Ectodomain Phosphorylation of β-Amyloid Precursor Protein at Two Distinct Cellular Locations*

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Jochen Walter, Anja Capell, Albert Y. Hung†, Hanno Langen‡, Martina Schnölzer‡, Gopal Thinakaran, Sangram S. Sisodia, Dennis J. Selkoe†, and Christian Haass**

From the Central Institute of Mental Health, Department of Moleculare Biology, J5, 68159 Mannheim, Germany, Hoffman-LaRoche Ltd., Pharmaceutical research, Gene Technologies, 4070 Basel, Switzerland, the Department of Cell Biology, German Cancer Research Center, 69120 Heidelberg, Germany, the Department of Neurology and Program in Neuroscience, Harvard Medical School and Center for Neurologic Diseases, Brigham and Woman’s Hospital, Boston, Massachusetts 02115, and the Neuropathology Laboratory, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205-2196

The β-amyloid precursor protein (βAPP) is a transmembrane protein that is exclusively phosphorylated on serine residues within its ectodomain. To identify the cellular site of βAPP phosphorylation, we took advantage of an antibody that specifically detects the free C terminus of β-secretase-cleaved βAPP containing the Swedish missense mutation (APP<sup>swe</sup>-β). This antibody previously established the cellular location of the β-secretase cleavage of Swedish wild type βAPP as a post-Golgi compartment (Haass, C., Lemere, C., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D. J. (1995) Nature Med. 1, 1291–1296). We have now localized the selective ectodomain phosphorylation of βAPP to the same compartment. Moreover, the phosphorylation sites of βAPP were identified at Ser<sup>198</sup> and Ser<sup>206</sup> of βAPP by trypic peptide mapping, mass spectrometry, and site-directed mutagenesis. Intracellular phosphorylation of βAPP was inhibited by Brefeldin A and by incubating cells at 20 °C, thus excluding phosphorylation in the endoplasmic reticulum or trans-Golgi network. Ectodomain phosphorylation within a post-Golgi compartment occurred not only with mutant Swedish βAPP, but also with wild type βAPP. In addition to phosphorylation within a post-Golgi compartment, βAPP was also found to undergo phosphorylation at the cell surface by an ectoprotein kinase. Therefore, this study revealed two distinct cellular locations for βAPP phosphorylation.

Alzheimer’s disease (AD) is the most common cause of age-related mental failure. It is now widely accepted that the deposition of the amyloid β-peptide (Aβ) within the brain parenchyma and in cerebrovascular blood vessels is an early and necessary feature of AD (2). Aβ is derived from the membrane-spanning β-amyloid precursor protein (βAPP; Ref. 3). βAPP can be proteolytically processed within two general pathways: an amyloidogenic and a nonamyloidogenic processing route (summarized by Haass and Selkoe (4)). Within the latter pathway, βAPP is constitutively cleaved by a protease referred to as α-secretase. This cleavage occurs near the middle of the Aβ region, thus inhibiting Aβ formation (5, 6) and resulting in the secretion of APP<sub>α</sub>-α (for terminology, see Fig. 2A) into the media of cultured cells (7). In the amyloidogenic pathway, βAPP is first cleaved by β-secretase at the N terminus of the Aβ domain and subsequently by γ-secretase at its C terminus, resulting in the constitutive secretion of Aβ (8–11).

One cellular mechanism for the generation of Aβ involves reinternalization of full-length βAPP from cell surface to endosomes (12), in which the β-secretase cleavage can occur (13). During recycling of endosomes to the cell surface (13), the resulting 12-kDa C-terminal fragment is cleaved by γ-secretase to release Aβ. Missense mutations, found in a few families with familial autosomal dominant AD, frame the Aβ domain (reviewed by Mullan and Crawford (14)). All familial autosomal dominant AD-linked mutations found in the βAPP gene have now been shown to influence directly Aβ generation. A mutation just before the N terminus of the Aβ region at the β-secretase cleavage site (the “Swedish” mutation; Ref. 15) results in a 3–6-fold increased production of Aβ (16–18). Missense mutations close to the α-secretase site also cause an increased production of Aβ, but the increase is paralleled by alternative N-terminal cleavages of Aβ (19). Mutations at the C terminus of the Aβ domain (just after the γ-secretase site) result in the generation of longer Aβ peptides ending at amino acid 42 instead of amino acid 40 (20). The former peptides have been shown to aggregate more rapidly (21), presumably leading to an accelerated amyloid plaque formation.

Recently, we (1) and others (22) showed that the increased production of Aβ from βAPP molecules bearing the Swedish mutation is due to a cellular mechanism distinct from that principally involved in Aβ generation from wild type βAPP. β-Secretase cleavage of βAPP appears to generally occur within the endocytic pathway (13). During reinternalization, only small amounts of full-length, uncleaved βAPP molecules are available, because substantial quantities of βAPP have already been cleaved by α-secretase. However, in the case of Swedish mutant βAPP, we found that β-secretase cleavage occurs at an earlier time point in βAPP trafficking, namely within the secretory pathway on the way to the cell surface, predominantly in a post-Golgi compartment, most likely secretory vesicles (1). Therefore, β-secretase cleavage of Swedish mutant βAPP, in...
contrast to the principal β-secretase cleavage of wild type βAPP, occurs in competition with α-secretase cleavage in the secretory pathway. βAPP matures by undergoing N- and O'-glycosylation, sulfation, and phosphorylation during transport from the endoplasmic reticulum to the cell surface (7, 8, 23). Protein phosphorylation is known to be involved in the regulation of cellular processes such as differentiation, metabolism, and signal transduction (for review, see Ref. 24). Besides many intracellular phosphoproteins, some phosphorylated secretory proteins have been described, e.g. fibronectin (25), fibrinogen (26), pro-lactin (27), chromogranin B, secretogranin II (28), and L-29, a soluble lecithin (29). Although the cellular locus for phosphorylation of most of these secretory proteins is not identified, it has been shown that chromogranin B and secretogranin II are phosphorylated in the secretory pathway within the trans-cisternae of the Golgi (28).

In addition to numerous intracellular protein kinases, ecto-protein kinases acting at the surface of intact cells have been characterized (30–32). These enzymes use extracellular ATP as cosubstrate to phosphorylate endogenous cell surface proteins as well as soluble proteins and have been implicated in a number of biological phenomena, including cell growth inhibition (33), long-term potentiation in neurons and synaptogenesis (34, 35), and parasite-host interactions (36, 37). Ubiquitously occurring casein-like ectoprotein kinases can be released from the cell surface upon interaction with extracellular protein substrates (38, 39), thus allowing them to act at a distance to their cellular origin.

In this study, we have determined the subcellular locations of the phosphorylation of βAPP. We used an antibody (192sw (40); see Fig. 2A) specifically detecting APPαβ1–40, the derivative we found to be generated in high quantities within a well defined post-Golgi secretory compartment (1). Through biochemical and cell biological experiments we demonstrate that intracellular phosphorylation of Swedish βAPP as well as wild type βAPP occurs within this compartment, i.e. after the trans-Golgi, most likely within secretory vesicles. Ectodomain phosphorylation was mapped to Ser199 and Ser206 of βAPP695, which represent potential phosphorylation sites for casein kinase (CK)-2 and CK-1, respectively. Further, we show that βAPP can be phosphorylated by an ectoprotein kinase activity on the cell surface. Therefore, our data demonstrate that βAPP undergoes ectodomain phosphorylation at two distinct cellular locations.

Materials and Methods

Cell Culture, Metabolic Labeling, and Drug Treatment—Kidney 293 cells were stably transfected with the wt βAPPαβ cDNA (9, 41) or with the βAPPas cDNA containing the Swedish double mutation (18). Chinese hamster ovary cells stably transfected with the amyloid precursor-like protein 2 (APLP2) cDNA (42) were used as described previously (43). Briefly, cells stably transfected with the Swedish βAPP mutation were pulse-labeled with [35S]methionine for 5 min in methionine and serum-free media. Cells were then chased for the indicated time points in media containing excess amounts of methionine and 10% fetal calf serum. Media were changed with antibody C7 (to detect full-length βAPP) and antibody 192sw (to detect intracellular βAPPαβ). Media were immunoprecipitated with antibody 192sw (to detect secreted βAPPαβ).

Phosphoamino Acid Analysis—Phosphoamino acid analysis was carried out by two-dimensional high voltage electrophoresis (47). Radiolabeled proteins electrotransferred onto polyvinylidene difluoride-membrane were hydrolyzed in 6 M HCl for 90 min at 110°C. Subsequently, supernatants were dried in a SpeedVac concentrator, and pellets were dissolved in 5 μl of pH 1.9 buffer (7.8% acetic acid, 2.5% formic acid) and spotted onto cellulose-TLC plates together with unlabeled phosphoamino acids (48). Digestion of radiolabeled βAPP was carried out for 24 h at 37°C with 0.5 mg/ml trypsin (Ref. 49). Phosphoamino acids were identified by autoradiography and comparison with ninhydrin-stained standard.

Phosphopeptide Mapping by Tryptic Digestion—In vivo, [32P]-phosphorylated βAPP was isolated by immunoprecipitation and SDS-PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell). Digestion of radiolabeled βAPP was carried out for 24 h at 37°C with 0.5 mg/ml trypsin (Ref. 40). After the tryptic digest was separated on Tris/Tricine gradient gels (10–20%; Novex), and radiolabeled peptides were visualized by autoradiography.

Matrix-assisted Laser Desorption/ Ionization-Mass Spectrometry—Approximately 10 μg of unlabeled βAPP together with a trace of βAPPαβ was digested with trypsin, and the resulting peptides were separated on 10–20% acrylamide, 0.1% trifluoroacetic acid. Molecular masses (isotopic average) of the eluted peptides were determined by a Vision 2000 (Finnigan) mass spectrometer equipped with a nitrogen laser and detected in reflection mode at an accelerating voltage of 5000 V. μl of the peptide solution was crystallized in matrices consisting of 1% 2,4-dihydroxybenzoic acid in 0.1% aqueous trifluoroacetic acid. All peptide spectra were externally calibrated by using the monoisotopic masses of sodium (M, 23.0) and fulleren C70 (M, 840.0). Peptides were identified by computer-assisted analysis using the Swiss-Prot sequence data bank and the special program package HUSAR (developed at the Department of Molecular Biophysics, German Cancer Research Center, Heidelberg).

Phosphorylation of Cell Surface Proteins by Ectoprotein Kinase—Phosphorylation was carried out as described earlier (30). Briefly, subconfluent monolayer cell cultures (5–7 × 106 cells/cm2), grown in Dulbecco’s minimum essential medium (10% fetal calf serum) were washed twice with prewarmed (37°C) isotonic phosphorylation buffer (30 mM Tris, pH 7.3, 70 mM NaCl, 5 mM magnesium acetate, 0.5 mM EDTA, 5 mM KH2PO4/K2HPO4, 290 ± 10 mosm) and incubated for 5 min at 37°C in the same buffer.

Phosphorylation was started by the addition of 0.5–1.5 μM γ[32P]ATP and allowed to proceed for 0–30 min at 37°C. Reactions were terminated by removing cell supernatants followed immediately by two washes of the cells with ice-cold phosphorylation buffer containing 2 mM unlabeled ATP. Subsequently, cells were lysed in presence of 2 mM ATP for 7 min on ice. Cell lysates (prepared as described by Haass et al. (43)) were centrifuged for 10 min at 14,000 × g, and cellular βAPP was isolated by immunoprecipitation as described above and separated by SDS-PAGE. Radiolabeled proteins were detected by autoradiography of dried gels. Cell viability during phosphorylation assays was evaluated by several criteria.

In Vitro Mutagenesis—The βAPP cDNA construct containing a stop codon at the α-secretase cleavage site was described previously (23). The C-terminal deletion construct of βAPP was described by Haass et al. (44). A cDNA construct containing a stop codon at the β-secretase site of wt βAPP was generated as described above, using the following annealed oligonucleotides: GATCTCTGAAAGTAGAAGTGGGGACG
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RESULTS

Ectodomain Phosphorylation of APLP2—In order to obtain a general validation of ectodomain phosphorylation of βAPP (23), we examined the phosphorylation of the highly related APLP2. APLP2, APLP1, and βAPP are members of a conserved gene family of homologous proteins (52–55). APLP2 is particularly similar to βAPP because it shares some of its characteristic biochemical properties and also matures through the constitutive secretory pathway, where its ectodomain is secreted into culture media (42, 55, 56). To analyze the potential phosphorylation of APLP2, Chinese hamster ovary cells stably transfected with the APLP2 cDNA were metabolically labeled with [35S]methionine or [32P]orthophosphate. Conditioned media were immunoprecipitated with antibody D2–1. As a control, APP−α was immunoprecipitated from βAPP-transfected kidney 293 cells labeled with [32P]orthophosphate. APLP2sw indicates the chondroitin sulfate glycosaminoglycan-modified form of APLP2.

The corresponding cDNAs were stably transfected into kidney 293 cells as described (1, 51), and single cell clones were isolated using cloning cylinders (51).

All mutations were confirmed by sequencing both DNA strands.

Intracellular Phosphorylation of Swedish Mutant βAPP—Little is known about the cell biology of ectodomain phosphorylation. In order to determine the subcellular locus for βAPP phosphorylation, we used an antibody (192sw; Fig. 2A) that specifically recognizes APPsw, which we previously detected in high quantities within the lysates of kidney 293 cells stably transfected with the Swedish βAPPsw cDNA and metabolically labeled with [32P]orthophosphate were immunoprecipitated with antibody C7 to detect full-length βAPP or with 192sw to detect intracellular APPsw. Conditioned media were precipitated with antibody 192sw to detect secreted APPsw (192sw, Medium).

Phosphorylation activity was detected in any of the AD brain extracts, both those from temporal and occipital regions, indicating a relative resistance of βAPP to protein phosphatase activities, suggesting a long lasting biological function of phosphorylated APPsw molecules.

Stability of Ectodomain Phosphorylation of βAPP—To assess the stability of βAPP phosphorylation, we examined protein phosphatase activity in AD brain extracts as well as in the conditioned media of cultured cells (57, 58). No βAPP dephosphorylation activity was detected in any of the AD brain extracts, both those from temporal and occipital regions, indicating a relative resistance of βAPP to protein phosphatase activities. In addition, APPsw did not undergo dephosphorylation in conditioned media (data not shown). These experiments demonstrate that ectodomain phosphorylation of βAPP is relatively resistant to protein phosphatase activities, suggesting a long lasting biological function of phosphorylated APPsw molecules.

Intracellular Phosphorylation of Swedish Mutant βAPP—Little is known about the cell biology of ectodomain phosphorylation. In order to determine the subcellular locus for βAPP phosphorylation, we used an antibody (192sw; Fig. 2A) that specifically recognizes APPsw, which we previously detected in high quantities within the lysates of kidney 293 cells stably transfected with the Swedish βAPPsw cDNA (1). To determine if ectodomain phosphorylation also occurs on intracellular APPsw, we radiolabeled kidney 293 cells expressing Swedish mutant βAPP with [32P]orthophosphate. Upon immunoprecipitation of cell lysates and media we detected phosphorylated intracellular and secreted APPsw, as well as phosphorylated intracellular full-length βAPP (Fig. 2B). This result indicates the occurrence of intracellular phosphorylation of the βAPP-ectodomain. To prove that APPsw−β was indeed produced de novo and not taken up by fluid phase endocytosis, we pulse-labeled kidney 293 cells stably transfected with the Swedish cDNA. The cells were then chased in the presence of excess unlabeled methionine. Aliquots of the cell lysates were immunoprecipitated either with antibody C7 (to detect maturation of full-length βAPP) or with antibody 192sw (to detect intracellular APPsw−β).

In addition, conditioned media were immunoprecipitated with antibody 192sw to detect secreted APPsw−β. As shown in Fig. 3, full-length βAPP was processed within 45 min from immature N-glycosylated form to mature N- and O-glycosylated form. Shortly after, the amount of full-length βAPP increased significantly with a concomitant decrease in the steady-state level of APPsw−β.
mediated uptake of secreted species. Shortly after that, the amount of intracellular APPs declined due to its secretion into the media. Full-length βAPP was detected with antibody C7 (top panel), and intracellular (middle panel) as well as secreted APPsw-β (bottom panel) was precipitated with antibody 192sw.

βAPP declines due to the secretion of APPsw. Consistent with our previous results (1), the highest level of intracellular APPsw-β was detected after 45 min. After this time point the levels of intracellular APPsw-β declined, and an increase of secreted APPsw-β in the media was observed (Fig. 3). The precursor product relationship clearly indicates that intracellular APPsw-β is produced de novo and not due to a fluid phase mediated uptake of secreted species.

Mapping of Phosphorylation Sites within βAPP—To determine which amino acids were phosphorylated in Swedish mutant βAPP, we performed phosphoamino acid analysis of intracellular as well as secreted APPsw-β. Both species are phosphorylated exclusively on serine residues (Fig. 4). This result is in line with recent studies showing that wt βAPP is constitutively phosphorylated solely on serine residues (23). It also confirms that phosphorylation of intracellular APPsw-β is an amino acid phosphorylation, not an incorporation of phosphate into sugar moieties of βAPP.

In order to identify the site(s) of βAPP phosphorylation, we performed tryptic peptide mapping of in vivo phosphorylated βAPP molecules. Kidney 293 cells stably transfected with wild type βAPP695 or cDNA constructs deleting large portions of the N-terminal half (AX construct (23) (Fig. 5A) or the C-terminal half (XB construct (23)) were labeled with [35S]methionine, and secreted APPsw-β was immunoprecipitated from cell lysates and conditioned media with antibody 192sw, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membrane. The bands were excised and subjected to two-dimensional phosphoamino acid analysis by separation with high voltage electrophoresis along the horizontal axis at pH 1.9 and along the vertical axis at pH 3.5. The mobilities of ninhydrin-stained phosphoamino acid standards are indicated. The origin is denoted by an arrowhead.

Serine phosphorylation of intracellular APPsw-β (cell lysate) and secreted APPsw-β (media). Kidney 293 cells stably transfected with the Swedish βAPP cDNA were metabolically labeled with [32P]orthophosphate or [35S]methionine. Secreted forms of the APPsw-β was eluted from Tris/Tricine gel and subjected to matrix-assisted laser desorption/ionization-mass spectrometry (see “Materials and Methods”). Three monoisotopic masses of 2286.5, 3673.5, and 4877.3 (± 10) were detected in the eluate. The masses of 2286.5 and 3673.5 could not be matched to tryptic peptides of βAPP and presumably represent peptides of autocatalytically cleaved trypsin, migrating close to the phosphorylated βAPP tryptic peptide. In contrast, the mass of 4877.3 matches that of the sequence of amino acids 181–224 of βAPP695 in a double phosphorylated form (4714.7 + 160 Da). Since the amino acid sequence of this peptide contains four serine residues, we searched for putative phosphor acceptor sites by computer-assisted analysis. Serine residues 198 and 206 were identified within an acidic sequence of this peptide, representing potential phosphorylation sites for CK-2 and CK-1, respectively (Fig. 5D). These serines were therefore mutated to alanines, and the corresponding cDNA constructs were stably transfected into kidney 293 cells. Single cell clones were metabolically labeled with [32P]orthophosphate or [35S]methionine, and secreted βAPPsw was immunoprecipitated from conditioned medium with antibody B5. Phosphate incorporation was quantified by phosphor imaging. As shown in Fig. 5E, phosphorylation of βAPP containing the S198A mutation was reduced by about 80%, while that of the S206A mutation was reduced by about 15%. Similar data were obtained after immunoprecipitation of full-length βAPP from cell lysates (data not shown). Taken together, these data might therefore indicate that both serines represent in vivo phosphorylation sites (see “Discussion” for details).

Phosphorylation of βAPP Occurs within Golgi-derived Vesicles—Ectodomain phosphorylation of βAPP was found on all types of secreted APPsw molecules, regardless of whether Swedish or wt βAPP was cleaved at either the α- or the β-secretase site. To produce APPsw molecules with defined C termini corresponding to α- or β-secretase-cleaved APPsw, we stably transfected kidney 293 cells with cDNA constructs containing stop codons at sites corresponding to these scissions. These transfecants were then metabolically labeled with [35S]methionine or [32P]orthophosphate, and their conditioned media...
Two Cellular Sites of \( \beta \)APP Phosphorylation

Fig. 6. Phosphorylation of truncated, soluble forms of \( \beta \)APP. Stop codons were introduced into wild type or Swedish \( \beta \)APP cDNA corresponding to the cleavage sites of \( \alpha \)-secretase (\( \alpha \)-stop), \( \beta \)-secretase (\( \beta \) wt-stop), or \( \beta \)-secretase of Swedish \( \beta \)APP (\( \beta \) sw-stop). Kidney 293 cells stably expressing these constructs were metabolically labeled with \([^{35}S] \)methionine (left panel) or \([^{32}P] \)orthophosphate (right panel) and precipitated from conditioned media with antibody B5. The arrow marked \( \beta \)APP, indicates APP\(_{\beta} \) derived from the transfected cDNA constructs. The unmarked arrow indicates APP\(_{\alpha} \), from endogenous \( \beta \)APP\(_{\alpha} \). Differences in the amounts of APP\(_{\beta} \) in these cell lines are due to different expression levels of \( \beta \)APP.

Fig. 5. Identification of the phosphorylation sites of \( \beta \)APP within its ectodomain. A, schematic of wild type \( \beta \)APP (WT) and the AX and XB constructs, missing large portions of the N-terminal and the C-terminal half of the \( \beta \)APP ectodomain, respectively. The APP\(_{\beta} \)-domain is represented by a striped bar, and vertical lines represent cellular membranes. The numbers above denote amino acid residues with the restriction sites used to generate the constructs indicated (23). B, kidney 293 cells stably transfected with wild type (WT) \( \beta \)APP695, AX, or XB were labeled with \([^{35}S] \)methionine or \([^{32}P] \)orthophosphate for 2 h. Quantification of protein expression and phosphate incorporation in the different forms of \( \beta \)APP were carried out by phosphor imaging. Bars represent means ± S.E. of three independent experiments.

were precipitated with antibody B5, which detects all secreted APP\(_{\beta} \) species. As shown in Fig. 6, APP\(_{\beta\alpha} \)-\( \alpha \), APP\(_{\beta\beta} \)-\( \beta \), and APP\(_{\beta\alpha\beta} \)-\( \beta \) were each secreted as phosphorylated species. Thus, membrane insertion of \( \beta \)APP is not necessary for its phosphorylation, and APP\(_{\beta} \) can be phosphorylated regardless of which \( \beta \)-secretase activity cleaved the precursor, indicating a general cellular mechanism for the ectodomain phosphorylation of mutant and wt \( \beta \)APP.

To determine whether phosphorylation of \( \beta \)APP occurs in the same compartment as the \( \beta \)-secretase cleavage of Swedish \( \beta \)APP (1), we investigated the effect of BFA on phosphorylation of Swedish \( \beta \)APP. BFA is known to cause a collapse of the Golgi network, resulting in a block of forward transport at the cis-Golgi compartment (61). Kidney 293 cells stably transfected with Swedish \( \beta \)APP were metabolically labeled with either \([^{35}S] \)methionine or \([^{32}P] \)orthophosphate in the absence or presence of BFA. Cell lysates were precipitated with antibody C7 (to detect full-length \( \beta \)APP) or antibody 192sw (to detect intracellular APP\(_{\beta\alpha\beta} \)-\( \beta \)), and conditioned media were precipitated with antibody 192sw (to detect secreted APP\(_{\beta\alpha\beta} \)-\( \beta \)). As reported previously, BFA treatment not only inhibited the maturation of full-length \( \beta \)APP but also completely inhibited the generation of intracellular APP\(_{\beta\alpha\beta} \)-\( \beta \) and its secretion (Fig. 7A; Refs. 1 and 44). Treatment with BFA also resulted in an inhibition of \( \beta \)APP ectodomain phosphorylation (Fig. 7B), clearly showing that phosphorylation does not occur within the endoplasmic reticulum or the early Golgi. The trace amounts of phosphorylated species detected after BFA treatment are due to \( \beta \)APP molecules that escaped the BFA block at the beginning of the experiment.

To determine whether ectodomain phosphorylation of \( \beta \)APP occurs within the trans-Golgi network, kidney 293 cells expressing Swedish \( \beta \)APP were incubated at 20 °C. Under such conditions, membrane proteins accumulate within the trans-Golgi network (45). As reported previously (1) incubation at 20 °C resulted in the accumulation of full-length N\(^{\prime} \)- and O\(^{\prime} \)-glycosylated \( \beta \)APP within cell lysates; no APP\(_{\beta\alpha\beta} \)-\( \beta \) was detected in cell lysates or conditioned media (Fig. 8A; Ref. 1). As shown above, after labeling with \([^{32}P] \)orthophosphate at 37 °C, mature phosphorylated \( \beta \)APP was precipitated from cell lysates and phosphorylated APP\(_{\beta\alpha\beta} \)-\( \beta \) from both lysates and media (Fig. 8B). In contrast, incubation of cells at 20 °C completely inhibi-
with antibody C7 to detect full-length wild type and Swedish
were metabolically labeled with [35S]methionine (immunoprecipitations, thus not allowing a quantitative comparison.
[32P]orthophosphate (lysates were precipitated with antibody C7 to visualize full-length
APPs from [35S]methionine-PAGE and radiolabeled proteins visualized by autoradiography. Differences
in the amounts of immunoprecipitated APPs from cell lysates with antibody C7 (Lyase) in the case of wt APP
(A) or with antibody B5 in the case of ΔC-APP (B). Secreted APPw*-α was precipitated from cell supernatants (media) with antibody 1736. The relatively higher amounts of APPw*-α in supernatants from transfectants expressing ΔC-βAPP as compared with that from transfectants expressing wt-βAPP is due to a higher rate of α-secretase cleavage, which is in close agreement with previous results (13, 44). C, phosphorylation of soluble APPw*-α by ectoprotein kinase on the surface of kidney 293 cells. APPw*-α was collected from supernatants of kidney 293 cells stably transfected with a cDNA construct containing a stop codon corresponding to the α-secretase site (compare Fig. 4) for 1 h. The supernatant was taken off and split into two halves. One half was incubated with untransfected kidney 293 cells (+ Cells), and the other half was incubated in a Petri dish without cells (− Cells). Both dishes were incubated for 15 min at 37 °C in the presence of 1 μM [γ-32P]ATP. APPw*-α was immunoprecipitated with antibody 1736 and separated by SDS-PAGE. D, two-dimensional phosphoamino acid analysis of cell surface βAPP, showing that βAPP is exclusively phosphorylated on serine residues by ectoprotein kinase.

FIG. 7. Brefeldin A inhibits phosphorylation of βAPP. Cells transfected with the Swedish (A) or wild type (A, C) cDNA were metabolically labeled with [35S]methionine (A, C) or [35P]orthophosphate (B, D) at 20 or 37 °C. Cell lysates were precipitated with antibody C7 to detect full-length wild type and Swedish βAPP, with antibody 192sw to detect intracellular APPw*-β, or with antibody 1736 to detect intracellular APPw*-α. Conditioned media were precipitated with antibody 192sw to detect secreted APPw*-β or with antibody 1736 to detect APPw*-α. Immunoprecipitates were separated by SDS-PAGE and radiolabeled proteins visualized by autoradiography. Differences in the amounts of immunoprecipitated APPs, from [35S]methionine- and [35P]orthophosphate-labeled cells are due to variabilities during immunoprecipitations, thus not allowing a quantitative comparison.

FIG. 8. Incubating cells at 20 °C inhibits βAPP phosphorylation. Cells transfected with the Swedish (A, B) or wild type (C, D) cDNA were metabolically labeled with [35S]methionine (A, C) or [35P]orthophosphate (B, D) at 20 or 37 °C. Cell lysates were precipitated with antibody C7 to detect full-length wild type and Swedish βAPP, with antibody 192sw to detect intracellular APPw*-β, or with antibody 1736 to detect intracellular APPw*-α. Conditioned media were precipitated with antibody 192sw to detect secreted APPw*-β or with antibody 1736 to detect APPw*-α. Immunoprecipitates were separated by SDS-PAGE and radiolabeled proteins visualized by autoradiography. Differences in the amounts of immunoprecipitated APPs, from [35S]methionine- and [35P]orthophosphate-labeled cells are due to variabilities during immunoprecipitations, thus not allowing a quantitative comparison.

FIG. 9. Cell surface phosphorylation of βAPP by ectoprotein kinase activity. Cell surface proteins of kidney 293 cells stably transfected with wt βAPP cDNA (wt) (A) or with a C-terminal truncated form of βAPP (ΔC) (B) were phosphorylated in the presence of 1 μM [γ-32P]ATP for 20 min at 37 °C. Full-length βAPP was immunoprecipitated from cell lysates with antibody C7 (Lyase) in the case of wt βAPP (A) or with antibody B5 in the case of ΔC-βAPP (B). Secreted APPw*-α was precipitated from cell supernatants (media) with antibody 1736.

Expected doublet of full-length βAPP from cell lysates representing the immature and mature forms of the precursor (Fig. 8C). Precipitation with antibody 1736, which specifically identifies APPw*-α and does not cross-react with full-length βAPP or APPw*-β, results in the detection of intracellular APPw*-α from cell lysates as well as secreted APPw*-α from conditioned media (Fig. 8C). The detection of intracellular APPw*-α is in good agreement with data published previously (51, 59, 60), indicating α-secretase cleavage within the secretory pathway. When cells were incubated at 20 °C, an accumulation of mature βAPP was observed; however, the generation of intracellular APPw*-α and consequently its secretion was completely inhibited (Fig. 8C). When cells were metabolically labeled with [35P]orthophosphate at 37 °C, we detected mature phosphorylated full-length βAPP, and precipitation of cell lysates with antibody 1736, specific for APPw*-α, resulted in the detection of intracellular phosphorylated APPw*-α (Fig. 8D). However, incubating the cells at 20 °C completely inhibited phosphorylation of wild type βAPP; no phosphorylated full-length βAPP or intracellular and secreted APPw*-α was detected (Fig. 8D). Taken together, these data show that intracellular ectodomain phosphorylation of wild type as well as Swedish βAPP occurs within a post-Golgi compartment, most likely within secretory vesicles, suggesting that this compartment represents a general subcellular site of ectodomain phosphorylation of βAPP.

Ectodomain Phosphorylation Can Occur on the Cell Surface—Because mature full-length βAPP is also present at the cell surface, we examined whether membrane-bound βAPP can be a substrate for ectoprotein kinases. Intact kidney cells, transfected with wild type βAPP cDNA, were incubated in the presence of 1 μM [γ-32P]ATP in the cell supernatant, allowing specific detection of ectoprotein kinase activity (30). Full-length βAPP was then precipitated from cell lysates and APPw*-α from cell supernatants. As shown in Fig. 9A (wt) cell surface-bound full-length βAPP was phosphorylated by ectoprotein kinase activity. Moreover, phosphorylated APPw*-α was recovered...
from cell supernatants (Fig. 9A, Media). Similar experiments with kidney 293 cells expressing Swedish βAPP showed that cell surface βAPPsw is also phosphorylated by ectoprotein kinase activity (data not shown). Cell surface phosphorylation was also investigated with cells expressing a C-terminal truncated form of βAPP, which inserts in cell membranes but does not undergo reinternalization (13, 44). As with full-length βAPP (Fig. 9A), the C-terminal truncated form of βAPP was also phosphorylated (Fig. 9B, ΔC), indicating that reinternalization of βAPP is not necessary for its phosphorylation. Again, phosphorylated APPsw-α was recovered from cell supernatants (Fig. 9B, Media). To prove whether phosphorylated APPsw-α does exclusively derive from its phosphorylated precursor or if soluble APPsw-α can be phosphorylated after proteolytic cleavage, cell-free supernatant containing APPsw-α was incubated with [γ-32P]ATP either in the absence or in the presence of untransfected intact kidney 293 cells. As shown in Fig. 9C, APPsw-α was phosphorylated only in the presence of intact cells, indicating that soluble APPsw-α could serve as a substrate for ectoprotein kinase. Thus, neither membrane insertion nor reinternalization is necessary for βAPP phosphorylation. However, βAPP was not phosphorylated in the absence of cells (Fig. 9C, Cells), showing that ectoprotein kinase activity is not cosecreted with βAPP-species. As revealed by two-dimensional phosphoamino acid analysis, phosphorylation of βAPP by ectoprotein kinase occurs exclusively on serine residues (Fig. 9D). The results clearly demonstrate that cell surface-bound βAPP and its soluble derivatives can be phosphorylated by membrane-associated ectoprotein kinase on the surface of intact cells.

**DISCUSSION**

In summary, our data show that full-length βAPP and its α- and β-secretase-cleaved derivatives can be phosphorylated at two different subcellular locations. In both cases, βAPP is exclusively phosphorylated on its ectodomain but not in the cytoplasmic tail. Ectodomain phosphorylation of βAPP has been demonstrated previously (23) and was further supported by the data presented here; βAPP can be phosphorylated on the cell surface by incubating cells with [γ-32P]ATP, and secreted APPs derived from recombinant cDNA constructs with stop codons inserted at the α- and β-secretase site of mutant and wild type βAPP still result in the secretion of phosphorylated APPs. Moreover, APPs incubated with living cells is phosphorylated by a cell surface ectoprotein kinase. Therefore, evidence from multiple experiments proves exclusive ectodomain phosphorylation of βAPP. Intracellular APPs and full-length βAPP molecules are phosphorylated within a post-Golgi compartment, most likely secretory vesicles. This is the cellular compartment to which we have localized the β-secretase activity cleaving Swedish βAPP (1). Therefore, phosphorylation of APP, occurs during or immediately before or after the secretory cleavages of βAPP.

The *in vivo* phosphorylation sites of βAPP were identified as serine residues 198 and 206 by phosphopeptide mapping, site-directed mutagenesis, and mass spectrometry. Moreover, in *in vitro* secreted APP, was detected exclusively in double phosphorylated form. Ser198 is followed by acidic amino acid residues and therefore represents a putative phosphorylation site for CK-2 (63), while Ser206 is preceded by a acidic domain and represents a CK-1 phosphorylation site (64). However, individual mutations of Ser198 and Ser206 differently affected the phosphorylation incorporation. The S198A mutation resulted in a reduction of phosphorylation of about 80%, while the S206A mutation reduced phosphorylation by about 15%. This might be explained by sequential phosphorylation events, in which the first phosphorylation at Ser198 facilitates the subsequent phosphorylation at Ser206 by acetylating this domain. A similar process has been described involving protein kinases A and CK-1 (65, 66).

Interestingly, in addition to the intracellular phosphorylation, our data also demonstrate a second cellular site for phosphorylation of membrane-bound βAPP: an ectoprotein kinase activity at the cell surface. In contrast to the intracellular phosphorylation of βAPP, which appears to be a constitutive event (23), phosphorylation by ectoprotein kinases could represent a regulated mechanism. Because ATP is known to be released into the extracellular environment by a variety of cellular stimuli (for review see Refs. 67 and 68), the availability of this cosubstrate for ectoprotein kinases could represent a biological regulation mechanism for phosphorylation of cell surface βAPP. Since α-secretase activity is present within cell lysates (51, 59, 60), as well as on the cell surface (12, 62), full-length surface βAPP will contribute to the pool of phosphorylated APP molecules in conditioned media. In addition, secreted derivatives of βAPP (APPsw-α and APPsw-β) released by α- or β-secretase into the cell supernatant also serve as substrates for ectoprotein kinase. Our study demonstrates for the first time the unusual phenomenon that βAPP and its principal secreted derivatives can undergo selective ectodomain phosphorylation at two distinct subcellular locations. It will now be important to determine whether both mechanisms result in the phosphorylation of identical amino acid residues or if βAPP is phosphorylated by different protein kinases on two or more sites within the same molecule. The functional consequences of this complex regulation of βAPP ectodomain phosphorylation are unknown so far. However, one might speculate that extracellular function(s) of βAPP, e.g. the modulation of neuronal excitability by APPs (69), could be regulated by selective ectodomain phosphorylation.

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