Secreted β-amyloid precursor protein stimulates mitogen-activated protein kinase and enhances τ phosphorylation

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ABSTRACT  Biological effects related to cell growth, as well as a role in the pathogenesis of Alzheimer disease, have been ascribed to the β-amyloid precursor protein (β-APP). Little is known, however, about the intracellular cascades that mediate these effects. We report that the secreted form of β-APP potently stimulates mitogen-activated protein kinases (MAPKs). Brief exposure of PC-12 pheochromocytoma cells to β-APP secreted by transfected Chinese hamster ovary cells stimulated the 43-kDa form of MAPK by >10-fold. Induction of a dominant inhibitory form of Raf in a PC12-derived cell line prevented the stimulation of MAPK by secreted β-APP, demonstrating the dependence of the effect upon p21™. Because the microtubule-associated protein τ is hyperphosphorylated in Alzheimer disease, we sought and found a 2-fold enhancement in τ phosphorylation associated with the β-APP-induced MAPK stimulation. In the ras dominant inhibitory cell line, β-APP failed to enhance phosphorylation of τ. The data presented here provide a link between secreted β-APP and the phosphorylation state of τ.

The cell surface glycoprotein β-amyloid precursor protein (β-APP) consists of a large extracellular domain, a single membrane-spanning region, and a short intracellular carboxyl terminus. The amyloid β (Aβ) peptide that forms amyloid fibrils in Alzheimer disease (AD) is proteolytically released as a 40- to 43-amino acid fragment from the parent molecule (1). Cleavage at another site results in secretion of the extracellular domain (secreted β-APP), which appears in the medium of cultured cells and in cerebrospinal fluid (2-4). Amounts of β-APP increase during neuronal differentiation (5-7) and in response to injury (8-10), suggesting possible physiologic roles for this molecule. Biological activities have been attributed to Aβ (11-13) and to secreted β-APP (14-18). Secreted β-APP at low (<1 nM) dosages has been reported in culture to increase cell survival and adhesion, increase neurite extension from neurons and PC-12 cells, and prevent intracellular calcium accumulation and cell death in neurons. How these functions are mediated is unclear.

Mutations in the β-APP gene (1) can give rise to the full spectrum of AD pathology, including deposition of hyperphosphorylated τ isoforms as paired helical filaments (PHFs; refs. 19 and 20). The sequence of steps connecting β-APP and its metabolites to the hyperphosphorylation of τ and the formation of PHFs is central to understanding the pathogenesis of AD. This connection is crucial because PHF-containing neuritic lesions correlate most closely with the presence of clinical dementia (21). Phosphorylation serves normally to regulate the binding of τ to microtubules. In AD, hyperphosphorylation of τ results in a decreased affinity of τ for microtubules and presumably a loss of neuronal microtubule stability (22, 23).

We sought to determine whether the mitogen-activated protein kinases (MAPKs) might be involved both in transducing the effects of β-APP and in regulating the phosphorylation state of τ. MAPKs (also known as extracellular signal-regulated kinases or ERKs) are a family of protein kinases 40–46 kDa that specifically phosphorylate serine and threonine residues 10% fixed rat serum (24). MAPKs are activated by a variety of trophic factors, often through stimulation of p21™ (25-27). Once active, MAPKs phosphorylate and regulate an array of cellular substrates involved in gene transcription, cell structure, and signal transduction. MAPK can be found associated with microtubules during purification (28, 29).

The enzymes responsible for regulating the phosphorylation state of τ in vivo are unknown. MAPKs are strong potential candidates, because they phosphorylate in vitro (30–33) several of the same Ser/Thr-Pro sequences that are phosphorylated in PHF τ in vivo (34–37). MAPKs have also been immunocytochemically localized to areas of the AD brain that develop immunoreactive dystrophic neurites (38) and in proximity to neurofibrillary tangles and senile plaques (39).

Here we report that secreted β-APP activates MAPKs at picomolar to low nanomolar concentrations. Associated with this activation is enhanced phosphorylation of the τ protein.

METHODS

Cell Culture. PC-12, GSrasl, and GSrasDN6 (the latter two lines provided by S. Hagegouw, State University of New York at Stony Brook) pheochromocytoma cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated horse serum (GIBCO/ BRL) and 5% fetal calf serum (Sigma) at 37°C in humidified air with 5% CO₂. Cells were plated at a density of 1 × 10⁶ cells per 60-mm plate 2 days prior to each experiment. Expression of transfected ras genes in GSras1 and GSrasDN6 cells was induced 16 hr before use by addition of 1 μM dexamethasone from a 5 mM stock in dimethyl sulfoxide (DMSO). Equal amounts of DMSO (final concentration, 0.02%) had no effect on control GSras1 and GSrasDN6 cells.

Chinese hamster ovary (CHO) cells were grown in DMEM supplemented with 10% fetal bovine serum (GIBCO/ BRL) and 1% nonessential amino acids (Sigma) at 37°C in humidified air with 5% CO₂. Cells were plated at 1:5 dilution and used as conditioned medium 24 hr later. This procedure resulted in concentrations of β-APP in the range of 1–10 nM as determined by comparison on immunoblot with purified β-APP. This is at least 5–10 times greater than the amounts produced by untransfected CHO or PC-12 cells.

Abbreviations: β-APP, β-amyloid precursor protein; MAPK, mitogen-activated protein kinase; AD, Alzheimer disease; PHF, paired helical filament; CHO, Chinese hamster ovary; NGF, nerve growth factor; Aβ, amyloid β; ERK, extracellular signal-regulated kinase.

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Immunocomplex Assay of MAPK. PC-12 cells were treated with either control medium (10% fetal calf serum in DMEM), conditioned medium (see above), conditioned medium immunocomplexed by immunoaffinity (Fig. 3) with anti-B5 (Athena Neurosciences, San Francisco; 2.4 μg of antibody per ml of medium), or control medium supplemented by purified β-APP, nerve growth factor (NGF) (2.5 S, GIBCO/BRL), or HPLC-purified synthetic Ab5_B40 (41). Following treatment, cells were washed twice with ice-cold phosphate-buffered saline with 1 mM sodium orthovanadate and then scraped into 100 μl of immunoprecipitation buffer (50 mM NaCl, 1 mM EDTA/10 mM Tris-HCl pH 7.4, 1% Triton X-100, 1% Nonidet P-40, 0.1% deoxycholate, and protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 10 μg of aprotinin per ml, 10 μg of pepstatin A per ml, 10 μg of leupeptin per ml, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 20 mM sodium fluoride). Lysates were centrifuged to remove cellular debris, normalized to 50–100 μg of protein (42), and precipitated for 1 hr at 4°C with 2.5 μl of anti-MAPK/ERK-1 antiserum kindly provided by J. Blenis (27). Precipitates were collected with 50 μl of 10% protein A-Sepharose, washed as described (27), and assayed for kinase activity for 30 min at 30°C. Kinase reaction mixtures contained 15 μg of myelin basic protein, 50 μM [γ-32P]ATP (1 μCi; 1 Ci = 37 GBq), 10 mM MgCl2, 5 mM benzamidine, 1 mM diethiothreitol, and 30 mM Hepes (pH 7.2) in a final volume of 30 μl. Reactions were terminated by boiling in SDS sample buffer, resolved on 15% polyacrylamide gels, and quantified by PhosphorImager analysis (Molecular Dynamics).

Immunoblots. Following treatment, cells were lysed in a boiling solution of 1 mM EDTA/5 mM EGTA/2% SDS/30 mM Tris-HCl, pH 6.8, and the above protease and phosphatase inhibitors, boiled for 10 min, and sheared by passage through a 26-gauge syringe. Lysate supernatants were normalized for protein, resolved on 10% polyacrylamide gels, and transferred by the semi-dry blotting system (Bio-Rad). After blocking for 16 hr at 4°C in phosphate-buffered saline and 3% bovine serum albumin, blots were probed for 4 hr at room temperature in the same solution with monoclonal antibodies to phosphotyrosine (4G10, UBI, 1:500) or MAPK (Zymed; 1:2500). Immunoreactive bands were visualized with anti-mouse antibody coupled to horseradish peroxidase (Promega) and the ECL system (Amersham).

Purification of Secreted β-APP. β-APP was purified by either immunoaffinity from serum-free medium (MCDB-302; Sigma) conditioned by CHO(β-APP711) cells. One method utilized affinity purification with monoclonal antibodies SA3 and IG7 (43) covalently attached to AminoLink gel resin (Pierce) according to the manufacturer’s instructions. β-APP bound to the column was eluted under high salt conditions (Gentle Elution Buffer; Pierce) and positive fractions were pooled, desalted, and concentrated using a Centricon C-30 microconcentrator. Ten × 150-mm plates of confluent cells yielded ~4 μg of β-APP. Protein concentration was estimated by comparison of Coomassie brilliant blue staining with a series of standard samples of bovine serum albumin. Duplicate immunoblots were analyzed with the anti-APP antibody Ab-10 (ref. 44; 1:10,000 dilution) or 22C11 (ref. 3; 1:10,000).

For some experiments, β-APP was purified from conditioned MCDB-302 medium by conventional chromatography modified from published methods (40). Medium concentrated with an Amicon YM-3 membrane was loaded onto a Pharmacia Mono-Q HRS/5 union-exchange fast protein liquid chromatography column equilibrated in 20 mM Tris-HCl (pH 7.4) and eluted at 1 ml/min with a 60-min linear gradient of 1 M NaCl. Fractions eluting at ~0.5-6.5 M NaCl contained the majority of β-APP as judged by immunoreactivity with 22C11. These fractions were pooled, concentrated by C-30 microconcentrator, and loaded onto a Tosohas 60-cm G300SW FPLC column developed in phosphate-buffered saline at a rate of 0.5 ml/min. β-APP eluted at 14–20 ml, coinciding with a single peak of UV absorbance, and was used for subsequent experiments following renaturation. Thirty × 150-mm plates of confluent cells yielded ~6.5 μg of β-APP.

Immunoprecipitation of Phosphorylated τ. PC-12 cells were radiolabeled for 165 min in DMEM with reduced phosphate (1% of normal) and 0.3–0.5 mCi of carrier-free [32P]orthophosphate (ICN) per ml. Incorporation of radiophosphate into nucleotides was measured by charcoal extraction (45). Following treatment, cells were lysed in 400 mM NaCl/0.2% Triton X-100/1 mM EDTA/5 mM EGTA/10 mM Tris-HCl, pH 7.6, and the above protease and phosphatase inhibitors, substituting 5 μM okadaic acid (GIBCO/BRL) for orthovanadate. After aliquots were taken for protein determination, the samples were heated to 90°C for 4 min, and the normalized supernatants were brought to 1% Triton X-100 and precipitated for 90 min with 30 μg of monoclonal antibody 5E2 (46). Precipitated samples were reconstituted with 50 μl of 10% protein G-Sepharose CL-4B (Pharmacia), washed twice in the above buffer containing 1% Triton X-100 and 0.1% SDS, washed once in 150 mM NaCl/10 mM Tris-HCl, pH 7.6, extracted in SDS sample buffer, resolved on 10% polyacrylamide gels, and quantified as above. All bands shown were absent in control immunoprecipitates without antibody or using 5E2 preabsorbed with purified τ. Immunoblots for τ were performed with 5E2 at 1:1000 dilution or AT8 (Immunogenetics, Ghent, Belgium) at 1:200.

RESULTS

β-APP Secreted by CHO Cells Activates MAPK. A preliminary assay to screen for β-APP activity utilized medium conditioned by CHO cells stably transfected with the full-length 695-, 751-, or 770-amino acid form of β-APP. Following exposure of PC-12 cells to conditioned medium, activity of MAPK/ERK-1 in the PC-12 cell lysates was measured in immunocomplexes using myelin basic protein as substrate. Medium enriched in β-APP695, β-APP751, or β-APP770 activated MAPK/ERK-1 approximately 25-, 18-, and 17-fold, respectively, at 10 min (Fig. 1A). Stimulation declined with time of exposure but remained at least 50% of the peak activity after 1 hr of treatment (data not shown). Medium from untransfected CHO cells, which contains small amounts of β-APP (see Methods) as well as other possible growth factors, stimulated MAPK to a much lesser degree (Fig. 1A, lane 3). Synthetic Ab5_B40 at 10 μg/ml (~1.75 μM) had no effect on MAPK activity (Fig. 1A, lane 2).

To confirm that the activity of the medium was due to secreted β-APP, this molecule was purified and added directly to PC-12 cells. Similar results were obtained using β-APP751 purified either by immunoadfinity (Fig. 2A) or conventional chromatography (not shown). Protein purified from CHO(β-APP711)-conditioned medium appeared as a single band of 120 kDa on gels stained with Coomassie brilliant blue (Fig. 2A, lane 1) and on immunoblots with antibodies to the carboxyl (Ab-10, lane 2) or amino terminus (22C11, not shown) of secreted β-APP. Purified β-APP stimulated MAPK in a dose-dependent manner with a nearly 2-fold activation at 20 μM and >10-fold at 2 nM (Fig. 2B). Prior immunoprecipitation of conditioned medium with anti-B5, an affinity-purified antiseraum raised against the peptide β-APP44-92, markedly diminished MAPK activation (Fig. 2C).

MAPKs are activated by phosphorylation on tyrosine and threonine residues (24). As an independent assay for MAPK activation, tyrosine phosphorylation in response to secreted β-APP was measured by anti-phosphotyrosine immunoblot. Treatment with conditioned medium (Fig. 3) or purified β-APP711 (not shown) resulted in increased tyrosine phos-
phorylation of proteins at 43 and 41 kDa. These proteins comigrate with immunoreactive MAPK (lanes 4 and 5) and most likely represent MAPK/ERK-1 and -2 (26, 27).

**Activation of MAPK by β-APP Is Dependent on p21<sup>ras</sup>.** Many growth factors that activate MAPK, such as NGF and fibroblast growth factor, do so through activation of the p21<sup>ras</sup> protein (25–27). The dependence of β-APP on p21<sup>ras</sup> was assayed in the PC-12-derived cell line GSrasDN6. GSrasDN6 cells express the dominant inhibitory Ha-Ras mutant RasS17N under control of the dexamethasone-responsive mouse mammary tumor virus promoter (26, 27). Induction of inhibitory ras, which had no discernible effect on kinase activity in untreated cells, prevented β-APP-stimulated MAPK activation as measured by immunocomplex kinase assay (Fig. 1C) or tyrosine phosphorylation (not shown). Similar results to those shown were also obtained for stimulation with purified secreted β-APP.

**β-APP Stimulates Intracellular Phosphorylation of τ.** MAPKs phosphorylate τ in vitro (30–33). To determine whether stimulation with secreted β-APP enhances phosphorylation of τ in intact cells, PC-12 cells were preincubated in [32P]orthophosphate and τ was immunoprecipitated with monoclonal antibody 5E2 following a heat extraction step. The treatments used did not significantly affect either the total amount of cellular τ as measured by immunoblot with 5E2 or incorporation of radiophosphate into the intracellular pool of nucleotides (data not shown). Increases in labeling of

**Fig. 1.** p21<sup>ras</sup>-dependent activation of MAPK/ERK-1 by medium enriched in β-APP. (A) MAPK/ERK-1 activity in lysates from PC-12 cells treated for 10 min with control (C) medium, medium supplemented with 10 μg of Aβ<sub>1-40</sub> peptide per ml, or conditioned medium from CHO cells that are untransfected or transfected with human β-APP<sub>192</sub>, β-APP<sub>231</sub>, or β-APP<sub>276</sub>. The arrowhead indicates the position of myelin basic protein serving as kinase substrate. (B) Dependence of MAPK stimulation on p21<sup>ras</sup>. MAPK/ERK-1 activity was measured in GSrasDN6 cells. The inhibitory form of ras was induced in lanes 2 and 4 16 hr before exposure to control or conditioned medium. Dex, dexamethasone.
precipitated protein thus represent increased incorporation of phosphate per mol of τ.

Fifteen-minute exposure to medium enriched in β-APP695 or β-APP770 (Fig. 4A, lanes 1–4) or purified β-APP751 (lanes 5 and 6) approximately doubled the amount of phosphate incorporated into τ. No significant increase was caused by medium from untransfected CHO cells (Fig. 4A) or by Aβ40 peptide (data not shown). To further support an association between MAPK activation and enhanced τ phosphorylation, two other treatments that activate MAPK were tested for their effect on τ phosphorylation: application of NGF and induction of the constitutively active Ha-Ras mutant RasQ61L in the Gsras1 cell line (26, 27). Both NGF and the constitutive activation of ras by dexamethasone in Gsras1 enhanced phosphorylation of τ (Fig. 4C). Immunoprecipitation of τ-phosphorylation, like activation of MAPK, was prevented in GsrasDN6 cells by the induction of inhibitory ras (Fig. 4B).

**DISCUSSION**

Our results demonstrate activation of a particular signal transduction system, the p21^ras^-dependent MAPK cascade, in response to secreted β-APP. The MAPK cascade, because of its central role in cell regulation (24), is a likely candidate for mediating at least some of the biological actions of β-APP (14–18). The low doses (<100 pM) of β-APP required to stimulate MAPK are consistent with concentrations required for its described biological effects. It is not clear, however, that each of the biological actions of β-APP is mediated by a single mechanism, as studies aimed at ascertaining the molecule’s active site have given discrepant results (18, 47).

Secreted β-APP is a normal component in the extracellular compartment of the nervous system (4). The factors that regulate its level are unknown. The fairly steep decline in MAPK activity following stimulation suggests that with constant circulating levels of secreted β-APP, MAPK may maintain a relatively low basal activity. Under conditions when β-APP increases, such as during development, after injury, and in Down syndrome (1, 5, 6, 8–10), MAPK may reset at a higher level.

The link between β-APP and τ, the two key proteins comprising the structural basis for senile plaques and neurofibrillary tangles, respectively, is a central missing element in the pathogenesis of AD. These experiments suggest such a link through activation of MAPK. Of the many kinases that phosphorylate τ in vitro (21), the proline-directed kinases such as MAPK are among the likeliest to do so in vivo, considering the prevalence of phosphorylated Ser/Thr-Pro sequences in τ from PHFs and fetal brain (23, 34–37, 48, 49).

Our data provide further evidence for this idea: activation of MAPK in intact PC-12 cells is invariably accompanied by enhanced τ phosphorylation. The increased phosphorylation of τ in our experiments is relatively modest compared with the robust activation of MAPK, without an evident shift in τ’s electrophoretic mobility. The relatively small increase in phosphorylation is likely due to the high baseline level of τ phosphorylation observed in cultured cells (see also ref. 23) compared to mature brain tissue. Thus, even unstimulated PC-12 cells display considerable reactivity on immunoblots with the phosphorylation-dependent τ antibody AT8 (S.M.G., unpublished results). Secreted β-APP generates further increases in AT8-reactive τ (S.M.G., unpublished results), indicating increased phosphorylation of at least one of the sites phosphorylated in PHF τ (35).

τ in the developing brain, like PHF τ, is hyperphosphorylated relative to normal adult τ (23, 48, 49). Phosphorylation of fetal τ could reflect activation of MAPK by neurotrophic factors during development, as many of its phosphorylation sites are also in vitro substrate sites for MAPK. Although fetal τ is phosphorylated at many of the same sites as PHF τ, it does not form PHFs. The abnormal properties of PHF τ may arise from phosphorylation at a wider array of sites within the molecule than occurs in fetal τ or, alternatively, from the occurrence of fetal-type phosphorylation within an adult cellular milieu. Persistent activation of MAPK in AD could increase phosphorylation of other cellular elements as well as τ, ultimately leading to the loss of cytoskeletal integrity and synaptic death that characterize the disease.

Complicating the problem of linking senile plaques and neurofibrillary tangles is the observation that, despite their concomitant occurrence in the AD brain, the two lesions are
spatially dissociated (21). Our findings raise the possibility that secreted β-APP might promote PHF formation at sites remote from where Aβ deposits as amyloid fibrils. The actual distribution of secreted β-APP in the AD brain has not been established and, as a soluble molecule in the extracellular space, has been difficult to study. Immunoreactive β-APP, in many cases lacking the Aβ epitope, has been noted within the abnormal neurites that surround the senile plaque core (50–52).

Though we have discussed the effects of MAPK primarily from the standpoint of τ and the cytoskeleton, aberrant activation of this enzyme in the nervous system is likely to have far-ranging consequences. The results of MAPK activation appear to differ between neuronal and nonneuronal cells, as activation of p21ras and MAPK in PC-12 cells causes neuronal differentiation rather than transformation (26, 27). It will be important to determine whether aberrant activation of signal transduction cascades can result in neurodegeneration.

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