced (24) apoptosis. Whether the ALG-2 pathway is dependent on the activation of these enzymes has yet to be analyzed. However, because we analyzed cell clones expressing residual amounts of ALG-2, in order to precisely assess how general and physiological the role of this protein is, ALG-2 knockout mice and cell lines should be generated. The other cDNA identified, ALG-3, is the mouse homolog of STM2, the familial Alzheimer’s disease gene on chromosome 1. This finding raises the possibility that, as has also been suggested by other studies (25), cell death plays an important role in the pathophysiology of Alzheimer’s disease and provides a clue to the physiological function of STM2. However, whether the truncated ALG-3 RNA confers resistance to cell death by being translated into a polypeptide (26) and whether the endogenous ALG-3 protein is involved in the death process remain to be determined.

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4. Polyadenylated poly[A] plus mRNA was prepared from 3DO cells stimulated with the monoclonal antibody to CD3e 2C11 (Pharmergen), which was immobilized on tissue culture plates (1 µg/ml) for 5 hours. Therefore, both constitutive transcripts and mRNAs regulated either positively or negatively by TCR stimulation were represented. After reverse transcription with random and oligo(dT) primers, the cDNA was cloned into the vector pLTP, which was designed to obtain eukaryotic expression of cloned cDNAs from cytomembranoglobulin (CMV) enhancer-promoter sequences and to replicate episomally in mouse cells (P. Vito and L. D’Adamo, unpublished data). The pLTP vector was derived from plasmid pCDNA (Invitrogen) as follows: The neomycin resistance gene was excised and a fragment of polymeera virus, encoding the large T antigen and the viral origin of replication, was cloned into a Bsp HI site. The cDNA library was transiently transfected into 3DO cells with DEAE dextran (450 mg/ml). Eighteen hours later, the transfected cells were stimulated with 2C11 immobilized on tissue culture plates to trigger Fas. After 12 hours, the living cells were recovered and lysed in 0.6% SDS and 10 mM EDTA to isolate circular non-integrated plasmids. The rescued vectors were transformed into XL2-Blue MRF’ ultracompotent cells (Stratagene) to obtain sub libraries. This procedure was repeated four times. Two groups have also described systems based on the selection of apoptotic genes by antisense RNA. After 2C11 PCD induction (T. G. Gabib, P. L. Mantel, R. Rosli, C. D. Cream, J. Biol. Chem. 269, 23615 (1994); L. P. Deuss, E. Feinest, H. Berisso, O. Cohen, A. Kimchi, Genes Dev. 9, 15 (1995).
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Alignment of auditory and visual receptive fields in the optic tectum of the barn owl (Tyto alba) is maintained through experience-dependent modification of auditory responses in the external nucleus of the inferior colliculus (ICX), which provides auditory input to the tectum. Newly learned tectal auditory responses, induced by altered visual experience, were found to be pharmacologically distinct from normal responses expressed at the same tectal sites. N-methyl-D-aspartate (NMDA) receptor antagonists administered systemically or applied locally in the ICX reduced learned responses more than normal responses. This differential blockade was not observed with non-NMDA or broad-spectrum antagonists. Thus, NMDA receptors preferentially mediate the expression of novel neuronal responses induced by experience during development.

Experience-dependent modification of neuronal responses tailors the function of neural circuits based on the sensory experience of the individual. Pharmacological studies of this process (1) have implicated the NMDA subtype of excitatory amino acid (EAA) receptor in the induction of experience-dependent synaptic modification. However, interpretation of these experiments is difficult because the specific effects of NMDA receptor blockade are usually confounded with nonspecific effects of blocking postsynaptic activity (2). We have used a different approach in a system in which normal and newly learned responses can be recorded simultaneously at single sites. Here we show that newly functional circuitry, once it has been induced by experience-dependent processes, is pharmacologically specialized: Transmission through this circuitry is preferentially mediated by NMDA receptors, relative to transmission through original circuitry (3).

Barn owls localize sounds using interaural timing difference (ITD) as a cue for sound source azimuth. In the ICX, where the owl's map of auditory space is synthesized, neurons are tuned to specific ITD values and are organized into an map of ITD, and hence of azimuthal space. The auditory tectal map is relayed topographically to the optic tectum, where it is aligned with the tectal map of visual space so that tectal neurons are tuned to the value of ITD produced by sounds at the locations of their visual receptive fields (VRFs) (4). This alignment is dynamically maintained by experience-dependent plasticity and can be altered systematically if owls are raised wearing prismatic spectacles that optically displace the visual field in azimuth (5, 6). During prism-rearing, tectal neurons develop novel responses to sounds with ITDs that correspond to the location of their optically displaced VRFs (schematized in Fig. 1A). At many tectal sites, these novel responses, which are to ITDs that are systematically displaced from the normal ITD range (5), first appear while responses to ITDs in the normal range continue to be expressed, creating a "transition state" ITD tuning curve (7) (Fig. 1A, middle panel). Transition state tuning curves are often abnormally broad and sometimes double-peaked. They are defined here as those ITD tuning curves that include both responses to ITD values that are normally appropriate for that tectal site, termed "normal responses," and responses to ITD values corresponding to the primitively displaced VRF, termed "learned responses" (8). Over subsequent weeks, normal responses are eliminated to produce a narrow ITD tuning curve centered on the learned ITD value (Fig. 1A, bottom panel).

The alteration of tectal ITD tuning can be accounted for by experience-dependent plasticity that occurs at the level of the ICX (6, 7). In the study reported here, we compared the pharmacology of ICX circuits mediating normal and newly learned responses in prism-reared owls. We did this by applying EAA receptor antagonists either systematically or locally into the ICX while recording responses at tectal sites displaying transition state ITD tuning. We focused on NMDA and non-NMDA subtypes of EAA receptors, because auditory transmission in the ICX of normal owls is known to be mediated through these receptors (9). ITD tuning was monitored in the optic tectum rather than in the ICX, because tectal VRFs allow unambiguous determination of normal ITD tuning for any given site (6, 8).

Systemic injection of the anesthetic and NMDA receptor antagonist ketamine HCl (10) at 10 to 15 mg per kilogram of body weight, a dose known to selectively antagonize NMDA receptors in the ICX (11),

Newly Learned Auditory Responses Mediated by NMDA Receptors in the Owl Inferior Colliculus

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