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Defective Ubiquitination of Cerebral Proteins in Alzheimer's Disease

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Alzheimer's disease (AD) is characterized by the presence of neurofibrillary tangles (NFT), senile plaques, and cerebrovascular deposits of amyloid-β. Ubiquitin has also been shown to be present in some of the inclusions characteristic of this disease. To obtain further insight into the role played by the ubiquitin pathway in AD, we investigated the capacity of postmortem samples of cerebral cortex from normal and AD patients to form high-molecular-weight ubiquitin–protein conjugates. Activity of the ubiquitin-activating enzyme (E1) and ubiquitin-conjugating enzymes (E2) involved in the ubiquitin pathway was also determined. In normal samples, the amount of high-molecular-weight ubiquitin–protein conjugates (HMW-UbPC) in cytosol increased with incubation time, whereas, in samples of AD cases, these were almost undetectable. The addition of an adult rat fraction, enriched in ubiquitinating enzymes, restored the capacity of AD brain cytosolic fraction to form conjugates. The trypsin-like proteolytic activity of the 26S proteasome was found to be decreased in AD cytosol brain. Assay of the activity of E1 and E2 by thiol-ester formation revealed a significant decrease in AD samples. Moreover, Western blotting using a specific antibody against E1 showed a dramatic drop of this enzyme in the cytosolic fraction, whereas normal levels were found in the particulate fraction, suggesting a possible delocalization of the enzyme. Our results suggest that a failure in the ubiquitination enzymatic system in brain cytosol may contribute to fibrillar pathology in AD. J. Neurosci. Res. 62:302–310, 2000.

Key words: Alzheimer disease; ubiquitin; ubiquitinating enzyme; 26S proteasome

INTRODUCTION

Alzheimer's disease (AD) is a heterogeneous group of disorders in terms of etiological factors. At least three different genes encoding β-amyloid precursor protein (βPP) and presenilins (PS) 1 and 2 are clearly related to early-onset, autosomal dominant variants of the disease (Goate et al., 1991; Sherrington et al., 1995; Levy-Lahad et al., 1995). A fourth gene, apolipoprotein E (apoE), is considered a strong susceptibility factor through allelic inheritance in late-onset forms of AD (Corder et al., 1993). However, most AD cases are not associated with any known genetic defect and are considered sporadic. However, in all AD patients regardless of etiology and age of onset, there is a progressive aggregation of abnormal proteins in the brain. The hallmark lesions in AD brains are the intraneuronal neurofibrillary tangles (NFT), composed of paired helical filaments, and the extracellular senile plaques, composed mainly of amyloid fibrils. Although the major fibrillar deposits are made of amyloid-β peptide of 42 or 43 residues (Aβ42–43; Masters et al., 1985) and hiperphosphorylated tau in intraneuronal NFT (Lee et al., 1991), respectively, several proteins are consistently associated with such deposits. Among a long list, βPP (Tagliavini et al., 1990) and apoE (Namba et al., 1991) are included, and recent reports point to the possible accumulation of PS1 and PS2 in NFT and senile plaques (Chui et al., 1998). In addition to genetic and postranslational factors involved in a higher tendency of some of these proteins to form fibrillar aggregates in AD brains, an impairment of protein degradation resulting from a defective proteolytic machinery remains as a possible contributor to fibrillar pathology.

Degradation of a protein via the ubiquitin pathway proceeds in two successive steps: 1) covalent attachment of multiple ubiquitin molecules to the protein substrate and 2) degradation of the targeted protein by the 26S proteasome complex with the release of free ubiquitin. The system consists of several components: Ubiquitin, a conserved protein of 76 residues, is activated in its C-terminal Gly to a high-energy thiol ester intermediate, a reaction catalyzed by ubiquitin-activating enzyme (E1). After activation, one of several ubiquitin-conjugating enzymes (E2) transfers activated ubiquitin from E1 to a member of the ubiquitin-protein ligase family (E3), to which the substrate protein is specifically bound. The first ubiquitin moiety is transferred to the ε-NH2 group of a Lys of the protein substrate. A polyubiquitin chain is synthesized by the

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progressive transfer of additional activated ubiquitin to Lys 48 of the previously conjugated ubiquitin molecule. The chain serves as a recognition marker for the proteasome (Hershko and Ciechanover, 1986; Ciechanover and Schwartz, 1998). Alterations in this process have been implicated in the pathogenesis of several diseases, both inherited and acquired. One of them is Angelman's syndrome (Kishino et al., 1997). There is a family of related inclusion bodies in certain pathologies of the central nervous system associated with ubiquitin and certain enzymes of the ubiquitin pathway. The first inclusions to be recognized as being associated with ubiquitin were the tau-containing NFT of AD. Kwak et al. (1991) reported that components of the 20S proteasome are present in cortical Lewy bodies and some NFT. It has been postulated that the ubiquitin-dependent degradation pathway may be compromised in AD, including defective polyubiquitination and impaired proteasomal degradation (Master et al., 1997; van Leeuwen et al., 1998). The aim of our study was to determine the in vitro formation of ubiquitin conjugates and to assess the activity of the enzymes involved in ubiquitin conjugation in brain samples from AD patients and normal controls.

MATERIALS AND METHODS

Materials

Frozen postmortem brain samples from normal controls (mean age 63.5 years, range 46–76 years; n = 7) and certified sporadic late-onset AD patients (mean age 77 years, range 60–87 years; n = 14) were provided by the National Neurological Research Specimen Bank, VAMC, (Los Angeles, CA), which is sponsored by NINDS/NIMH, National Multiple Sclerosis Society, Hereditary Disease Foundation, Comprehensive Epilepsy Program, Tourette Syndrome Association, Dystonia Medical Research Foundation, and Veterans Health Services and Research Administration, Department of Veterans Affairs. Human E1 cDNA was kindly provided by A.L. Schwartz. Carrier-free Na-^{125}I, acrylamide, and pGex2T expression vector were from Amersham Corp. (Amersham, England). Carbobenzoxy L-leucyl-L-leucyl-L-leucinal (MG 132) and lactacystin were purchased from Calbiochem-Novabiochem (San Diego, CA); N,N′-bismethylene acrylamide, restriction enzymes, and pGem T vector were from Promega Corporation (Madison, WI). All reagents, unless otherwise specified, were purchased from Sigma Chemical Co. (St. Louis, MO).

Ubiquitin Labeling

Native ubiquitin was radiolabeled with carrier-free Na-^{125}I by the chloramine T method (Ciechanover et al., 1980), with slight modifications. The tubes with the highest specific radioactivity were kept in 50 μl aliquots at −20 °C. The specific activity obtained ranged between 2.9 and 10.0 mCi/mg.

Isolation of the Soluble Fraction and Preparation of Fraction II

Cerebral cortex from normal and AD cases was homogenized (glass-teflon homogeneizer), in 20 mM Tris HCl, pH 7.4, 140 mM NaCl, and centrifuged at 105,000*g* for 90 min at 4 °C to separate the supernatant (soluble fraction). A fraction enriched in ubiquitinating enzymes (fraction II) was prepared using a soluble fraction isolated from adult rat brain as described elsewhere (Ciechanover, 1978). The soluble fraction was passed through a DEAE cellulose column equilibrated in 3 mM PO_4KH_2, pH 7.4, 1 mM dithiothreitol (DTT), washed with buffer A (3 mM PO_4KH_2, pH 7.0, 1 mM DTT), and eluted with buffer B (10 mM Tris HCl, pH 7.1, KCl, 1 mM DTT). Proteins in fraction II were precipitated with $(NH_4)_2SO_4$, dialyzed against 10 mM Tris HCl, pH 7.4, 1 mM DTT, ATP 0.5 mM, and kept in 200 μl aliquots at −80 °C. Protein concentration was determined by the method of Lowry (1951) using bovine serum albumin as standard.

Assay of Ubiquitin Conjugation

The conjugation of ^{125}I-ubiquitin to soluble proteins was assayed as previously described (Adamo et al., 1994) using 5×10^5 cpm of ^{125}I-ubiquitin and 1 μg of unlabeled ubiquitin in an incubation medium in the presence and absence of ATP. When ATP was omitted, creatine phosphate and creatine phosphokinase were replaced with 1 mM 2-deoxyglucose and 4 U hexokinase (Jahgen et al., 1986). Samples were incubated for the indicated times at 37 °C. Ten microliters of 5× Laemmli sample buffer were used to stop the reaction, and the sample was immediately boiled for 2 min. The incubation, as described above, was also done with the addition of 50 μg of fraction II, enriched in the enzymes E1, E2(s), and E3(s).

Electrophoretic Analysis and Autoradiography

High-molecular-weight ^{125}I-ubiquitin-protein conjugates (HMW-UbPC) were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970). The concentration of acrylamide/bis used for SDS-PAGE varied in different experiments and is indicated in the text. After electrophoresis, the gels were stained with 0.25% Coomassie brilliant blue R-250. Autoradiography was carried out by exposure of the gels to 3 M radiographic film for 5 days at −70 °C. The level of ^{125}I-ubiquitin conjugates was quantified by densitometry of the autoradiograms.

26S Proteasome Proteolytic Activities

Cerebral cortex from normal and AD cases was homogenized in solubilization buffer (12.5 mM KCl, 135 mM Tris-acetate, pH 7.5, 80 mM EGTA, 6.25 mM β-mercaptoethanol, and 0.17% octyl-β-D-glucopyranoside), and centrifuged at 105,000*g* for 90 min. Soluble fraction and total homogenate were used to assay the three main 26S proteasome proteolytic activities: peptidyl glutamyl hydrolase (PGp hydrolase) and trypsin- and chymotrypsin-like activities. To determine trypsin- or chymotrypsin-like activity, 100 μg of proteins were incubated with 100 μl of 100 mM HEPES HCl, pH 7.5, containing 50 μM substrate (Boc-Leu-Ser-Thr-Arg-7-amino-4-methylcoumarin or Ala-Ala-Phe-7-amido-4-methylcoumarin, respectively) for 30 min at 37 °C. The reaction was stopped by addition of 100 μl 220 mM sodium acetate buffer, and nondegraded protein was precipitated for 30 min at 4 °C. A clear supernatant was obtained by centrifugation at 400*g* for 30 min at 4 °C. A 200 μl aliquot of the supernatant was mixed with 2 ml of distilled water, and the fluorescence was measured in a Hitachi F1200 spectrofluorometer at excitation and emission wavelengths

of 370 nm and 430 nm, respectively. PGp hydrolase-like activity was measured in a similar manner using Clz-Leu-Leu-Glu-β-naphtylamide as the substrate, followed by precipitation of intact protein by the addition of 300 μl of absolute ethanol. After precipitation and centrifugation, 250 μl of the supernatant were diluted with 2.0 ml of distilled water, and the fluorescence was measured at excitation and emission wavelengths of 333 nm and 450 nm, respectively. Protein-free blanks were included, and a standard curve was prepared with 7-amino-4-methylcoumarin or β-naphthylamine. The three proteolytic activities were assayed in the presence and absence of 50 μM lactacystin, a specific inhibitor of the proteasome.

Production of Anti-E1 Polyclonal Antibody

A region between amino acids 184 and 234 of the human E1 sequence was amplified by PCR using E1 cDNA cloned in pGem T as a template. The following primers were used: E1 forward 5′-CCGGATCCGTTACCAAGGACAACCC-3′ and E1 reverse 5′-AATTCATAAGGACCCAGGACTTT-3′. After digestion with Bam H1 and EcoRI, the 150 bp insert was subcloned into pGex2T vector, and the resulting construct was designated pGex2T-E1a. GST-E1a fusion protein was expressed and purified as described elsewhere (Smith and Johnson, 1988). After immunization of New Zealand rabbits, antiserum (anti E1) was tested for reactivity against GST-E1a and E1 in the soluble brain fractions by Western blot.

Immunoblotting

After SDS-PAGE, the proteins were electrophoretically transferred to nitrocellulose membranes, blocked with PBS containing 5% low-fat milk, 0.1% Tween 20, and incubated overnight at 4 °C with anti-E1. The membranes were washed with PBS 5% low-fat milk, 0.1% Tween 20, and the bands detected with anti-rabbit IgG-alkaline phosphatase conjugate and nitroblue tetrazolium/5-bromo-4-cloro-3-indolylphosphate. Immunoreactive bands were quantified by densitometric analysis.

Determination of the Activities of Enzymes Involved in Ubiquitin Conjugation

The activities of E1 and E2s were determined using their property to form thiol esters with ubiquitin as described elsewhere (Johnston et al., 1991). This assay was done in a medium containing 50 mM Tris HCl, pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 2 mM 5′-adenosine, β,γ-imino triphosphate (AMP-PNP), ^{125}I-ubiquitin (1×10^6 cpm), and soluble fraction or cortex total homogenate (100 μg proteins) in a final volume of 50 μl. Following the incubation at 37 °C for 15 min, the reaction was stopped by addition of 2× Laemmli buffer (containing 5% β-mercaptoethanol) or thiol ester buffer (50 mM Tris, 4% SDS, 8 M urea, pH 6.8). AMP-PNP is an ATP analog that allows the formation of ubiquitin conjugates while precluding ATP-dependent degradation by the 26S proteasome. After standing at room temperature for 20 min, proteins present in the mixture were boiled and separated by SDS-PAGE. Gels were dried and exposed as described above. The activities of E1 and E2 were quantified by determining the difference between the density of the 120 and 25 kDa bands in the samples treated with thiol ester buffer and the density of the same bands in the sample reduced with β-mercaptoethanol.

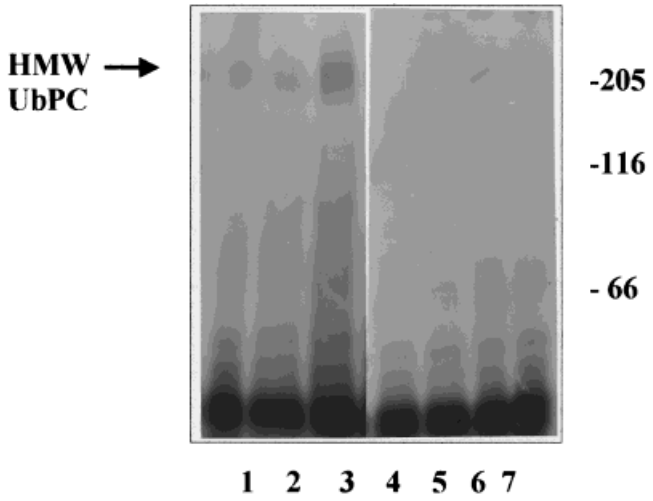

Fig. 1. Time course of HMW-UbPC formation in normal and AD cerebral cortex. Cytosolic proteins (100 μg) obtained from normal (**lanes 1–3**) and AD (**lanes 4–7**) samples were used for the conjugation assay, in the presence of radioactive ubiquitin and ATP. Autoradiography of a 5–8% gradient SDS-PAGE; lanes 1, 4: 10 min; lanes 2, 5: 15 min; lanes 3, 6: 30 min; lane 7: 45 min. Arrow, high-molecular-weight ubiquitin–protein conjugates (HMW-UbPC). Right, molecular mass markers in kilodaltons.

To test the specificity of the antiserum against E1, the soluble fraction was mixed with the antiserum and preimmune antiserum overnight at 4 °C. Immune complexes were precipitated with protein A agarose. After centrifugation, the supernatant was used to estimate the activity of E1, as described above.

Statistical Analysis

Differences between groups were compared by the Student's *t*-test and were considered significant at $P < 0.01$.

RESULTS

The formation of ^{125}I-ubiquitin–protein conjugates in the soluble fraction of postmortem human brain cortex was assayed by SDS-PAGE and autoradiography showing the presence of HMW-UbPC of ~200 kDa, as described elsewhere (Jahgen et al., 1986). When ATP was depleted from the reaction mixture, no radiolabeled HMW-UbPC was seen, indicating that these components reflect true polyubiquitin conjugates of soluble brain proteins and not aggregated species of ^{125}I-ubiquitin alone (data not shown). A time-course experiment showed that maximal HMW-UbPC formation was reached after 30 min of incubation, so this time point was used for further experiments (Fig. 1, lanes 1–3).

Cytosolic fractions from brains of 46–76-year-old normal subjects showed no differences in the amount or the pattern of HMW-UbPC, suggesting that, under the experimental conditions used for our studies, ubiquitination of brain proteins did not change with age (data not shown). Next, we studied the formation of HMW-UbPC

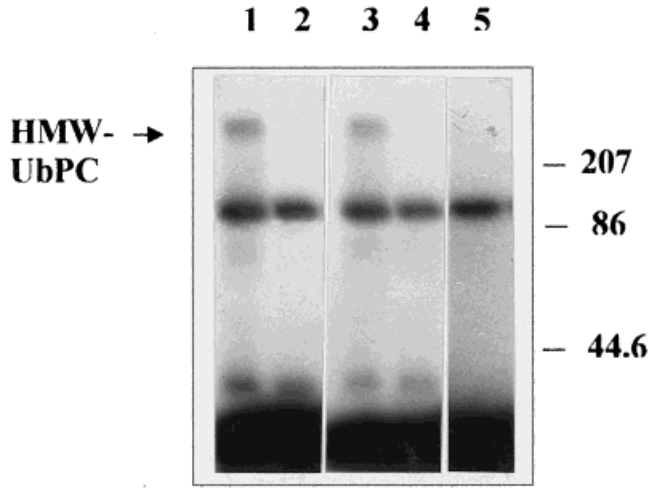

Fig. 2. Formation of HMW-UbPC in normal and AD cerebral cortex. Cytosolic proteins (100 μg) were used for the conjugation assay in the presence of radioactive ubiquitin and ATP. Autoradiography of a 12.5% SDS-PAGE. Incubation in medium containing ATP for 30 min; **lane 1:** normal frontal cortex; **lane 2:** AD frontal cortex; **lane 3:** normal temporal cortex; **lane 4:** AD temporal cortex, **lane 5:** assay carried out in the absence of brain proteins. The radioactive band of ~75 kDa most likely represents a nonspecific association between ^{125}I-ubiquitin and bovine serum albumin, used to stabilize tracer preparation. Arrow, HMW-UbPC. Right, molecular weight mass in kilodaltons.

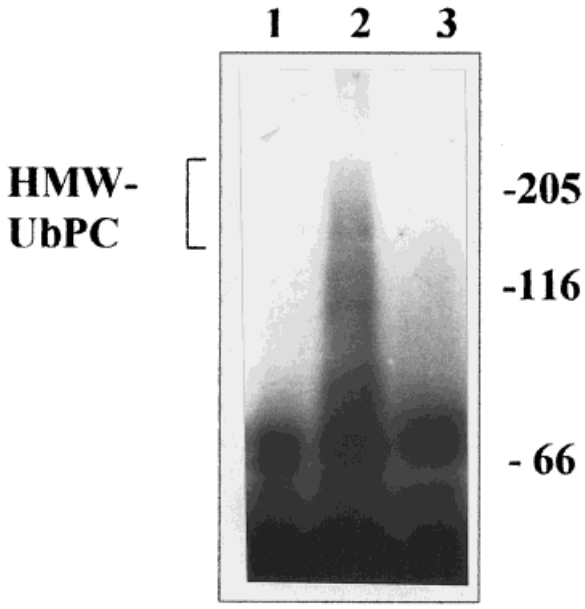

Fig. 3. Effect of fraction II on conjugate formation in AD cerebral cortex. Autoradiography of a 5–15% gradient SDS-PAGE. Cytosolic proteins (100 μg) were used for the conjugation assay in the presence of radioactive ubiquitin and ATP, in the absence (**lane 1**) or presence (**lane 2**) of fraction II (50 μg). Fraction II proteins (50 μg) were conjugated to ubiquitin in the presence of ATP (**lane 3**). Right, molecular mass markers in kilodaltons.

in cytosolic fractions obtained from sporadic, late-onset AD patients with neuropathological confirmation. No HMW-UbPC were detected, even after a 45 min incubation (Fig. 1, lanes 4–7). Formation of ^{125}I-ubiquitin–protein conjugates was not detected in temporal and frontal cortex samples from 12 of 14 independent cases of AD (Fig. 2). Densitometric analysis showed that the mean optical density of HMW-UbPC bands in AD and control samples was 2.1 ± 0.5 and 23 ± 1.8 arbitrary units, respectively. The broad range of ages studied (60–87 years) suggested that the absence of HMW-UbPC was not influenced by age in the AD group. The postmortem intervals were less than 24 hr in normal controls and less than 10 hr in most of the AD cases. Therefore, it is unlikely that the low levels of HMW-UbPC in AD brains can be attributed to autolysis. The lack of capacity to form HMW-UbPC found in the AD samples was corrected by the addition of fraction II obtained from rat brain cortex cytosol, enriched in the enzymes E1, E2(s), and E3(s), that catalyze the steps for the formation of ubiquitin-protein conjugates (Fig. 3). No HMW-UbPC were detected when rat fraction II was analyzed alone. These results suggested that the absence of HMW-UbPC in AD brains was not related to abnormalities in the substrate proteins present in the cytosolic fraction.

To test whether the lack of detection of HMW-UbPC in AD samples was due to a much faster rate of degradation, we studied the effect of proteasome inhibition on the accumulation of ubiquitin conjugates. When the proteasome inhibitor MG 132 (Lee et al., 1996) was added to the incubation media, we detected a significant increase in the levels of HMW-UbPC in normal samples. On the contrary, the almost undetectable levels of HMW-UbPC present in AD samples were not influenced by the addition of MG 132 (Fig. 4). Because these results could reflect a gross depletion of proteasomes from the AD cytosolic fractions (Lowe et al., 1990; Kwak et al., 1991; Fergusson et al., 1996), the three major proteasome proteolytic activities were examined in the total homogenates and in the cytosol from control and AD brains. The results show that, except for the trypsin-like activity, which shows a decrease of 50% in the AD cytosolic fraction, the other activities were within normal values (Table I). These results suggest that the lack of accumulation of HMW-UbPC in AD cytosol in the presence of MG 132 was not due to the absence of proteasomes in the fraction. However, they are consistent with a partial depletion of cytosolic proteasome components that may localize to NFT, as reported previously (Lowe et al., 1990; Fergusson et al., 1996). To investigate the possible presence of inhibitor(s) of ubiquitination in AD brains, control and AD cytosolic fractions were mixed and assayed for the presence of HMW-UbPC, as described above. The formation of HMW-UbPC in control samples was not inhibited by AD brain cytosol (data not shown).

To explore the possibility that the ubiquitinating enzymes were directly involved, we studied the ability of

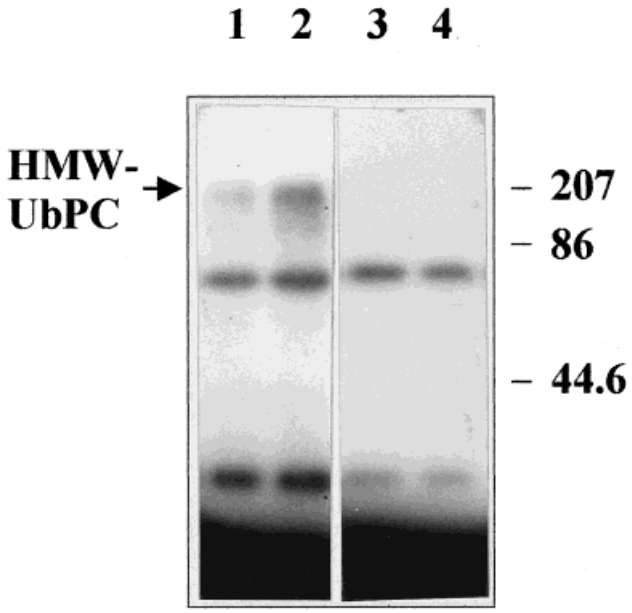

Fig. 4. Effect of the proteasome inhibitor MG 132 on ubiquitin–protein conjugate formation in AD and normal cerebral cortex. Autoradiography of a 12.5% SDS-PAGE. Cytosolic proteins (100 μg) were used for the conjugation assay in the presence of radioactive ubiquitin and ATP, in the absence (**lanes 1,3**) or presence (**lanes 2,4**) of MG 132 (100 μM). Lanes 1, 2: Controls; lanes 3, 4: AD samples. Right, molecular mass markers in kilodaltons. The radioactive band of ~75 kDa most likely represents a nonspecific association between ^{125}I-ubiquitin and bovine serum albumin, used to stabilize tracer preparation.

cytosolic brain fractions from normal and AD subjects to form thiol–ester conjugates between ^{125}I-ubiquitin and the E1, E2(s), and E3(s) components of the ubiquitination cascade. The thiol–ester assay was performed in the presence of AMP-PNP, a βγ nonhydrolyzable ATP analog that allows the formation of ubiquitin conjugates while precluding ATP-dependent degradation of the conjugates by the 26S proteasome (Johnston and Cohen, 1991). Incubation of normal brain soluble samples with AMP-PNP and ^{125}I-ubiquitin showed the presence on SDS-PAGE of several radiolabeled components, including two bands of ~120 kDa and ~25 kDa, respectively, which were resistant to boiling in 8 M urea and which disappeared after the addition of β-mercaptoethanol (Fig. 5). The 120 kDa band is consistent with conjugates between E1 (110 kDa) and ubiquitin (8.5 kDa), whereas the 25 kDa species may reflect ubiquitin thiol–ester complexes with one or more components of the E2 family of enzymes with known molecular masses of 14–35 kDa (Varshavsky, 1997). Identical results were obtained with cytosolic brain fractions from two patients with Angelman's syndrome, known to be caused by mutations in the UBE3A/E6AP gene of the E3 ligase family, which were used as an additional control (data not shown). Densitometric analysis revealed a dramatic decrease in the levels of the 120 kDa and 25 kDa components in AD samples as compared to controls. In some cases, these components were not detected, and overall estimates were 12% ± 2.1% and 2% ± 0.6% of controls for the 120 kDa and 25 kDa bands, respectively ($P < 0.01$).

To investigate the presence and levels of E1 in AD brains, we prepared antiserum in rabbits against a fusion protein GST-E1 comprising the active site of the enzyme. To confirm that the antiserum contained antibodies against E1, we tested its ability to immunoprecipitate E1 from the soluble fraction. After incubation with anti-E1, the soluble fraction lost its capacity to form the thiol–ester complex ^{125}IUb-E1 (Fig. 6), indicating that E1 was depleted from the fraction by the antiserum. A similar assay with preimmune antiserum as a control showed the presence of the thiol–ester complex (data not shown).

Western blot of the soluble fraction from normal cerebral cortex with anti-E1 detected two components of apparent molecular masses of 110 and 90 kDa. The former corresponds to the molecular weight of intact E1, whereas the latter may represent a partially degraded component. The specificity of the reaction was confirmed by complete adsorption of both bands with GST-E1a, whereas immunoreactivity was not modified by incubation with GST alone. Moreover, an antiserum against an unrelated GST fusion protein showed no reactivity against the 110 kDa and 90 kDa components (Fig. 7).

When the proteins of the soluble fraction from AD brains were tested with anti-E1, a dramatic decrease in the levels of the 110 kDa band was observed compared to controls. In some cases, these components were not detected, and overall estimates were 28% of control for the 110 kDa band (Fig. 8, lanes 1, 3). When this experiment was done with samples of the 105,000*g* pellet, AD brain samples showed levels of 110 kDa bands similar to those of controls (Fig. 8, lanes 2, 4).

DISCUSSION

A growing body of evidence suggests that the ubiquitin-dependent degradation pathway may be altered in AD brains in several ways. Antibodies against ubiquitin and proteasome components can label NFT, and tau–ubiquitin conjugates have been shown to accumulate in AD brains (Perry et al., 1986). Interestingly, monoubiquitin, as opposed to polyubiquitin–tau complexes, seems to predominate, suggesting a partial defect in the ubiquitination of this cytoskeletal protein in AD (Morishima-Kawashima et al., 1993). βPP soluble isoforms have been shown to be degraded after ubiquitin tagging (Gregori et al., 1994), and Aβ itself seems to bind to the 20S proteasome and inhibit its chymotrypsin-like activity (Gregori et al., 1995). More recently, PS1 and PS2 have been shown to form multiubiquitin complexes that may target their proteasomal degradation in cell culture transfection studies (Kim et al., 1997; Fraser et al., 1998). PS1 appears to be necessary for the γ-proteolytic cleavage of βPP that generates Aβ. Several mutations in PS1 and PS2, associated with familial AD, have been proposed to lead to a gain of function related to an increased production of the longer

TABLE I. Proteasome Proteolytic Activity in Normal and AD Brain Samples†

	Soluble fraction			Homogenate		
Activity	Normal	AD	Percentage	Normal	AD	Percentage
Trypsin-like	1,045.0 ± 212.6	507.9 ± 69.9	48.6★	2,376.9 ± 373.8	1,677.9 ± 222.9	70.6
Chymotrypsin-like	1,733.8 ± 533.1	1,539.7 ± 155.2	86.8	2,063.4 ± 214.2	1,862.1 ± 69.6	90
PGp hydrolase	54.8 ± 14.6	38.3 ± 7.6	70	91.1 ± 4.14	86.3 ± 5.13	95

†Samples of total homogenate and soluble fraction from normal and AD brain were used to determine the three major proteolytic activities of the proteasome. Assays were run using specific substrates for each case and in the presence and absence of 50 μM lactacystin. To obtain the true proteasome proteolytic activity, the nonspecific (lactacystin resistant) activity was subtracted from the total activity. Results are expressed as pmoles/mg · min.
★The differences between controls and AD trypsin-like activity were statistically significant ($P < 0.01$, unpaired *t*-test).

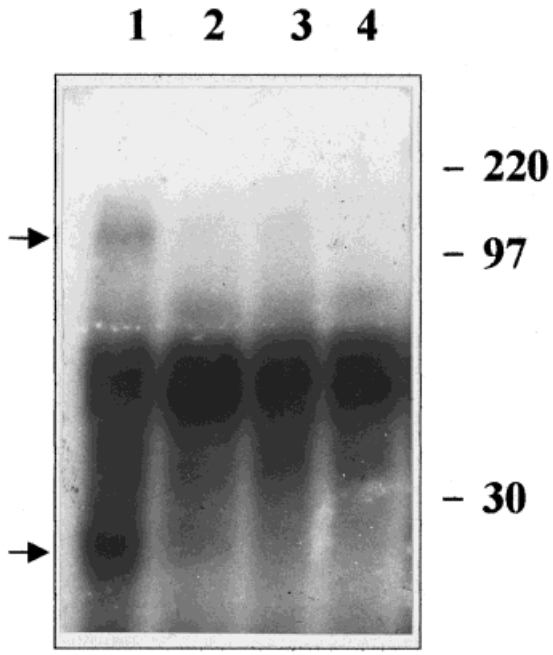

Fig. 5. Activities of E1 and E2(s) in normal controls and AD brain cytosol samples. E1 and E2(s) were determined by a thiol–ester assay. Equal amounts of protein (100 μg) were used in each assay. Autoradiography of a 12.5% SDS-PAGE. **Lanes 1,2:** Controls; **lanes 3,4:** AD samples. After incubation at 37 °C for 15 min, the reaction was stopped by the addition of 5× Laemmli buffer containing 5% β-mercaptoethanol (lanes 2, 4) or thiol–ester buffer without β-mercaptoethanol (lanes 1, 3). The arrows indicate the positions of E1-ubiquitin (~120 kDa) and E2-ubiquitin (~25 kDa). Right, molecular mass markers in kilodaltons.

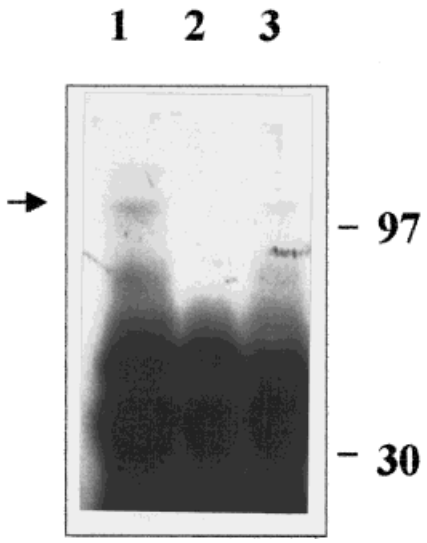

Fig. 6. Immunoprecipitation of E1 from the soluble fraction. Cytosolic proteins (100 μg) were mixed with anti-E1 antibody, and the immunocomplexes were precipitated with protein A agarose. E1 activity was determined in the supernatant (**lane 3**) and in the cytosolic fraction without adsorption of the antibody, in the absence (**lane 1**) and presence (**lane 2**) of β-mercaptoethanol. Right, molecular mass markers in kilodaltons.

amyloidogenic Aβ isoforms of 42 or 43 residues (Sheuner et al., 1996). Whether an impaired or a slower rate of degradation of wild-type PS results in overproduction of Aβ42–43 in sporadic AD remains to be studied. Although the proteolytic pathway of apoE in the brain is poorly understood, a recent report describes proteasomal degradation of apoE overexpressed in cultured macrophagic cells (Duan et al., 1997). This finding raises the possibility that apoE may partially enter the ubiquitin protein degradation pathway. Furthermore, postranscriptional dinucleotide deletions in the mRNA of ubiquitin B has been recently proposed as a novel pathogenic mechanism in sporadic AD that may result in a defective ubiquitination (van Leeuwen et al., 1998).

Our results showing very low levels of HMW-UbPC in AD brain cytosol raised several questions, including 1) a much faster rate of deubiquitination or degradation in AD brains compared to normals, 2) the presence of inhibitor(s) of ubiquitin conjugation, and 3) a defect in the enzymes involved in ubiquitination. The appearance of stable HMW-UbPC when fraction II from rat brain cytosol was added to AD samples, the presence of HMW-UbPC when normal and AD-soluble fractions were mixed, the lack of capacity of MG 132 to increase HMW-UbPC in AD brain, and the slight decrease in AD samples in the three proteasome proteolytic activities strongly argue against the first two possibilities. Our findings with the thiol–ester assay are consistent with a defect in E1 activating enzyme and, perhaps, one or more of the

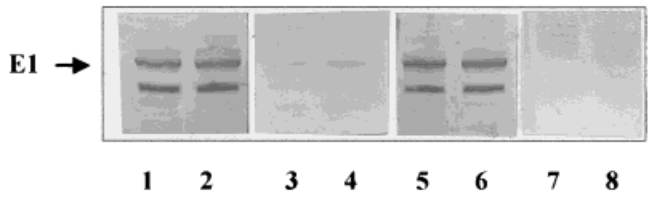

Fig. 7. Western blot of cytosolic proteins (100 μg) from normal brain. **Lanes 1,2:** probed with antiserum against GST-E1a (anti-E1); **lanes 7,8:** probed with an antiserum raised against an unrelated GST fusion protein. Anti-E1 was treated with GST-E1a (**lanes 3,4**) and with GST alone (**lanes 5,6**).

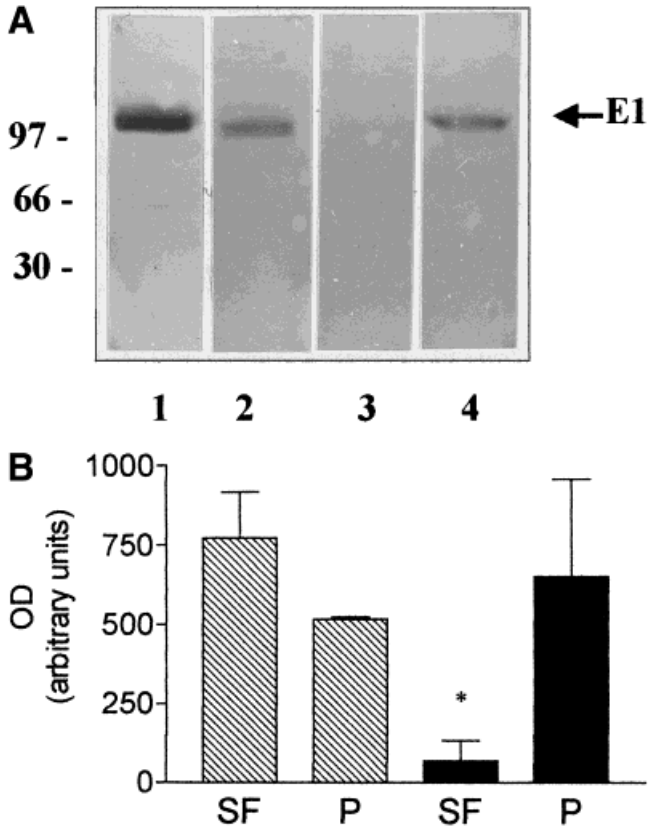

Fig. 8. **A:** Western blot of cytosolic proteins (100 μg; **lanes 1,3**) and pellet (**lanes 2,4**) from normal (lanes 1, 2) and AD (lanes 3,4) cerebral cortex, probed with anti-E1. Right, molecular mass markers in kilodaltons. **B:** Band of 110 kDa present in the soluble fraction (SF) and in the pellet (P) analyzed by densitometry. Hatched bars, controls; solid bars, AD. Results represent the mean ± SD of four normal and four AD brain samples.

E2 conjugating enzymes of the ubiquitin system in the brain cytosol of AD patients (Pickart and Rose, 1985). This assay has been extensively used to study the activity of the ubiquitination enzymatic system (Cook and Chock, 1992; Hatfield and Vierstra, 1992; Liu et al., 1992; Pickart et al., 1994; Hatfield et al., 1997; Shang et al., 1997), because the only proteins known to form thiol–esters with ubiquitin are E1 (Ciechanover et al., 1980; Hatfield and Vierstra, 1992) and the members of the E2 family (Pickart and Rose, 1985; Haas and Bright, 1988), although it has been reported that E3s may also have such an activity (Berleth et al., 1992). Results of the Western blot using a specific antibody against E1 were consistent with the thiol–ester assay, showing a marked decrease of E1 levels in the AD cytosolic fraction, whereas normal levels of this enzyme were formed in the particulate fraction. The displacement of key components of the ubiquitin degradation pathway to an insoluble compartment (i.e., fibrillar deposits) has been proposed in AD (Lowe et al., 1990; Fergusson et al., 1996).

Ms73 belongs to a family of ATPases that act as regulatory subunits of the 26S proteasome. In hippocampal sections from AD patients, Ms73 has been coimmunolocalized with NTF, dystrophic neurites, and neuropil threads (Fergusson et al., 1996). Furthermore, it has been shown that PGP9.5 (a ubiquitin carboxyl-terminal hydrolase) is enriched in ubiquitinated inclusion bodies (Lowe et al., 1990). The findings in the present study suggest that E1 may be localized in fibrillar inclusions present in AD brain.

This study raises the additional question of whether an impaired ubiquitination resulting from an enzymatic defect is a primary event or secondary to the disease process. The biochemical demonstration of multiubiquitin complexes in AD brains has been addressed only partially. However, the current evidence for the presence of ubiquitinated tau (Cook and Chock, 1992) and other ubiquitin adducts (Master et al., 1997) speaks against the former possibility. The presence of ubiquitin conjugates in certain inclusions found in AD (Perry et al., 1989; Morishima-Kawashima et al., 1993) does not preclude the possible existence of a defective ubiquitinating machinery in the cytosol of AD brains, in that, among other explanations, the delocalization of E1 and possibly E2(s) could be a late event occurring during the development of the disease. However, in light of the heterogeneity of the disease, we propose that a defect in ubiquitination may play a pathogenic role in a subgroup of sporadic AD patients. In any case, a secondary impairment of ubiquitin-dependent degradation resulting from a decrease in normal E1 and E2(s) activities in the brain cytosol, as a consequence of their delocalization to the particulate fraction, may contribute to the abnormal accumulation of proteins in AD and this deserves future research as a possible disease mechanism.

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