Involvement of Cell Cycle Elements, Cyclin-dependent Kinases, pRb, and E2F-DP, in B-amyloid-induced Neuronal Death*

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Alzheimer’s disease (AD) is a neurodegenerative disorder marked by progressive loss of memory and impairment of cognitive ability. Patients with AD display neuropathological lesions including amyloid plaques, neurofibrillary tangles, and eventual neuronal loss in brain regions associated with cognitive function (1). The amyloid plaque, an invariant pathological hallmark of AD, is composed of B-amyloid (AB), a 39–43-amino acid residue hydrophobic peptide that assembles into insoluble aggregates (2, 3). It is derived by alternative proteolysis from an integral membrane localized B-amyloid precursor protein (BAPP) (4). The importance of AB in progression of AD is underscored by the identification and linkage of mutations of BAPP and the presenilins in familiar cases of AD (5–7). Presenilins are transmembrane proteins thought to be involved in BAPP processing and trafficking (4). Additional support for the importance of AB in AD comes from reports demonstrating the neurotoxic properties of aggregated AB (7) The mechanism by which AB causes neuronal death is not well understood.

Recently, several groups have reported abnormal up-regulation of a variety of cell cycle proteins in brains from AD patients (8–10). However, it is unclear whether these deregulated cell cycle events contribute to neurodegeneration in AD or whether they are byproduct of a stressed brain. The cell cycle is a tightly regulated process controlled by sequential activation of cyclin-dependent kinases by up-regulation of its obligate activating cyclin partner (11). Generally, it is thought that Cdk4/6 and Cdk1 complex regulate the G1 to S transition, whereas Cdk2/cyclin E and Cdk3 control G2 to M transitions. One important target substrate for the CDKs is the tumor suppressor, retinoblastoma protein (pRb), which is phosphorylated by activated Cdk4/6/cyclin D (13).

Once hyperphosphorylated, Rb is released from the transcriptionally activating factor complex E2F-DP, which then activates genes required for S phase transition (14). Interestingly, it is up-regulation of several of the above discussed cell cycle control elements that have been reported in brains of AD patients. They include cyclin B, D, and E, Cdc2, and Cdk4 (8–10). In addition, increased Cdc2 activity has also been shown in AD brains (9).

In this report, we test the hypothesis that cell cycle elements play an obligate role in neuronal death evoked by AB. We provide support that certain cell cycle-related elements, Cdk4/6 and E2F-DP, are required for AB-induced neuronal death and that this signaling pathway may be a required component of neurodegeneration in AD.

EXPERIMENTAL PROCEDURES

Generation of Recombinant Sindbis—Recombinant sindbis virus expressing FLAG epitope-tagged DN Cdk2, 3, 4, and 6 as well as FLAG-tagged mutants of DP1 containing either the E2F-binding domain or DNA-binding domain deletions were constructed as described previously (15, 16). Control nonexpressing vectors were generated by eliminating the initiating codon of each inhibitor and in the case of Cdk4, Cdk3, and Cdk6, introducing a premature stop codon. All mutations, deletions, and FLAG tags were introduced by polymerase chain reaction as described previously and confirmed by sequencing (15, 16). Viral particles were generated by in vitro transfection and transfection into baby hamster kidney cells and titrated by plaque assay as described previously (15, 16).

Culture and Survival of Neuronal PC12 Cells—PC12 cells were differentiated by treatment with nerve growth factor as described previously (17). The cells were plated onto 96-well plates (coated with collagen I) at a density of 5,000 or 20,000 cells/well. Prior to addition of peptides, cultures were rinsed once with RPMI 1640, and the medium was changed to RPMI 1640 plus penicillin/streptomycin AB (1–40, Bachem CA, Torrence, CA) at a density of 100 μg/ml. TUNEL and LDH assays were performed according to the manufacturers instructions (TUNEL, in situ cell death detection kit, Roche Molecular Biochemicals; LDH, Promegs CytoTox 96 nonradioactive cytotoxicity assay kit).

Culture and Survival of Cortical Neurons—Rat cortical neurons were cultured from embryonic day 18 rats as described previously (16). The neurons were plated into 24-well dishes (approximately 200,000 cells/well) coated with polylysine in serum-free medium (N2;Dulbecco’s modified Eagle’s medium (1:1) supplemented with 6 mg/ml D-glucose, 100 μg/ml transferring, 25 μg/ml insulin, 20 nM progesterone, 60 μM putres-
the means expressed as a percentage of cells plated on day 0 and are reported as evaluated as described previously (16). All experimental points are expressed as a percentage of cells plated on day 0 and are reported as the means ± S.E. (n = 3).

Western Blot Analyses—Cortical neurons were dissociated and cultured as described above. At appropriate times during AB treatment or 24 h after infection (expression analyses), the neurons were harvested in sample buffer, and 50 μg of protein were loaded onto SDS-polyacrylamide gels and transferred onto nitrocellulose membrane as described previously. Blots were probed with anti-phospho-Rb antibody (New England Biolabs, 1:1000 dilution), anti-cyclin D1 (Santa Cruz, 1:300 dilution), or anti-FLAG antibody (10 μg/ml; VWR).

RESULTS

Inhibition of AB-evoked Death by the Pharmacological CDK Inhibitor Flavopiridol—The study of neuronal death in AD in vivo is made difficult by the lack of significant neuronal loss in mice models of AD expressing mutant BAPP or presenilins and the difficulty in genetic manipulations of neurons in vivo. Given this, we utilized an in vitro model of AB-induced neuronal death of cultured embryonic day 18 cortical neurons and neurally differentiated PC12 cells. Although these systems may not completely reflect the in vivo processes involved in AD, we feel that such studies provides valuable insight into AB-induced death signaling events. Reports from many groups as well as our own experience has indicated that aggregated AB of various lengths, including 1–42, 1–40, and 25–35, cause the apoptotic death of CNS neurons (18, 19). As shown in Fig. 1, AB (1–40), treatment results in the death of cortical neurons (70% death at 36 h). Equivalent treatment of neuronal PC12 cells also results in an increase in TUNEL positive neuronal PC12 cells and LDH release (Fig. 2).

To test for the obligate role of certain cell cycle elements in neurodegeneration, we reasoned that inhibition of such regulatory signaling would lead to neuronal survival. Because CDKs are both up-regulated in AD brains and play a central role in cell cycle progression, we first assessed the ability of the pharmacological CDK inhibitor flavopiridol to protect neuronal PC12 cells and cortical neurons from AB-evoked death. Flavopiridol (L86–8275, (−)-cis-5,7-dihydroxy-2-(2-chlorophenyl)-8(4-(3-hydroxy-1-methyl)-piperidinyl)-4H-benzopyran-4-one) has been shown to inhibit CDK activity with high specificity and is less effective against epidermal growth factor receptor kinase and protein kinases C and A (20). Flavopiridol reduced TUNEL staining (Fig. 2A) and LDH release (Fig. 2B) in neuronal PC12 cells treated with AB. Similarly, flavopiridol protected primary cortical neurons from AB-evoked death (75% survival with flavopiridol cotreatment versus 25% in the controls; Fig. 1). Most importantly, the concentration of flavopiridol required for neuroprotection (Fig. 1B) is the same dose reported to be required for inhibition of cell cycle progression (21). As shown in Fig. 3, cortical neurons treated with AB alone showed profound blebbing and shrunken phase bright morphology and degenerated processes, whereas neurons cotreated with flavopiridol demonstrated a flatter, more phase dark morphology typical of live cortical neurons.

Requirement for Cdk4 and 6 in Death of Cortical Neurons Evoked by AB—Although the above studies suggest the importance of CDKs in AB-evoked neuronal death, we also targeted CDKs using a more molecular biological approach to identify individual CDKs critical for regulating neuronal death. To do this, we expressed kinase dead dominant negative (DN) forms of CDKs via the recombinant sindbis virus. We have previously utilized this neurotropic viral vector to target genes to both sympathetic and cortical neurons and to evaluate neuronal apoptotic signaling mechanisms (15, 16). The DN CDK constructs were FLAG epitope-tagged to permit detection of the expressed protein. Expression of the CDKs were confirmed by Western blot analyses (Fig. 4A).

Expression of both DN Cdk4 (Fig. 5B) and DN Cdk 6 (Fig. 5C) protected cortical neurons from death evoked by AB, whereas DN Cdk2 (Fig. 5A) and DN Cdk3 (data not shown) did not. To control against the effects of the virus, we infected cultures with recombinant virus containing each respective DN CDK construct with the initiation codon deleted and a premature stop codon inserted. No effect on neuronal survival was observed with the control viruses. These results suggest that Cdk4/6 play an important role in neuronal death evoked by AB.

Phosphorylation of pRb/p107, a Substrate of Cdk4/6—The only reported substrates of Cdk4/6 activity is pRb and its re-
lated family member p107 (12, 13, 22). If, as our pharmacological and DN CDK expression studies suggest, Cdk4/6 activity is required for death of neurons, we would predict that phosphorylation of pRb/p107 would increase during death of cortical neurons evoked by AB. To test this, we utilized an antibody directed against the phospho-epitope of pRb at serine 795 and its equivalent site in p107, sites known to be phosphorylated by Cdk4/6. As would be expected if Cdk4/6 was active, there was a transient increase in pRb/p107 phosphorylation 4 h after AB treatment of cortical neurons (Fig. 6).

Because an up-regulation of cyclin D1 may increase Cdk4/6 activity, we next determined whether an elevation in cyclin D1 protein could be detected during AB treatment of cortical neurons. Cyclin D1 protein was readily detectable in cultures of untreated control cortical neurons by Western blot analyses. However, cyclin D1 levels remained relatively constant during the time course of AB treatment (Fig. 6).

Requirement for E2F-DP Complex in Death of Cortical Neurons Evoked by AB—Phosphorylation and subsequent release of pRb/p107 would lead to E2F activation (12–14). Accordingly, we examined whether inhibition of E2F-DP results in increased neuronal survival. To test this, we expressed a mutant form of DP1, an obligate binding partner to the E2F family members, which contains deletions in the DNA-binding domain (DP1 103–126) (23). This construct has been previously shown to act in a dominant negative fashion to inhibit cell cycle progression and E2F activity (23). As shown in Fig. 7, expression of DN DP1 protected cortical neurons from AB-induced death (75% survival with DN DP1 versus 35% without). No protective effect was observed with the control “stop” viruses or a mutant form of DP1, which has the E2F-binding domain deleted (DP1 233–272). The latter construct does not inhibit E2F transactivation (23) and was used as a control. Both DP1 constructs (DP1 103–126 and DP1 233–272) were FLAG epitope-tagged, and expression was confirmed by Western blot analyses (Fig. 4B). This findings taken together with the Cdk4/6 data suggest that Cdk4/6 activity may act to induce AB-evoked neuronal death via the actions of E2F-DP1 members.

DISCUSSION

Previous evidence indicated that a variety of cyclins and CDKs are up-regulated in brains of AD patients (8–10), sug-
suggesting that elements that normally control cell cycle progression in proliferating cells may also modulate neuronal death. Accordingly, we examined 1) whether CDKs play a required role in death of cortical neurons evoked by AB and 2) whether certain signaling elements downstream of CDKs (pRb and E2F1DP) are modified and/or required for death.

Involvement of CDK in Death of Neurons Evoked by AB—We show presently that the CDK inhibitor flavopiridol and expression of DN Cdk4/6 but not DN Cdk2 or 3 block death of PC12 cells and/or cortical neurons evoked by AB. These results imply that Cdk4/6 activity is an obligate signaling component of AB-induced neuronal death. In support of this observation, several groups have suggested that CDKs may play an important role in neuronal death. For example, NGF deprivation leads to increased Cdc2 activity and cyclin B expression in neuronal PC12 cells (24) as well as elevated cyclin D1 transcript levels in sympathetic neurons (25). Cyclin D1 protein is also increased in cis-platinum-treated sensory neurons (26).

Furthermore, expression of the CDK inhibitor p21 is required for survival of differentiated neuroblastoma cells (27), and the CDK inhibitor p16 protects these cells from death caused by trophic factor deprivation (28). Pharmacological cell cycle blockers suppress death of sympathetic and cortical neurons evoked by trophic factor deprivation (21, 29) and/or DNA damaging conditions (17, 30). Finally, virally mediated expression of CDK inhibitors such as p16 and p27 and of dominant negative forms of Cdk4 and 6 inhibit the death of neurons because of the above mentioned apoptotic stimuli (15, 16).

In contrast to the up-regulation of cyclin D1 in AD brains and in several other neuronal death paradigms (see above), we do not observe increased expression of this protein with AB treatment. Cyclin D1 is readily detectable in our untreated control cultured neurons. The failure to see an increase in cyclin D1 levels may reflect on the embryonic nature of our culture system and suggests that activation of Cdk4 may occur via post-translational mechanisms. In support of this, we have observed an increase in cyclin D1-associated kinase activity in dying cortical neurons evoked by DNA damage (15). This increase is observed without change in levels of cyclins, CDKs or CDK inhibitors.2 CDK activation may proceed through a different mechanism in the adult brain, perhaps requiring cyclin and/or CDK synthesis.

Rb and E2F1DP1 as Mediators of Neuronal Death Evoked by AB—We observed a transient increase in pRb/p107 phosphorylation during AB treatment of cortical neurons consistent with the requirement for Cdk4/6 activity. These results are intriguing in light of recent observations that pRb may play a

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overexpression of Cdk2DN (A), Cdk4DN (B), Cdk6DN (C), and respective controls on the time course of survival of AB-treated cortical neurons. The asterisks denote the significance (Student’s t test) with respect to AB treatment + control virus treatment.
Each point is the mean ± S.E. of data from three cultures and is expressed relative to the number of neurons present in each culture at the time of AB treatment (100 μg/ml). Control viruses for each vector were generated by removal of the start codon and introduction of a premature stop site. Effects of over-expression of DN DP1 (DNA-binding domain deletion) (A) and DP1(233) (E2F-binding domain deletion) (B) and respective controls on the time course of survival of AB-treated cortical neurons. The asterisk denotes significance (Student’s t test) with respect to AB treatment + control virus treatment.

role in apoptosis as well as cell cycle control. For example, overexpression of pRb in several cell contexts results in increased survival (31–33), whereas loss of Rb is associated with increased death (34, 35). In addition, mice lacking in pRb display massive neuronal loss during development (36). Finally, phosphorylation of Rb has been shown to occur in dying neurons treated with cis-platinum (26) and camptothecin.3

One consequence of the phosphorylation of pRb/p107 is release from and activation of E2F (23). As a transcriptional activator, E2F is known to interact with multiple proteins including c-Abl (40). In addition, E2F and DP are also reported to physically interact with p53 both in vivo and in proliferating cells (41, 42). The existence of such interactions in neurons and/or their potential function/importance are unknown and cannot be ruled out.

The findings presented here represent a significant insight into the potential mechanisms underlying the loss of neuronal function and viability in the brains of those suffering from Alzheimer’s disease. The action of amyloid to induce a state of neuronal de-differentiation, indicated by an attempted return to cell cycle, may be an early event in the progressive loss of neuronal and synaptic function. In this context, intervention and reversal of this process may lead not only to an alteration in the progression of cognitive decline in the Alzheimer’s patient, but the potential to restore neuronal function and improve cognition.

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