Signal Transduction through Tyrosine-phosphorylated C-terminal Fragments of Amyloid Precursor Protein via an Enhanced Interaction with Shc/Grb2 Adaptor Proteins in Reactive Astrocytes of Alzheimer's Disease Brain*

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The proteolytic processing of amyloid precursor protein (APP) through the formation of membrane-bound C-terminal fragments (CTFs) and of soluble β-amyloid peptides likely influences the development of Alzheimer's disease (AD). We show that in human brain a subset of CTFs are tyrosine-phosphorylated and form stable complexes with the adaptor protein ShcA. Grb2 is also part of these complexes, which are present in higher amounts in AD than in control brains. ShcA immunoreactivity is also greatly enhanced in patients with AD and occurs at reactive astrocytes surrounding cerebral vessels and amyloid plaques. A higher amount of phospho-ERK1,2, likely as result of the ShcA activation, is present in AD brains. *In vitro* experiments show that the ShcA-CTFs interaction is strictly confined to glial cells when treated with thrombin, which is a well known ShcA and ERK1,2 activator and a regulator of APP cleavage. In untreated cells ShcA does not interact with either APP or CTFs, although they are normally generated. Altogether these data suggest that CTFs are implicated in cell signaling via Shc transduction machinery, likely influencing MAPK activity and glial reaction in AD patients.

The cytoplasmic region of the amyloid precursor protein contains an NP*XY* motif, which is present in the cytodomains of several tyrosine kinase receptors and in non-receptor tyrosine kinase (1, 2). In tyrosine kinase receptors the tyrosine residue of this motif is phosphorylated upon tyrosine kinase activation, and the NP*X*pY motif (where pY is phosphotyrosine) functions as a docking site for the phosphotyrosine-binding domain present in several adaptor proteins interacting with tyrosine kinase receptors and non-receptor tyrosine kinase, such as the pro-

teins belonging to the Shc family (3, 4). In APP[1] and in APP-related proteins APLP1 and APLP2 the NPTY motif interacts with several adaptor proteins, such as Fe65 (5), X11 (6), mDab1 (7), and JIP-1 (8), but this interaction has been demonstrated to be independent of tyrosine phosphorylation (9, 10). Recent data (11) show that in human brain CTFs can be tyrosine-phosphorylated and that *in vitro* the APP cytodomain is tyrosine-phosphorylated by the non-receptor tyrosine kinase Abl, which phosphorylates a tyrosine residue upstream (Tyr-682), the NPTY motif (11, 12). This phosphorylation generates a motif pY*XX*P that is recognized by the SH2 domain of Abl itself and that might be a docking site for SH2-containing adaptors such as Shc and Grb2 proteins (11). Here we describe that in human brain tyrosine-phosphorylated CTFs represent docking sites for Shc and Grb2 proteins and generate stable complexes with these adaptors that are up-regulated in AD cases. ShcA formation is strictly confined to activated astroglial cells only, and its levels are highly enhanced in AD brains in comparison to control subjects. In AD brains it is also up-regulated in the expression of Erk1,2 kinase, likely as a consequence of ShcA activation. *In vitro* experiments show that thrombin triggers the ShcA-CTFs interaction and Erk phosphorylation in cultured astrocytes, whereas in neuronal cells these complexes are undetectable. Therefore, our data suggest an involvement of APP in cell signaling through its CTFs and correlate APP metabolism and ShcA to the reactive gliosis and inflammatory phenomena that occur in AD.

EXPERIMENTAL PROCEDURES

Brain Samples Preparation and Western Blotting—Cerebral cortex was obtained at autopsy from clinically and neuropathologically verified (according to the Consortium to Establish a Registry for Alzheimer's Disease (CERAD)) (13) cases of sporadic AD ($n = 6$, age 72 ± 7, postmortem interval, 5.2 ± 2.4 h), control subjects ($n = 6$, age 73 ± 9, postmortem interval, 4.7 ± 2 h) in which AD had been excluded by clinical and autopsy examination, including immunohistochemical analysis. Monoclonal antibodies 4G8 and 6E10, specific for residues 17–21 and 6–10 of Aβ, were purchased from Signet Pathology Systems (Dedham, MA). The polyclonal antibody F25608 (a gift from Dr. P. Gambetti, Case Western Reserve University, Cleveland, OH) and the monoclonal antibody 643/695 Jonas (Roche Diagnostics) are specific for the C terminus of APP and CTFs. The monoclonal antibody for the N terminus of APP mAb348 was purchased from Chemicon (Temecula,

[1] The abbreviations used are: APP, amyloid precursor protein; CTFs, C-terminal fragments; AD, Alzheimer's disease; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GFAP, glial fibrillary acidic protein; Aβ, beta amyloid; AP, alkaline phosphatase.
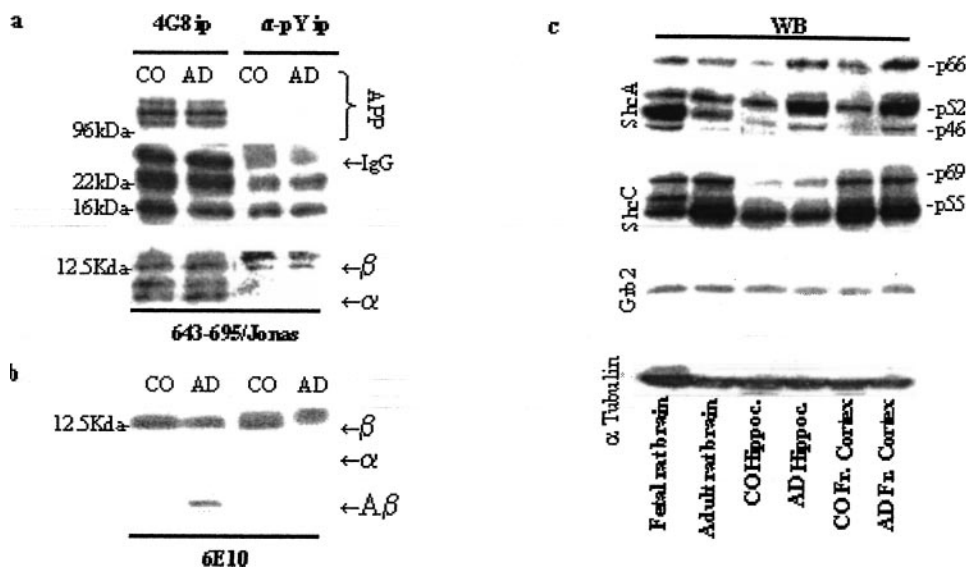
FIG. 1. **Tyrosine phosphorylation of APP and CTFs.** *a*, in human brain samples both APP and a subset of CTF polypeptides migrating at 22, 16, and 12.5 kDa are specifically immunoprecipitated by an antibody for the Aβ region of APP (4G8). A subset of CTFs is also immunoprecipitated by an anti-phosphotyrosine antibody (pY-20). Immunostaining with an anti-APP C-terminal antibody, besides the above-mentioned bands, also shows other CTFs bands migrating at around 10 and 8 kDa (α-secretase products) immunoprecipitated by the 4G8 antibody only. Tyrosine-phosphorylated APP is almost undetectable, and a signal corresponding to APP was obtained only after prolonged exposure (not shown). No significant differences are present between AD and non-demented control tissues (*CO*) in APP or CTF content. *b*, the identity of 12.5-kDa migrating bands as β-secretase-derived CTFs is ascertained by 6E10 immunostaining of immunoprecipitated peptides as in *a*. 6E10 antibody recognizes only β-secretase-derived CTFs in both AD and control cases, whereas α-derived CTFs, which were previously precipitated by 4G8 antibody, remain undetectable. 6E10 specificity for β-secretase APP fragments was confirmed also by staining of two or three Aβ bands normally present in AD brain samples. *c*, Western blottings (*WB*) of brain samples from frontal cortex (*COFr*) and hippocampus (*Hippoc*) from Alzheimer, control, and cortical rat brain homogenates are probed with anti-ShcA, anti-ShcC, anti-Grb2, and anti-α-tubulin as control for the amount of protein loaded. A significantly higher amount of ShcA was present in AD samples *versus* controls. As control we analyzed rat brain extracts, in which high ShcA levels in fetal and high ShcC levels in the adult animals are present.

CA). Monoclonal antibodies to phosphotyrosine (pY-20), anti-ShcC, anti-Grb2, and anti-α-tubulin were from Transduction Laboratories (Lexington, KY). The phospho-ERK-specific antibody was from New England Biolabs (Beverly, MA). Anti-ShcA antibodies were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY), and from Transduction Laboratories. All the chemicals were from Sigma unless otherwise specified. Brain cortical samples were homogenized in a Tris-buffered saline buffer supplemented with 1% Triton X-100 and protease inhibitors (Complete™, Roche Diagnostics) and spun down at $68,000 \times g$ 30 min. The supernatant was adjusted to pH 8, 0.5% sodium deoxycholate, and after protein counting (Bradford method, Bio-Rad) an equal amount of protein was immunoprecipitated with the antibodies indicated coupled to protein A-Sepharose. Protein A beads were then loaded and electrophoresed by Tris-Tricine SDS-PAGE and electroblotted on polyvinylidene difluoride membrane, and proteins were probed with specific antibodies in Tris-buffered saline plus 0.02% Tween 20 with 5% low fat milk and detected by ECL (Amersham Biosciences AB).

Immunohistochemistry—Immunohistochemistry was carried out in formalin-fixed and paraffin-embedded brain specimens from frontal cortex and hippocampus. Briefly, deparaffined sections were pretreated in citrate buffer at pH 5 in a microwave and probed with the indicated antisera. Development reaction was carried out with AP-labeled secondary antibody visualized by fast red chromogen (Dako, Glostrup, Denmark).

Cell Culture—Primary cultures of rat cortical neurons were obtained from 18- to 20-day-old Sprague-Dawley embryos as published previously (14). Briefly, cortices were excised, trypsinized, and resuspended in Neurobasal medium supplemented with 2% B27 (Invitrogen), 0.5 mM glutamine, and antibiotics. Cells were seeded in poly-L-lysine-coated wells, and after 8–9 days the cell population was determined to be at least 95% neuronal by MAP2 immunostaining. Primary cultures of rat cortical type 1 astrocytes were prepared from 2-day-old Sprague-Dawley pups. Cortices were dissected out, trypsinized, and cells cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics. Astrocyte-enriched cultures were obtained from mixed glial cultures by the shaking off method and were composed of greater than 95% glial fibrillary acidic protein-positive cells. Untreated and thrombin-treated cells were lysed in a Hepes buffer containing 1% Nonidet P-40 and 0.5% sodium deoxycholate, pH 7.4. After a 10-min centrifugation at $1500 \times g$, cell lysates were either cold methanol-precipitated, and the resulting pellets analyzed by Western

blotting after protein counting (Bio-Rad protein assay), or immunoprecipitated and analyzed as described above.

RESULTS

Characterization of the Expression of Tyrosine-phosphorylated CTFs and Shc in Human Brain—We have described recently (11) the presence in human brain of tyrosine-phosphorylated CTFs in both AD and non-AD control subjects. Here we analyze the pattern of tyrosine phosphorylation identified in APP and CTFs of AD and age-matched normal brains. Brain extracts were immunoprecipitated with antibodies recognizing the Aβ region of APP or phosphotyrosine residues. Immunoprecipitated proteins were then blotted with the anti-APP C-terminal antibody 643–695/Jonas. APP holoproteins migrating at 96–110 kDa were immunoprecipitated by 4G8 antibody, whereas no detectable APP was observed by immunoprecipitating brain extracts with anti-Tyr(P) antibody (Fig. 1*a*). Only in prolonged exposures of overloaded samples is a weak signal detectable corresponding to APP bands when immunoprecipitated with antiphosphotyrosine antibody (data not shown), suggesting that in human brain the levels of tyrosine-phosphorylated APP are low. On the contrary, several low molecular mass bands, migrating at 22, 16, and at 12.5 kDa, were easily immunoprecipitated by both antibodies (Fig. 1*a*). In particular, the 12.5-kDa migrating band, previously identified as the β-secretase product C99, was immunoprecipitated by both antibodies, whereas other CTFs bands migrating below 12.5 kDa and the α-secretase products at 8 kDa (15–17) were immunoprecipitated by 4G8 antibody but not by anti-Tyr(P) (Fig. 1*a*). The identity of 12.5-kDa migrating bands as β-secretase-derived C99 is confirmed by 6E10 immunostaining of similarly immunoprecipitated brain extracts (Fig. 1*b*), where the antibody detects specifically only β-secretase-cleaved CTFs, in force of its selectivity for residues 6–10 of Aβ (18). CTFs bands migrating at 16 and 22 kDa and recognized by the anti-Tyr(P)

Signal Transduction through Tyrosine-phosphorylated CTFs
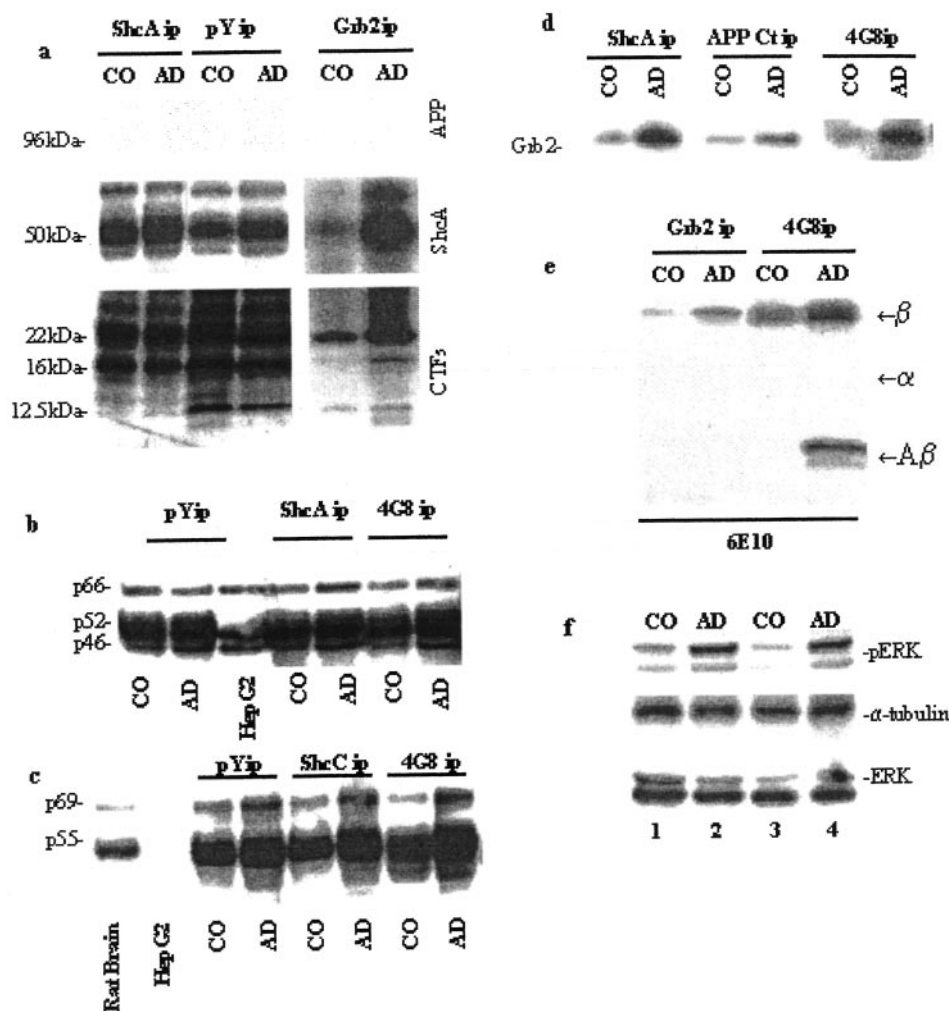
FIG. 2. **Co-immunoprecipitation of CTFs with ShcA in human brain.** *a*, brain extracts from AD and control cases (*CO*) were immunoprecipitated with anti-ShcA, anti-phosphotyrosine, and anti-Grb2 antibodies. Immunostaining of electrophoresed proteins with antisera to APP, ShcA, and CTFs shows that ShcA co-immunoprecipitates only with tyrosine-phosphorylated CTFs that migrate at 22, 16, and 12.5 kDa and does not precipitate either APP or α-secretase-generated CTFs. Anti-Grb2 antibody co-immunoprecipitates ShcA and CTFs (mainly the 22- and 16-kDa fragments and more weakly the C99 at 12.5 kDa) in brain extracts from control (*CO*) and AD cases. The amount of ShcA and CTFs linked to Grb2 protein is higher in AD in comparison to control subjects. *b* and *c*, conversely, brain extracts from AD and control cases immunoprecipitated with anti-ShcA, anti-phosphotyrosine, and 4G8 antibodies show the co-precipitation of ShcA proteins (*b*) and ShcC (*c*). HepG2 cell line and adult rat brain extracts are loaded as positive control for ShcA and ShcC, respectively. *d*, brain extracts immunoprecipitated with anti-ShcA, anti-APP C-terminal antibody, and 4G8 were examined for the presence of Grb2 protein by immunoblotting. Grb2 is present as a 24-kDa migrating band in both AD and control cases (*CO*) co-immunoprecipitated by both ShcA and anti-C-terminal APP antibodies. The identity of 12.5-kDa migrating bands precipitated by Grb2 as β-secretase-derived CTFs is confirmed by 6E10 immunostaining of immunoprecipitated peptides as in *b*. 6E10 antibody recognizes only β-secretase-derived CTFs in both AD and control cases and a subset of Aβ peptides only in AD extracts precipitated by 4G8. CTFs and Aβ peptides derived from α-secretase cleavage remain undetectable. *f*, Western blotting of brain extracts probed for phospho-ERK1,2, unphosphorylated ERK1,2, and tubulin as protein loading control. In AD samples an enhanced level of phospho-p42 and p44 is present in comparison to control (*CO*) both in frontal cortex (*lanes 1* and *2*) and in hippocampus (*lanes 3* and *4*).

antibody correspond to previously identified APP fragments (19–22), which have been suggested to be generated by a non-β-secretase cleavage. These data suggest that CTFs derived from different proteolytic pathways undergo different metabolic destiny and that those derived from α-secretase cleavage are not tyrosine-phosphorylated.

The tyrosine phosphorylation of APP, although very weak, and of CTFs suggests that they can be involved in tyrosine kinase-dependent signaling, by functioning as docking molecules for phosphotyrosine-interacting proteins. Shc proteins possess SH2 and phosphotyrosine-binding domains that may recognize the pYENP or NPTpY motifs in the APP C terminus, respectively (3, 4). Therefore, they likely represent candidates as interacting proteins with the tyrosine-phosphorylated C terminus of APP. To examine this point, we first analyzed brain extracts by Western blotting, looking at the expression of ShcA

and ShcC in AD and non-AD control subjects. The results are summarized in Fig. 1*c*, where the levels of ShcA proteins were analyzed in hippocampal and frontal cortex extracts. p46, p52, and p66 ShcA isoforms were detected in normal and Alzheimer's brains, but their levels were significantly increased in the latter. Immunoblotting with anti-ShcC and anti-Grb2 antibodies showed also that these proteins were expressed in human but without significant difference between AD and control subjects (Fig. 1*c*). The presence of ShcA in human senile brain contrasts with previous observations (3, 23) that ShcA accumulates in rat fetal brains, whereas ShcC is more abundant in rat adult brains as shown in Fig. 1*c* as control. This might due in part to the fact that in our samples, cells from small meningeal vessels, which may contain ShcA, likely were also present, but this also suggests that Shc isoforms may undergo an aging-specific change of expression (3). Moreover, our data are in

agreement with previous observations indicating that proliferating glia and injured cells may also express abnormally high levels of ShcA (3, 23, 24).

Tyrosine-phosphorylated CTFs Interact with Shc-Grb2 Adaptors, Enhanced CTFs-ShcA-Grb2 Complexes and ERK1,2 Activation in AD—To verify if APP or CTFs may interact with Shc proteins, we immunoprecipitated protein extracts from Alzheimer's and normal brains with anti-ShcA, anti-phosphotyrosine, and anti-Grb2 antibodies. Immunoprecipitated proteins were analyzed by Western blotting, and immunodetection was carried out with the anti-APP C-terminal antibody 643–695/Jonas. This experiment demonstrated that tyrosine-phosphorylated CTFs migrating at 22, 16, and 12.5 kDa were co-immunoprecipitated with ShcA protein, whereas neither full-length APP nor α-secretase-derived CTFs (see also Fig. 1a) were complexed with ShcA (Fig. 2a). Conversely, immunoprecipitation of brain extracts with anti-phosphotyrosine and Grb2 antibodies pull down ShcA as well as the 22-, 16-, and 12.5-kDa bands of CTFs. Interestingly, a higher amount of ShcA and CTFs was co-immunoprecipitated in Alzheimer's than in control samples by anti-Grb2 antibody, in agreement with the observation that ShcA levels are increased in AD brains and suggesting that in AD the activation of the CTFs-ShcA-Grb2 transduction machinery is enhanced. Only under prolonged exposure is a weak signal detectable corresponding to APP bands when immunoprecipitated with anti-phosphotyrosine and anti-Grb2 antibodies, suggesting that tyrosine-phosphorylated APP may be involved in such complexes, although weakly (data not shown). Samples immunoprecipitated with anti-ShcC antibody showed a strong CTFs signal corresponding to the 22-kDa fragment and a weak signal at 16 kDa only (data not shown) in both AD and control cases. The immunoprecipitation of brain extracts with anti-phosphotyrosine and anti-CTFs antibodies followed by immunoblotting with anti-ShcA or anti-ShcC antisera showed that both ShcA (Fig. 2b) and ShcC (Fig. 2c) were co-immunoprecipitated by these antibodies. As negative control we used antibodies for CD26 and for tau proteins which were unable to co-precipitate Shc adaptors (data not shown). Considering that both ShcA and ShcC co-immunoprecipitated with CTFs, we examined the presence of CTFs-Shc-Grb2 complexes by searching Grb2 in the proteins co-immunoprecipitated by anti-ShcA and by anti-APP antibodies. The 24-kDa migrating band identified by anti-Grb2 immunostaining was co-immunoprecipitated by anti-ShcA, anti-C-terminal APP 643–695/Jonas, and by the 4G8 antibodies (Fig. 2d). To ascertain the identity of 12.5-kDa migrating bands as β-secretase-derived CTFs, we probed brain samples immunoprecipitated with 4G8 and Grb2 antibodies with the 6E10 antibody (Fig. 2e). 6E10 recognizes β-secretase-derived CTFs precipitated by both antibodies both in control and in AD subjects, although in AD the amount of CTF pull-down by Grb2 is higher than in control (Fig. 2e). Altogether these data indicate that in human brain a complex involving CTFs, ShcA, and Grb2 is present and that this complex is more present in AD than in control brain extracts. To investigate whether the activation of this pathway would lead to the activation of MAPK (3, 25, 26) in AD, we looked at the phosphorylation status of ERK1,2 proteins in human brain. A higher amount of phospho-ERK1 and -2 was detected in AD cases in comparison to control subjects as demonstrated by Western blotting with anti-phospho-ERK1,2 antibody (Fig. 2f).

ShcA Localization at Reactive Astrocytes Around Plaques, Enhanced Expression in AD—To identify the cellular localization of ShcA signaling, we analyzed by immunohistochemistry hippocampal sections of formalin-fixed and paraffin-embedded brain samples from control and AD patients. The immuno-staining demonstrated that ShcA signal is significantly more intense in AD brain than in non-demented control samples (Fig. 3, a and b) and occurred mainly at perivascular astrocytes, white matter astrocytes, and also at reactive astrocytes surrounding amyloid plaques (Fig. 3, c and d). In normal brains ShcA staining appeared very weak and when barely present was localized in non-activated astrocytes as well (Fig. 3a). Under our conditions, neurons remained unstained by anti-ShcA antibody both in AD and in non-AD subjects. As hypothesized previously (3), in the adult normal human brain ShcA is mainly expressed in astrocytes and may be overexpressed under degenerative conditions where astrogliosis is present or in glial brain tumors (3, 23). To ascertain the identity of ShcA-positive cells as astrocytes, we probed adjacent slides from AD subjects with anti-ShcA and anti-GFAP antibodies. Although astrocytes are cells with a typically fine and flat morphology (4–6 μm thick) and eventually difficult to detect in adjacent slides (5 μm thick), in our conditions, activated astrocytes were heavily stained by anti-ShcA antibody and were also labeled in the adjacent section by anti-GFAP antibody (Fig. 3, e–h). Our data suggest that the interaction between CTFs and ShcA, which may occur in glial cells, is likely related to astrogliosis and to the reactive inflammatory phenomena observed in AD (26), as also suggested by ERK activation (Fig. 2f). The phosphorylation and interaction of CTFs with Shc, besides AD, may occur in age-matched control brain as well, although to a lesser extent. This could suggest that either normal brain aging is accompanied by a certain degree of astrogliosis or that in normal conditions a basal level of proliferative activity through ShcA signaling exists.

Thrombin Triggers the CTF-ShcA Interaction in Cultured Rat Astrocytes but Not in Neurons—To investigate the conditions whereby phosphorylated CTFs interact with Shc adaptor proteins, we have carried out a series of experiments *in vitro* in primary cultures of rat astrocytes and cortical neurons. These cells possess high levels of APP, ShcA, and CTFs (Fig. 4a) with significant differences in amount and electrophoretic pattern. The APP profile in astrocytes showed an increased amount of Kunitz protease inhibitor-containing isoforms and a lower amount of "neuronal" APP695 isoform in comparison to neurons, as described previously (27) (Fig. 4a, *upper panel*). ShcA expression was enhanced in astrocyte cultures in comparison to neuronal cultures treated with AraC, in order to minimize the glial component; this overexpression is mainly concerned with p66 and p46 isoforms. The electrophoretic profile of CTFs showed a higher amount of 16-, 22-, and 32-kDa migrating forms in astrocytes than in neurons and a relatively more abundant presence of 8–12.5-kDa migrating CTFs in neurons than in astrocytes (Fig. 4a).

In both cell types the immunoprecipitation with anti-ShcA antibody in normal conditions was completely ineffective in co-immunoprecipitating APP or CTFs (Fig. 4b). It is well known that thrombin, a major coagulant and inflammatory mediator, regulates cleavage and secretion of APP as well as Shc phosphorylation through specific protease-activated receptors, which are present in astrocytes (28–30). Furthermore, thrombin is a well known ERK1,2 activator in astrocytes, and it has been related to the inflammatory response in AD (31–33). Brief (15 min) thrombin treatments do not significantly alter the total amount of APP holoproteins, Shc proteins, or CTFs (Fig. 4a). Only in thrombin-treated astrocytes CTFs migrating at 12.5, 22, and 32 kDa were all co-immunoprecipitated by anti-ShcA antibody and detected by 4G8 staining (Fig. 4b). CTFs co-immunoprecipitated by ShcA antibody were also recognized by anti-phosphotyrosine staining (Fig. 4b) as shown previously for CTFs detected in human brain. On the contrary,

CONTROL ShcA staining / AD ShcA Staining / AD 4G8 staining / AD Shc A staining / AD GFAP Staining / AD ShcA Staining / AD GFAP Staining / AD ShcA Staining

FIG. 3. **Immunohistochemical localization of ShcA in adult human brain samples.** Immunohistochemistry was carried out in formalin-fixed and paraffin-embedded brain specimens from frontal cortex and hippocampus. ShcA is barely detectable in control samples (*a*, ×10 magnification), and only few glial cells possess a weak positive staining. On the contrary in AD samples ShcA antibody heavily stains activated astrocytes (*b*, ×10 magnification). In AD the ShcA staining is highly enhanced and localized mainly in astrocytes around vessels and plaques (*white arrows* in *b* and *inset* in *b*). ShcA immunolocalization in reactive astrocytes cells around amyloid plaques is shown in *c* where 4G8 staining identifies amyloid plaques (*black arrow*) and in *d* where immunoprobing of an adjacent slice with anti-ShcA antibody detects reactive glial cells (*black arrow*) around the same plaque identified in *c* (×20 magnification). *e* and *f*, ShcA immunostaining colocalize with anti-GFAP staining in astrocytes present in adjacent slides (the same shapes of *arrows* identify the same cell in adjacent slides, ×20 magnification) from an AD subject. Higher magnification (×40) of ShcA and GFAP staining in adjacent slides from an AD case are shown. Three astrocytes (*a–c*) are identified and are stained by both antibodies.
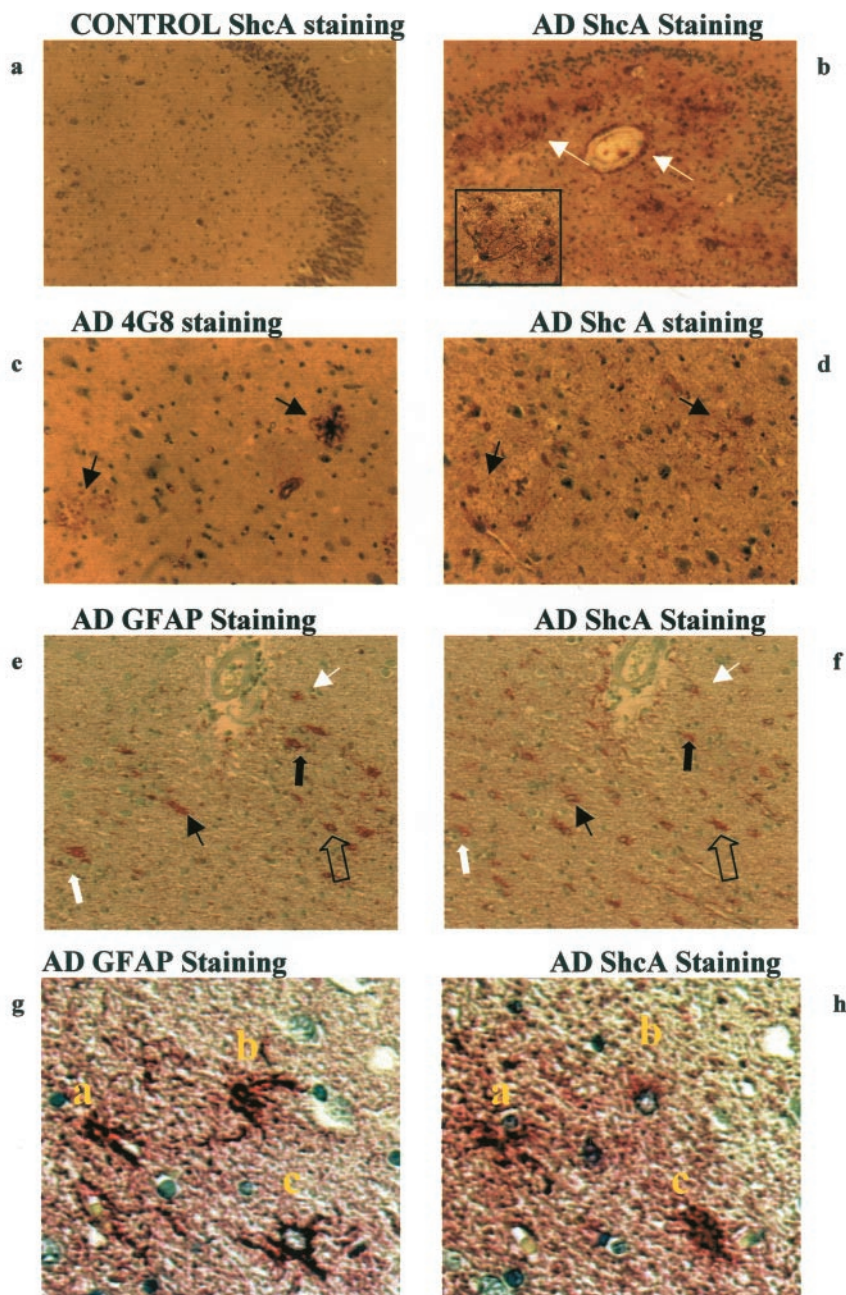
anti-ShcC antibody was ineffective in co-immunoprecipitating CTFs (data not shown). As shown above in human brain and also in cultured rat astrocytes, APP holoproteins were not co-immunoprecipitated with ShcA and CTFs (Fig. 4*b*). Similar results were obtained also when other glial-derived cells (C6) were used (data not shown). In primary cultures of post-mitotic rat cortical neurons, the thrombin treatment was unable to induce the formation of such complexes, and neither APP nor CTFs were co-immunoprecipitated by anti-ShcA antibody in control or in thrombin-treated neurons (Fig. 4*b*), although both APP and CTFs are abundantly represented in cell extracts (Fig. 4*a*). Also in these cells, α-secretase-derived CTFs were not tyrosine-phosphorylated and were not bound by either ShcA or ShcC (Fig. 4, *a* and *b*). Thrombin treatment, as reported previously (31), activated ERK1,2 proteins with a peak of activation at 15–20 min (data not shown). These data therefore confirm that the interaction between CTFs and ShcA is an event strictly associated with astroglial cells when mitogenically

stimulated *in vitro* and correlate the presence of such complexes to the gliosis present in AD brain.

DISCUSSION

Our data show that a subset of CTFs interact with Shc and Grb2 proteins, suggesting that they may transduce an intracellular signal through SH2 or phosphotyrosine-binding domain interacting adaptors. The effect of such interaction is likely linked to the activation of the MAPK pathway, as shown here in AD brain and in thrombin-treated astrocytes as well. The enhanced level of ShcA protein in Alzheimer's patients, the peculiar staining of activated astrocytes around amyloid plaques, and the increased CTFs-ShcA interaction in AD subjects altogether suggest that the activation of this pathway may play a role in Alzheimer's disease. The fact that the levels of tyrosine-phosphorylated APPs are weak and that only β-amyloid-bearing CTFs are tyrosine-phosphorylated and linked to Shc suggests that *in vivo* tyrosine phosphorylation

FIG. 4. Tyrosine-phosphorylated CTFs interact with ShcA proteins in thrombin-treated primary cultures of rat astrocytes. *a*, Western blotting (60 µg of protein in each lane) of neuronal and astroglial cell lysate shows basically no differences in the expression of APP (*upper panel*), ShcA (*middle panel*), and CTFs (*lower panel*) between control (*CO*) and thrombin-treated cells for 15 min at the concentration indicated. *b*, in thrombin-treated astrocytes only is present the co-precipitation of ShcA together with a subset of CTFs, which are stained by both 4G8 (*left*) and anti-phosphotyrosine (*right*) antibodies. Thrombin treatment in cultured neurons does not generate ShcA-CTFs or ShcA-APP co-precipitation.
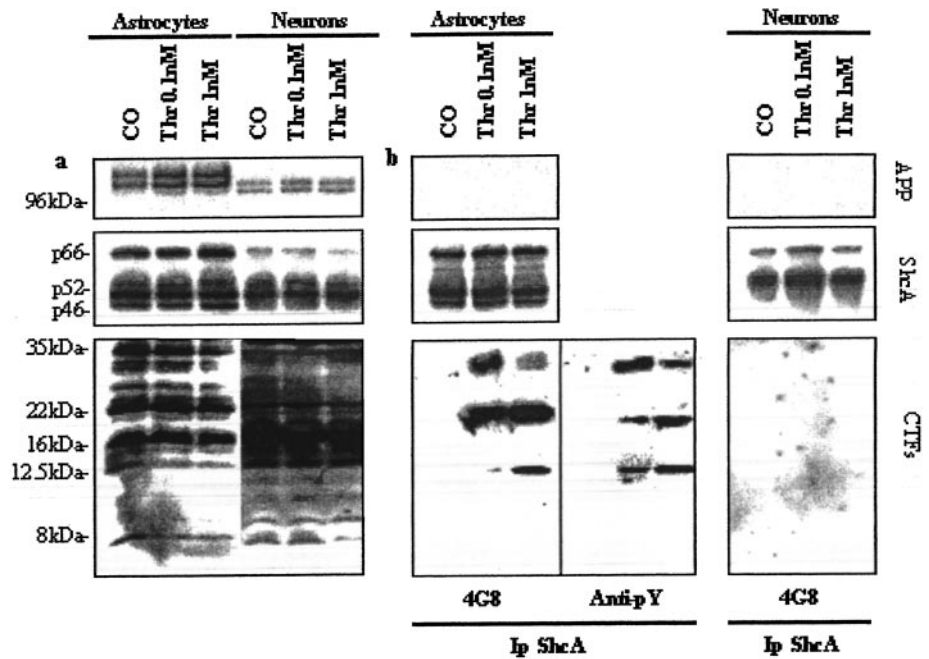
and cleavage of APPs are related events and that likely tyrosine phosphorylation renders APP a suitable substrate for a non-α-secretase enzymatic cleavage. In fact, α-secretase-cleaved derivatives remain unphosphorylated at tyrosine residues and do not interact with the Shc-Grb2 adaptors, both in human brain and cultured cells (Figs. 1, 2, and 4). We must also remember that CTFs are *per se* considered amyloidogenic, neurotoxic when overexpressed *in vitro* and *in vivo*, and that their expression is increased in Down's syndrome years before the formation of plaques (11, 34, 35). In this study we provide evidence that, besides their role as β-amyloid precursors, CTFs may be directly involved in intracellular signaling likely related to glial activation. We cannot exclude that phosphorylated APP holoprotein might be first bound by Shc adaptors and then cleaved to form CTFs. We have described recently (12) that residue Tyr-682 of APP may be phosphorylated upon activation of Abl kinase, forming a pYENP motif that is recognized and bound by the SH2 domain of Abl itself. The same motif seems involved in the binding with Shc proteins as suggested by a recent study *in vitro* (36). Therefore, tyrosine phosphorylation of APP might constitute a regulatory event for cleavage of APP and interaction with its cytosolic adaptors. We cannot exclude that also other phosphorylation sites described previously (37) on APP (likely present also on CTFs) may also contribute to the regulation of APP/CTFs-Shc interaction, although tyrosine phosphorylation at residue 682 seems pivotal for the interaction with Shc (12, 36). The fact that thrombin may trigger the CTFs-ShcA interaction suggests that the signaling activity through CTFs is tightly regulated and that a cascade of events such as kinase(s) activation, APP/CTFs phosphorylation, and Shc interaction is required. The increased ERK1,2 phosphorylation, described here in AD and also present in thrombin-activated astrocytes, suggests that ShcA activation is likely responsible for the induction of a glial associated mitogenic pathway. ShcC, which is co-precipitated with CTFs in human brain as reported (3), is expressed at very low levels or virtually absent in cultured proliferating astrocytes, and in the human adult brain is likely produced in neurons or in meningeal vessels with no significant differences between AD and control subjects (Fig. 1c). At this point it is still unclear if APP and CTFs participate to the same Shc-mediated signaling addressed to the MAPK activation or if their role is linked
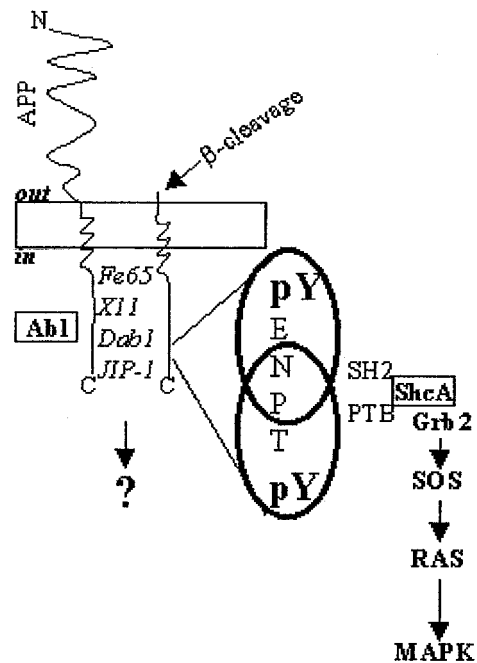

FIG. 5. Schematic hypothesis on proteins interacting with the cytosolic domain of holoAPP and CTFs. Abl and ShcA interact with the YENPTY region of APP and CTFs, upon phosphorylation of Tyr residues on APP. *Fe65*, *X11*, *Dab1*, and *JIP-1* interact with APP independently of the Tyr phosphorylation status. ShcA binding to CTFs occurs at the YENPTY motif, likely upon Tyr-682 phosphorylation. The putative signaling via CTFs-ShcA interaction may contribute to a mitogenic stimulus through MAPK activation in glial cells.

to the activation of a different intracellular signal, considering also that previous data describe phosphorylated-APP as an integrin-like molecule involved in cell motility and cell adhesion (38, 39) and that other adaptors and cytosolic proteins meet at the C terminus of APP (1, 40) (Fig. 5). This is the first identification of a signaling activity involving APP in astrocytes that to date have been merely considered as "reactive" cells to a primary amyloidosis of neuronal origin (26). A pathogenic role of thrombin in inflammation and AD has been proposed previously (30–33, 41) considering also that the cen-

tral nervous system is exposed to thrombin upon breakdown of the blood-brain barrier (26, 32, 41, 42). This occurs in acute conditions such as head injury and stroke and may also occur in chronic neurodegenerative diseases such as AD (32, 42). Thrombin, besides ShcA activation, is also involved in secretion, proteolysis of APP in platelets, and in cell cultures (28, 29, 43) and, as for the hemostatic enzyme factor Xa, may cleave APP generating potentially amyloidogenic C-terminal fragments (28, 44). Finally, the Kunitz protease inhibitor-containing isoform of APP/PN2, which is mainly produced by astrocytes, is a potent inhibitor of factors IXa, Xa, and XIa, which are involved in the formation of thrombin, itself suggesting an autoregulatory mechanism (44, 45). Therefore, our data correlate APP processing to glial proliferation and to the inflammatory response typical of AD and suggest that the activation of a mitogenic pathway through a CTFs-ShcA interaction by abnormally high levels of thrombin, possibly due to a compromised blood brain barrier, may trigger astrocyte reaction and possibly neurodegeneration.

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Signal Transduction through Tyrosine-phosphorylated C-terminal Fragments of Amyloid Precursor Protein via an Enhanced Interaction with Shc/Grb2 Adaptor Proteins in Reactive Astrocytes of Alzheimer's Disease Brain

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