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Alzheimer and β-amyloid-treated fibroblasts demonstrate a decrease in a memory-associated GTP-binding protein, Cp20

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ABSTRACT **The two proteins most consistently identified in the brains of patients with Alzheimer disease (AD) have been β-amyloid and tau, whose roles in the physiology or pathophysiology of brain cells are not fully understood. To identify other protein(s) involved in AD that have been implicated in physiological contexts, we undertook to analyze a specific memory-associated protein, Cp20, in fibroblasts from AD and control donors. Cp20, a GTP-binding protein that is a member of the ADP-ribosylation factor family, was significantly decreased in fibroblasts from AD patients. Normal control fibroblasts exposed to 10 nM β-amyloid, the same concentration that induced AD-like K^+ changes in control fibroblasts, showed a similar decrease in Cp20. Since it has been previously demonstrated that Cp20 is a potent regulator of K^+ channels, these findings suggest that changes in this memory-associated protein may explain previously observed differences in AD K^+ channels and suggest a pathophysiologic involvement linked to soluble β-amyloid metabolism that could contribute to the characteristic memory loss of AD.**

The two proteins most consistently identified in the brains of patients with Alzheimer disease (AD) have been β-amyloid, concentrated in typical plaque lesions, and tau, the main component of neurofibrillary tangles (1–5). The roles of β-amyloid and tau in the physiology or pathophysiology of brain cells, however, are not fully understood (4–10). Recent observations of AD-specific changes in K^+ channels (11, 12), receptor-mediated, inositol trisphosphate-induced intracellular Ca^{2+} release in human fibroblasts (13), and related K^+ channel changes in human olfactory neuroblasts (14), together with other observations (15–17), suggested that there may be other key proteins that are critical for AD but also contribute to storage of associative memory. To identify other proteins involved in AD that have physiological functions, we undertook to analyze a specific memory-associated protein, Cp20, in fibroblasts from AD and control donors. Cp20, a high-affinity substrate for protein kinase C (15), shows specific differences of phosphorylation in neurons of mollusks and mammals that undergo associative learning (18–20). This GTP-binding protein, which induces a number of memory-specific neuronal changes [e.g., K^+ current reduction, focusing of synaptic terminal branches (20, 21)], also regulates retrograde axonal transport (22) and is a member of the ADP-ribosylation factor (ARF) protein family that has been implicated in the trafficking of particles between the Golgi compartment and the endoplasmic reticulum (23). In addition, it was recently found that Cp20 activated DNA transcription in nerve cells (T.J.N., J.L.O., H. Kim, and D.L.A., unpublished observations), which correlates with previous findings showing increased mRNA synthesis in conditioned *Hermissenda* (25, 26). Moreover, Cp20 was found to be highly enriched in ribosomes and nuclei of both squid optic lobe and sea urchin oocytes (T.J.N., J.L.O., H. Kim, and D.L.A., unpublished observations). Taken together, these observations indicate that Cp20 may be an important component of a signal transduction pathway that includes regulation of gene transcription and is essential in memory storage processes, and perhaps also relevant for AD pathophysiology. We report here that Cp20 is consistently and markedly decreased in the fibroblasts of both AD patients and nonaffected close relatives of AD patients, but not in aged-matched controls who are not members of families with hereditary AD. Incubation of normal fibroblasts with low concentrations of soluble β-amyloid reproduced the AD phenotypes for Cp20.

METHODS

Cell Lines and Cell Culture. Human skin fibroblasts were grown to confluence in 75-cm^2-growing-surface culture flasks (Falcon) containing Dulbecco's modified Eagle's medium (DMEM; GIBCO), supplemented with 10% fetal bovine serum (GIBCO). Cells from 13 AD individuals [AG06840, AG06844*, AG06848*, AG08170, AG7637, and AG08527* (familial AD, family 964; 4 men, 2 women); AG04401 (familial AD, family 747; 1 woman); AG07376, AG07377, AG06262, AG05770*, AG06263, and AG07375 (non-familial AD; 5 men, 1 woman); 60.40 ± 6.05 years (mean ± SD); *, autopsy confirmation], 9 age-matched control (AC) individuals [GM04260, GM04560, GM03524, AG07303, AG08044, AG09878, AG07141, AG07310, and AG06241 (all apparently normal, without known family history of AD; 3 men, 6 women), 62.89 ± 5.16 years], and 4 "escapees" (Es) [AG06838†, AG06842†, and AG07665‡ (members of family 964); AG08265† (member of family 2090), 67.25 ± 6.85 years; †, immediate relative affected (parents and/or siblings); ‡, uncle affected] were used for Cp20 and total protein assessments. Additional information regarding these cell lines can be found elsewhere (27, 28).

β-Amyloid Treatment. The same AC cell lines were grown in duplicate. One set of cells was treated with 10 nm β-amyloid [in dimethyl sulfoxide (DMSO)] and the other with DMSO alone for 48 hr. The total DMSO concentration was <0.1% in both groups. β-Amyloid-(1–40) (Bachem) was prepared in DMSO (230 μM) and later diluted in distilled water (Picopure; Hydro, Rockville, MD) to reach the final incubation concentration of 10 nM. This low β-amyloid concentration has been shown to have specific AD-like effects on a 113-pS K^+ channel, without altering basal levels of intracellular Ca^{2+} or causing other nonspecific cell damage (12).

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Abbreviations: AD, Alzheimer disease; AC, age-matched control; Es, "escapee(s)."

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Cell Homogenization and Protein Extraction. Culture medium was removed by aspiration and replaced with ≈20 ml of cold (4°C) phosphate-buffered saline (PBS). The cells were scraped from the flasks and centrifuged at 10,000 × *g* for 10 min at 4°C. The supernatant was discarded, and the pellet washed with 1 ml PBS and then inverted for 2–3 min to remove any remaining PBS. Pellets were washed with 1 ml of homogenization buffer [50 mM NaF/1 mM EDTA/1 mM EGTA/10 mM Tris·HCl, pH 7.4, containing leupeptin (20 μg/ml) and pepstatin (50 μg/ml)], transferred to Eppendorf tubes, and centrifuged at 10,000 × *g* for 10 min at 4°C. Supernatant was discarded, tubes were inverted for 2–3 min, and then 50–75 μl of homogenization buffer was added. The pellet was finally sonicated for 10–20 sec (ultrasonic homogenizer, Cole–Parmer). The crude protein extract was stored at −80°C for later analysis.

Protein Assay, Immunoblotting, and Total Protein Analyses. Protein concentration was determined by an established dye-binding assay (29) for all homogenates. For immunoblots (30), SDS/PAGE was carried out in a 4–20% acrylamide gradient gel of thickness 1.5 mm (NOVEX, San Diego). Sample volume was adjusted to give a protein concentration of 1 μg/μl. Novex sample buffer (16 μl) was added to 16 μl of sample, and the solution was heated to 85°C for 2 min, loaded onto the gel, and subjected to 115 mV for ≈1.5 hr. The Rainbow molecular weight standard (Amersham) was also loaded. The resolved proteins were electrophoretically transferred (51.2 mA for 2 hr) to an unmodified nitrocellulose paper (8 cm by 8 cm; Pierce). Transfer buffers were as follows: anode, 40 mM 6-aminohexanoic acid/25 mM Tris, 20% methanol, pH 9.4; cathode, 25 mM Tris, 20% methanol, pH 10.4, and 300 mM Tris, 20% methanol, pH 10.4. The nitrocellulose paper was exposed overnight to SuperBlock (Pierce) and then incubated at room temperature for 1.5 hr with 10 ml of solution containing the Cp20 monoclonal antibody (23) (1:1000 dilution) and SuperBlock. After five rinses with SuperBlock, the nitrocellulose paper was incubated for 1 hr at room temperature with 40 ml of alkaline phosphatase-conjugated protein A (1:500 dilution, Cappel) in SuperBlock. After two washes with SuperBlock two washes with PBS, and two washes with APS (100 mM Tris/100 mM NaCl/5 mM $MgCl_2$, pH 9.4), the nitrocellulose paper was stained for about 7–10 min with a staining solution containing 40 ml of APS, 3 mg of nitro blue tetrazolium (Pierce), and 5 mg of 5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Pierce). The staining reaction was stopped by rinsing with distilled water. Immunoblots were then digitized on a flatbed scanner and analyzed with imaging software written in the laboratory (TNIMAGE by T. J. Nelson; available by file transfer protocol to las1.ninds.nih.gov) for quantitative analysis. To correct for any difference in overall staining between gels, the integrated values of the band(s) of interest were normalized to the average background intensity of the blots. To study overall protein composition, an aliquot of each sample was analyzed by SDS/PAGE and the gel was exposed to the staining solution [0.1% (wt/vol) Coomassie blue R-250/40% (vol/vol) methanol/10% (vol/vol) acetic acid] for 20 min and slowly destained (7.5% acetic acid/15% methanol) for about 24 hr. Molecular size was determined by comparison with Mark12 standards (NOVEX, San Diego). Quantitative analysis of the gel was conducted with methods similar to those used for analyzing the immunoblots. Measurements of the regions of interest were normalized to the total densitometric area per lane.

Monoclonal Antibodies. Cp20 was purified from 20 squid optic lobes as described (23) and injected into mouse spleen. The spleen lymphocytes were fused with P3X63-Ag8.653 mouse myeloma cells. Hybridoma cells were selected by ELISA using plates coated with optic lobe extract. The hybridoma was cloned by limiting dilution and cultivated in serum-free medium. The IgM fraction was purified by precipitation with $(NH_4)_2SO_4$ and dialyzed against PBS. The antibody was previously shown to specifically recognize Cp20 in several species, including *Hermissenda*, rabbit, rat, sea urchin, and squid, as well as HPLC-purified Cp20 (23) (see Fig. 1).

HPLC Analysis. Fibroblast extracts were injected into an AX-300 anion-exchange HPLC column (1 cm by 25 cm; SynChrom, Lafayette, IN) and eluted with a gradient of 0–0.6 M potassium acetate (pH 7.4) over 20 min, followed by 0.6 M potassium acetate for 40 min, as described previously (20). Fractions (0.5 min) were collected and applied to nitrocellulose with a dot blot apparatus. The nitrocellulose was incubated with 5% bovine serum albumin for 16 hr and reacted with antibody as described above. Quantitative analysis of the stained blot was conducted as detailed above.

Isoelectric Focusing. Sample (10 μl) containing 20 μg of protein was mixed with 5 μl of 50% urea/0.05% methyl red and applied in duplicate lanes to a 1% agarose thin-layer isoelectric focusing gel containing 5% (vol/vol) Ampholines (pH 3–10). The samples were focused in a Multiphor II apparatus (Pharmacia LKB) at 5°C, blotted onto nitrocellulose, and stained with antibody as described above.

RESULTS

Immunoblot analyses revealed significant differences in Cp20 content in fibroblasts from AD patients as compared with the controls. A distinct dark band was observed in the 20-kDa region of immunoblots of all 9 AC cell lines, whereas it was almost absent or greatly decreased in all 13 familial and non-familial AD cell lines (Fig. 1). The 20-kDa band was also decreased or absent in immunoblots from four "escapees" (Es), clinically normal individuals, who were close relatives of patients with familial AD (32). Quantitative analysis of the immunoblots (Fig. 1 *D* and *E*) confirmed that Cp20 levels were significantly higher in the controls than in the AD and Es cell lines ($P < 0.001$; ANOVA, Bonferroni post-test). No significant differences were found between AD and Es cell lines. To rule out a generalized effect on all proteins of ≈20 kDa, a total protein analysis was conducted on Coomassie blue-stained SDS/polyacrylamide gels. Visual inspection (Fig. 2*A*) of the 20-kDa region, confirmed by quantitative analysis (Fig. 2 *B* and *C*), showed no between-group differences ($P > 0.05$, not significant; ANOVA, Bonferroni post-test). Analysis of the 66- to 33-kDa region also revealed no between-group differences ($P > 0.05$, not significant; ANOVA, Bonferroni post-test). Two additional protein bands in the high molecular mass region (≈200 kDa) also showed no significant differences between experimental groups ($P > 0.05$, not significant; ANOVA, Bonferroni post-test). To test the specificity of the anti-Cp20 monoclonal antibody and to confirm that the protein detected in human fibroblasts was indeed Cp20, cell extracts were subjected to thin-layer isoelectric focusing. After blotting onto nitrocellulose and staining with antibody, a single band was visible which had a pI of 5.5, identical to that of squid Cp20. Antibody staining of anion-exchange HPLC fractions also indicated a single antibody-reacting protein, which had a retention time identical with that of dephosphorylated, purified squid and *Hermissenda* Cp20 (20). The identity of the dephosphorylated form of Cp20 in squid has been confirmed previously by phosphorylation experiments with [γ-^{32}P]ATP and protein kinase C (T.J.N. and D.L.A., unpublished observations) (Fig. 1 *A* and *B*).

Since previous observations indicated that treatment with low concentrations of β-amyloid induced an AD-like K^+ dysfunction in control cells (12), we treated 9 AC cell lines with 10 nM β-amyloid for 48 hr. Following the same immunoblotting procedure and analysis, we found that Cp20 was significantly decreased in β-amyloid-treated cells as compared with their nontreated counterparts ($P < 0.003$; Wilcoxon test) (Fig. 3 *A* and *B*). Total protein analysis revealed that the β-amyloid

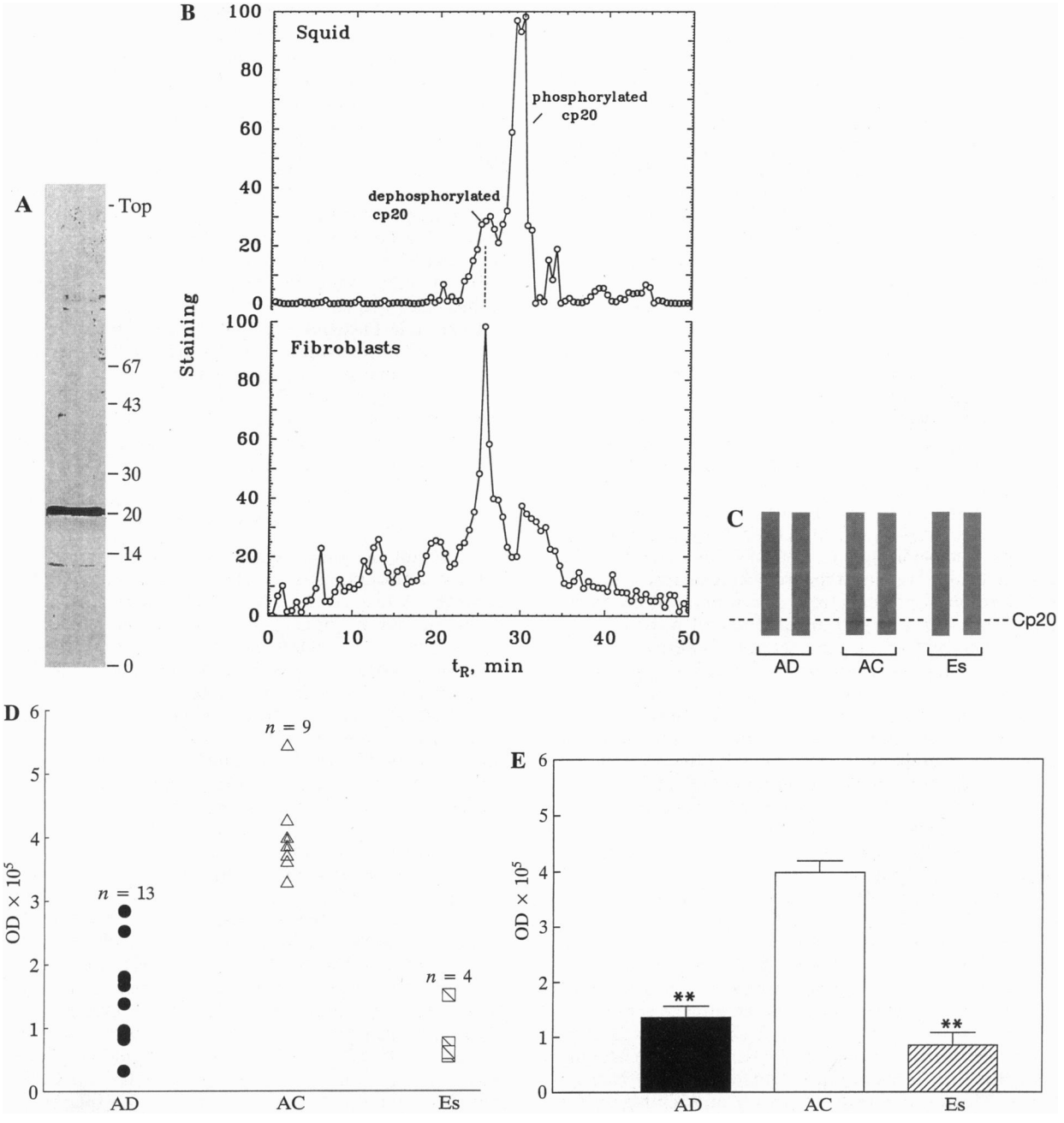

FIG. 1. Western blot analyses of Cp20. (*A*) Western blot of monoclonal anti-Cp20 reaction with Cp20 purified from squid optic lobe (stain, horseradish peroxidase/diaminobenzidine). Molecular size markers (kDa) are at right. (*B*) Retention time (t_R) of the dephosphorylated Cp20 extracted from human fibroblasts was identical to the t_R of Cp20 purified from squid. The identity of the dephosphorylated form of Cp20 in squid has been previously confirmed (T.J.N. and D.L.A., unpublished observations; see also text). (*C*) Representative Western blots showing the stained protein band corresponding to Cp20 (index line). Visual inspection indicates that Cp20 is decreased in AD and Es fibroblast cell lines. (*D*) Graphic representation of quantitative analysis of each cell line shows clearly significant differences, with no overlap, for controls (△) compared with AD (●) and Es (▨) (**, $P < 0.001$; ANOVA, Bonferroni post-test). No significant differences were found between AD and Es fibroblasts. (*E*) Bar graph representing the group data, further illustrating the significant Cp20 differences between the control fibroblasts and the AD and Es cell lines.

treatment was not a generalized effect on all proteins in the 20-kDa region ($P > 0.1$; Wilcoxon test (Fig. 3 *C* and *D*). In addition, no between-group differences were observed in the 60- to 33-kDa region or the 200-kDa region.

DISCUSSION

These results clearly demonstrate that Cp20, a memory-associated protein that induces a number of molecular and cellular changes that have been observed during memory acquisition and storage (20–23), is markedly decreased in fibroblasts from AD patients. This is a specific extension of our previous findings (11–13) that cellular steps (K^+ channel regulation, Ca^{2+} release) in memory storage are altered in AD. Since Cp20 is an extremely potent regulator of K^+ channels (20), its absence or reduction in AD could have some relationship to the previously observed differences of K^+ channels for AD fibroblasts (11, 12) and olfactory neuroblasts (14). The previously demonstrated regulation by Cp20 of retrograde axonal transport, as well as its sequential homology with the ARF protein Sar1p (which is involved in vesicle trafficking; ref. 23), suggests that its absence could also influence the

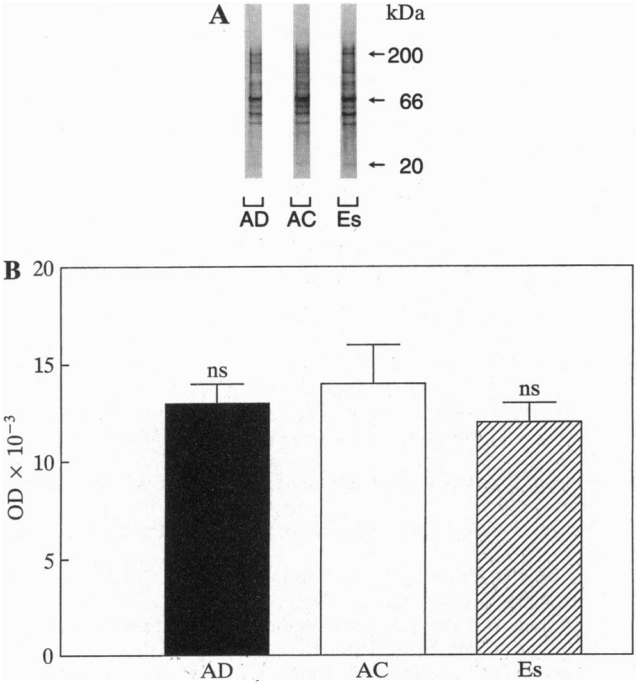

FIG. 2. Coomassie-stained SDS/polyacrylamide gels of AD, Es, and AC fibroblasts. (*A*) Protein profiles in all three groups studied. Three regions were analyzed in detail in order to detect generalized protein changes in AD and Es fibroblasts, with particular attention to the protein bands with molecular masses similar to Cp20 (≈20 kDa). (*B*) Quantitative analysis of the Cp20 region confirmed visual impressions that there were no between-group differences around the 20-kDa region. Similar analysis also showed no between-group differences for proteins of 36–66 kDa (see text). ns, Not significant.

predisposition to and/or development of the proteinaceous plaques and neurofibrillary tangles that characterize AD pathology in the human brain. These pathological processes, like Cp20, directly or indirectly involve vesicle trafficking (33–37) and, possibly, alterations of microtubule-associated proteins (4, 5). Phosphorylation of tau (a potentially pathological event) by mitogen-activated protein kinase can be promoted by amyloid precursor protein (the protein from which β-amyloid originates) and prevented by inhibition of Ras proteins (5, 38, 39). The Ras involvement in this process is intriguing, since Ras proteins and Cp20 share functional properties (40) and also some degree of amino acid sequence homology (23). Moreover, one of the suggested normal functions for tau is to participate in microtubule elongation and shaping axonal morphology (41), which may be related to dendritic changes induced by Cp20 during memory acquisition (21). It is also interesting that G_o, a heterotrimeric GTP-binding protein involved in membrane trafficking and axonal transport (42), associates with the cytoplasmic domain of the amyloid precursor protein (43). Cp20 also induces mRNA transcription in neuronal cells (T.J.N., J.L.O., H. Kim, and D.L.A., unpublished observations). Other researchers have found that mRNA translation is reduced by 40% in AD cerebral cortical tissues (31). Thus, Cp20 alterations could affect cellular growth or repair mechanisms either by inhibiting synthesis of new proteins or by its other effects on axonal transport and/or intracellular vesicle trafficking, contributing to AD pathology.

Since Cp20 was also decreased in clinically normal close relatives of individuals with familial AD (Es) the observed loss of Cp20 could diagnostically mark AD as well as genetic predisposition to AD even in the absence of clear clinical symptoms of the disease. Preliminary results have revealed a similar Cp20 decrease in olfactory neuroblasts from AD patients (unpublished observations). Olfactory neuroblasts are

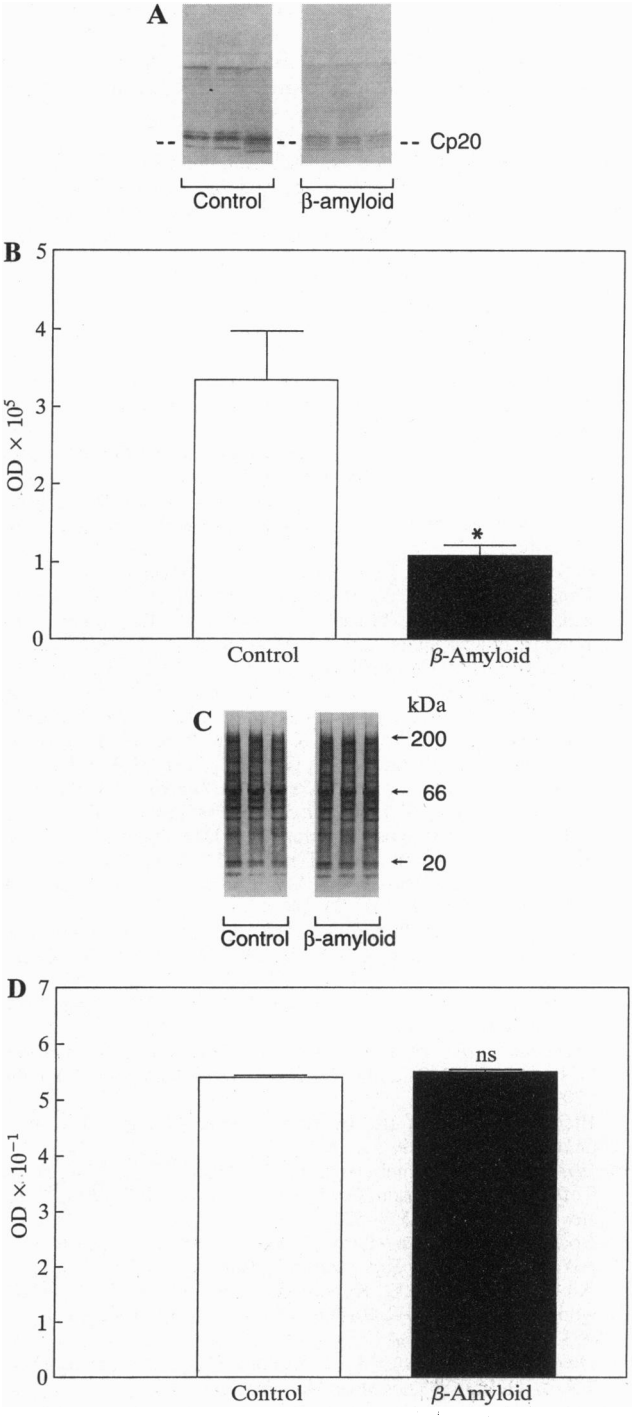

FIG. 3. β-Amyloid decreases Cp20 in control fibroblasts. (*A*) Western blots of AC fibroblasts treated with β-amyloid for 48 hr (*Right*) and of the same untreated cell lines (*Left*). A decrease in Cp20 (index line) can be clearly observed in the β-amyloid-treated cells as compared with the untreated counterparts. (*B*) Bar graphs represent the quantitative analysis showing significant difference (*, $P < 0.003$; Wilcoxon test) between β-amyloid-treated and untreated cells. (*C*) Total protein profiles (Coomassie blue staining) reveal no differences between treated and untreated cell lines. (*D*) Quantitative analysis of protein bands around 20 kDa (molecular mass of Cp20) confirms that β-amyloid did not cause a general decrease of ≈20-kDa proteins. Analysis of other bands (see text) also showed no β-amyloid effects. ns, Not significant.

closely related to central nervous system neurons. They express neuron-specific enolase and make direct synaptic contacts with the central nervous system (24). These results from

a different cell type, and from a different patient population, strengthen the hypothesis that this protein might have a significant role in AD pathophysiology, as well as provide a biochemical marker and a potential diagnostic tool for the disease.

Note Added in Proof. Recent analyses of human brain tissue have revealed similar Cp20 differences between AD patients and normal controls (unpublished observations), further indicating that the Cp20 changes observed in peripheral tissues are indeed systemic reflections of a disease process that primarily (and clinically) affects the brain.

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