The Protease Inhibitor, MG132, Blocks Maturation of the Amyloid Precursor Protein Swedish Mutant Preventing Cleavage by β-Secretase*

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Received for publication, September 26, 2000, and in revised form, November 16, 2000
Published, JBC Papers in Press, November 17, 2000
DOI 10.1074/jbc.M008793200

Amyloid (Aβ) peptides found aggregated into plaques in Alzheimer's disease are derived from the sequential cleavage of the amyloid precursor protein (APP) first by β- and then by γ-secretases. Peptide aldehydes, which inhibit cysteine proteases and proteasomes, reportedly block Aβ peptide secretion by interfering with γ-secretase cleavage. Using a novel, specific, and sensitive enzyme-linked immunosorbent assay for the β-secretase-cleaved fragment of the Swedish mutant of APP (APPSw), we determined that the peptide aldehyde, MG132, prevented β-secretase cleavage. This block in β-secretase cleavage was not observed with clasto-lactacystin β-lactone and thus, cannot be attributed to proteasomal inhibition. MG132 inhibition of β-secretase cleavage was compared with the serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF). AEBSF inhibition of β-secretase cleavage was immediate and did not affect α-secretase cleavage. With MG132, inhibition was delayed and it decreased secretion of α-cleaved APPSw as well. Furthermore, MG132 treatment impaired maturation of full-length APPSw. Both inhibited intracellular formation of the β-cleaved product. These results suggest that peptide aldehydes such as MG132 have multiple effects on the maturation and processing of APP. We conclude that the MG132-induced decrease in β-secretase cleavage of APPSw is due to a block in maturation. This is sufficient to explain the previously reported peptide aldehyde-induced decrease in Aβ peptide secretion.

Evidence continues to accumulate supporting the hypothesis that amyloid plaques in the brain have a causative role in the generation of Alzheimer's disease (for review, see Ref. 1). Increased brain levels of amyloid peptide and cognitive decline are strongly correlated (2). Amyloid plaques largely consist of peptides of 40 (Aβ40)3 and 42 (Aβ42) amino acids in length that

are derived by the enzymatic processing of a type I transmembrane protein called amyloid precursor protein (APP). Two enzymatic cleavages of APP are necessary to produce amyloid peptides. First, β-secretase cleaves APP to create the amino-terminal end of the peptide. A double mutation (K651N/M652L; 751 isoforming number) just amino-terminal to this β-secretase cleavage site has been identified in a Swedish pedigree of familial Alzheimer's disease (3). This double mutation of APP, known as the “Swedish” mutation (APPSw), elevates intracellular and secreted levels of Aβ peptide from 6- to 8-fold (4). This appears to be a consequence of increased cleavage of APPSw by β-secretase compared with wild type APP. Following β-secretase cleavage, γ-secretase subsequently cleaves the COOH-terminal membrane-bound fragment (CTF) of APP within the transmembrane sequence to release the Aβ peptide. Thus, inhibitors that specifically block the cleavage of APP by these secretases have enormous therapeutic potential.

Several laboratories have now cloned an enzyme that cleaves APP and APPSw at the β-secretase site (5–7). Referred to as BACE (β-site APP cleaving enzyme), this enzyme is a member of a unique family of transmembrane aspartic proteases. A second related protein designated ASPI1 or BACE2 has also been identified (6, 8, 9). However, the expression pattern of BACE2 in the brain appears to exclude it from playing a major role in Alzheimer's disease (9). The mature, fully glycosylated form of BACE has a half-life in the cell of greater than 9 h (10). BACE appears to localize to the Golgi apparatus (5). Despite about 40% amino acid similarity between BACE and pepsin proteases (9), the cysteine residues in BACE involved in intramolecular disulfide bonds are not conserved with other pepsin family members (10). Such fundamental structural differences may explain why β-secretase is insensitive to pepstatin, a specific inhibitor of pepsin proteases (7). The search for such a specific inhibitor of β-secretase cleavage of APP as a possible treatment for Alzheimer's disease has intensified with the discovery of BACE.

Before the cloning of the aspartic protease, BACE, cellular studies using a serine protease inhibitor, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF), suggested that it

protein; APPSw, APP bearing the Swedish mutation (K651N/M652L); BACE, β-site APP-cleaving enzyme; ELISA, enzyme-linked immunosorbent assay; APPSwβ, soluble β-secretase-cleaved APPSw fragment; iAPPSwβ, intracellular soluble β-secretase-cleaved APPSw fragment; APPSwα, soluble α-secretase-cleaved APPSw fragment; APPα, soluble α-secretase-cleaved APP fragment; APPβ, soluble β-secretase-cleaved APP fragment; CHO, Chinese hamster ovary; HEK293, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus Tween 20; ER, endoplasmic reticulum; BFA, brefeldin A.

* This work was supported in part by a Michigan Alzheimer's Disease Research Center pilot grant (to J. R. G.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Recipient of an Institute of Gerontology training fellowship (through Grant T32AG000114 from NIA, National Institutes of Health).

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3 The abbreviations used are: Aβ peptide, a 40- or 42-amino acid peptide derived from APP; p3, peptide derived from α- and γ-secretase cleavage of APP; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; CTF, COOH-terminal fragment; APP, amyloid precursor protein; APPSw, APP bearing the Swedish mutation (K651N/M652L); BACE, β-site APP-cleaving enzyme; ELISA, enzyme-linked immunosorbent assay; APPSwβ, soluble β-secretase-cleaved APPSw fragment; iAPPSwβ, intracellular soluble β-secretase-cleaved APPSw fragment; APPSwα, soluble α-secretase-cleaved APPSw fragment; APPα, soluble α-secretase-cleaved APP fragment; APPβ, soluble β-secretase-cleaved APP fragment; CHO, Chinese hamster ovary; HEK293, human embryonic kidney; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline plus Tween 20; ER, endoplasmic reticulum; BFA, brefeldin A.

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MG132 Block in Maturation Prevents APPSw β-Secretase Cleavage

MG132, a proteasomal inhibitor, was one of the most potent inhibitors of Aβase activity (11). There is little information published about Aβ peptide secretion (12–14). Of these peptide aldehydes, MG132 was one of the most potent inhibitors of Aβ peptide secretion (15). Studies focusing specifically on the secretion of Aβ40 and Aβ42 revealed peptide aldehydes to have a complex effect on APP processing. Curiously, at low concentrations, these peptide aldehydes produced an increase in Aβ40 and Aβ42 peptide secretion, whereas, at higher concentrations, a decrease in Aβ peptide secretion was observed (14–17). The increase in Aβ peptide secretion at low concentrations of peptide aldehydes has been postulated to result from inhibition of degradation of the CTFs generated by BACE cleavage, making more of them available for γ-secretase cleavage (17). The inhibition of Aβ peptide secretion with higher concentrations of peptide aldehydes is attributed to an impairment of γ-secretase cleavage of the CTFs (12, 13, 17, 18).

By developing an ELISA specific for the soluble β-secretase-cleaved amino-terminal fragment of APPSw (APPStβ; see Fig. 1A), we now report that the peptide aldehyde, MG132, prevents β-secretase cleavage of APPSw in a concentration-dependent manner. Furthermore, MG132 is not inhibiting secretion of APPSwβ into the medium since it blocked intracellular production of APPSwβ as well. MG132 is compared with AEBSF and the specific proteasomal inhibitor, clasto-lactacystin β-lactone, on β-secretase cleavage. MG132 impairs maturation, blocking β-secretase cleavage of APPSw in the late Golgi apparatus. This offers an alternative explanation as to how higher concentrations of a peptide aldehyde can decrease Aβ peptide secretion.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Protease Inhibitors**—The mouse monoclonal antibodies referred to in Fig. 1A as 22C11 and BIOSOURCE were obtained from Roche Molecular Biochemicals and BIOSOURCE International (monoclonal antibody P2-1), respectively. The mouse monoclonal antibody 8E5, which recognizes the first 19 amino acids of the Aβ peptide, was obtained from Senetek, Inc. The anti-APP COOH-terminal rabbit polyclonal antibody was obtained from Chemicon International, Inc., and the mouse monoclonal antibody to the amino terminus of APP, LN27, was purchased from Zymed Laboratories Inc. The mouse monoclonal antibody, 8E5, was a generous gift from Dr. Dale Schonback (Schonback Pharmaceuticals). The rabbit polyclonal antibody 6E4, was raised against a synthetic peptide corresponding to the last 19 amino acid residues of the carboxyl terminus of APP (CMQQNGY-ENPTYKFFEQMQN) that was cross-linked to keyhole limpet hemocyanin via an amino-terminal cysteine. The polyclonal antibodies, 931 and 932, were raised against a synthetic peptide corresponding to the last 19 nonessential amino acids, and penicillin/streptomycin/fungizone as described (19). The proteins were then washed and incubated in complete medium containing excess methionine and cysteine for the chase times shown. Pulse-labeling and chase were complete, the conditioned medium was collected and cells washed in PBS. Cells were lysed in 1 ml of lysis buffer (0.5% Nonidet P-40, 0.5% deoxycholate in 50 mM Tris, 150 mM NaCl, and 5 mM EDTA, pH 8.0) and insoluble cell debris removed by centrifugation as described (19). The resulting cleared supernatant of the cell lysate was then subjected to further analysis.

**Immunoprecipitation, Immunoblotting, and Gel Electrophoresis**—Full-length APP and APPSw were isolated from the cell lysate supernatants by immunoprecipitation using the 945 rabbit antisera to the carboxyl terminus of APP. Except where noted, lysates were incubated with 4 µl of 945 antisera for 90 min at 4 °C and protein-antibody complexes were isolated by incubation with protein A-Sepharose for 30 min at 4 °C. Intracellular APPSwβ was similarly isolated from cell lysates with 4 µl of 931 rabbit antisera. The 931 antisera was also used to isolate secreted APPSwβ from 0.5 ml of conditioned medium that had Nonidet P-40 and deoxycholate added to a final concentration of 0.5%. Immunoprecipitations using the mouse monoclonal antibodies, 8E5 or 6E10, were conducted as described above except that protein-antibody complexes were isolated using protein G-agarose (Roche Molecular Biochemicals).

Isolated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using an 8% separating gel. Radiolabeled proteins in SDS gels were detected by fluorography using Amplify (Amersham Pharmacia Biotech). Immunoblot analysis of isolated proteins was conducted essentially as described (19). Briefly, immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PROTRAN membrane (Amersham Pharmacia Biotech). The membranes were then washed and incubated with a sheep anti-mouse IgG antibody conjugated to horse-radish peroxidase as described by the manufacturer (Amersham Pharmacia Biotech). The membranes were again washed and signals detected by chemiluminescence using the ECL system (Amersham Pharmacia Biotech).

**Metabolic and Pulse-Chase Labeling**—Cells stably or transiently expressing APP or APPSw were preincubated in methionine/cysteine-free medium for 15 min prior to labeling. Cells were metabolically labeled by incubating in 2 ml of medium containing [35S]methionine and [35S]cysteine (Tran35S-label; ICN Pharmaceuticals) at 50 µCi/ml for 1 h. In pulse-chase studies, cells were incubated in methionine/cysteine-free medium for 15 min and then pulsed for 12 min with Tran35S-label (100 µCi/ml). Cells were then washed and incubated in complete medium containing excess methionine and cysteine for the chase times shown. Pulse-labeling and chase were complete, the conditioned medium was collected and cells washed in PBS. Cells were lysed in 1 ml of lysis buffer (0.5% Nonidet P-40, 0.5% deoxycholate in 50 mM Tris, 150 mM NaCl, and 5 mM EDTA, pH 8.0) and insoluble cell debris removed by centrifugation as described (19). The resulting cleared supernatant of the cell lysate was then subjected to further analysis.

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Isolated proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) using an 8% separating gel. Radiolabeled proteins in SDS gels were detected by fluorography using Amplify (Amersham Pharmacia Biotech). Immunoblot analysis of isolated proteins was conducted essentially as described (19). Briefly, immunoprecipitated proteins were resolved by SDS-PAGE and transferred to PROTRAN membrane (Schleicher & Schuell). Membranes were subsequently blocked in gelatin wash buffer (0.1% gelatin, 15 mM Tris, pH 7.5, 130 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100). Membranes were subsequently incubated with mouse monoclonal antibody 22C11 to detect the aminoterminal end of APP molecules from cell lysates or conditioned medium. Membranes were alternatively incubated with 6E10 to detect APPSwA in conditioned medium. Membranes were subsequently washed and incubated with a sheep anti-mouse IgG antibody conjugated to horseradish peroxidase as described by the manufacturer (Amersham Pharmacia Biotech). The membranes were again washed and signals detected by chemiluminescence using the ECL system (Amersham Pharmacia Biotech).

**Full-length APP and APPSw Sandwich ELISAs**—The 945 and 931 antibodies were used as capture antibodies for the full-length APP and APPSw Sandwich ELISAs, respectively. Each full-length APPSw was purified against the appropriate peptide that had been cross-linked to CNBr-activated Sepharose 4B (Amersham Pharmacia Biotech) following the manufacturer’s instructions. The anti-APP COOH-terminal antibody, 945, was affinity-purified with the same 20-amino acid synthetic peptide used above to inoculate rabbits. The 931 antibody was affinity-purified using a synthetic peptide identical to the last seven amino acids of the neoepitope of the β-secretase-cleaved APPSw soluble fragment (CEIESVNL). The 945 and 931 antibodies that bound to the immobilized peptide were eluted using ActiSep Elution Medium (Stegene). The eluted antibodies were desalted using a PD-10 column (Amersham Pharmacia Biotech) as directed by the manufacturers. The affinity-purified capture antibodies (945 and 931) were diluted to 1.2 µg/ml in PBS and 100 µl added per well to a 96-well Nunc-Immuno Maxisorp plate (Nalge Nunc International). After incubation to allow binding of antibody, SuperBlock (Pierce) was added to each well to block nonspecific binding sites. The wells were repeatedly rinsed and then stored at 4 °C in PBS, 0.05% Tween 20 (PBST) until ready for use.

For the full-length APP ELISA, the protein concentration of the cell lysate supernatant was determined using the BCA protein assay (Pierce). Ten micrograms of protein from each well were incubated into each well. The total volume in the well was adjusted to 100 µl with PBS, and samples were incubated overnight at 4 °C. On the following day, the samples were incubated an additional 1 h with constant shaking. The wells were then washed four times with PBS/T and then incubated with 100 µl of diluted detector antibody (the mouse monoclonal antibody, 8E5, at 0.25 µg/ml except where noted). All antibodies were...
Antisera raised in two rabbits (931 and 932) against APPSw specifically immunoprecipitates the PAGE. The resulting autoradiograph shows that 945 specifically recognizes only the neoepitope derived with \( \beta \)-secretase cleavage of APPSw. Although both APP\( \alpha \) and APPSw were detected in the 8E5 immunoprecipitates, neither form was immunoprecipitated using the 931 antibody (Fig. 2C, panel 1). Taken together, these results demonstrate that the 931 antibody specifically recognizes only the neoepitope derived with \( \beta \)-secretase cleavage of APPSw. It does not cross-react with full-length APPSw (compare Fig. 4, A and C, chase at 0 min), soluble APP\( \alpha \), APP\( \beta \), or APPSwa.

Characterization of ELISAs for Detection of Full-length APP and the Soluble \( \beta \)-Secretase Cleaved APPSw Amino-terminal Fragment—Since 931 specifically recognized the neoepitope of \( \beta \)-secretase-cleaved APPSw in conditioned medium, it was used to create an enzyme-linked immunosorbent assay (ELISA) to quantitatively measure the amount of secreted APPSw. A similar ELISA was also developed to measure the amount of full-length APP or APPSw present in cell lysates using 945. To identify the best detector antibody to use in the ELISA, the affinity-purified 945 was coated on 96-well plates to capture the \( \beta \)-secretase-cleaved protein and just 25 \( \mu l \) of conditioned medium was loaded per well. Each experiment was repeated at least three times, and the indicated values are averages of triplicate measurements \( \pm \) S.D.

RESULTS

Characterization of Polyclonal Antibodies to APP and APPSw—As shown in Fig. 1, \( \beta \)-secretase cleavage of APPSw generates a large soluble amino-terminal fragment (APPSw\( \beta \)) that is secreted into the medium and a CTF that is subsequently cleaved by \( \gamma \)-secretase to derive the \( \beta \) peptide. Two novel rabbit polyclonal antisera were generated to perform the experiments described below. The binding sites of these and other antibodies are also shown in Fig. 1. The first polyclonal antiserum (945) was raised to the last 20 amino acids of the APP carboxyl terminus. The specificity of the anti-COOH-terminal antibody, 945, is shown in Fig. 2A. CHO cells stably expressing APPSw (CHOAPPSw) were metabolically labeled for 1 h with \( ^{3}S \)-label. Following cell lysis, equal amounts of supernatant were incubated with 1, 2, or 4 \( \mu l \) of 945 antisera or with 4 \( \mu l \) of preimmune serum followed by protein A-Sepharose. The immunoprecipitated APPSw was compared with that obtained using the Chemicon anti-COOH-terminal antibody by SDS-PAGE. The resulting autoradiograph shows that 945 specifically immunoprecipitates the N-glycosylated immature (I) and completely glycosylated mature (M) forms of APPSw.

Characterization of ELISAs for Detection of Full-length APP and the Soluble \( \beta \)-Secretase Cleaved APPSw Amino-terminal Fragment—Since 931 specifically recognized the neoepitope of \( \beta \)-secretase-cleaved APPSw in conditioned medium, it was used to create an enzyme-linked immunosorbent assay (ELISA) to quantitatively measure the amount of secreted APPSw. A similar ELISA was also developed to measure the amount of full-length APP or APPSw present in cell lysates using 945. To identify the best detector antibody to use in the ELISA, the affinity-purified 945 was coated on 96-well plates to capture full-length APP from cell lysates of either CHO cells or CHO cells stably expressing APPSw. After rinsing, triplicate wells were incubated with no detector, 8E5 (0.25 \( \mu g/ml \)), BIOSOURCE (0.25 \( \mu g/ml \)), or LN27 (0.5 \( \mu g/ml \)) mouse monoclonal antibodies and developed as described under “Experimental Procedures.” The signal level observed using CHO cell lysates with these detector antibodies did not significantly differ from background, demonstrating the specificity of the ELISA for the stably expressed, human APPSw (Fig. 2D). Full-length APPSw was specifically detected in CHOAPPSw lysates with 8E5 and the BIOSOURCE mouse monoclonal antibodies showing the
When using 6E10 as the detector antibody, no signal above background was observed (data not shown). This indicated that when using 6E10 as the detector antibody, no signal above background was observed (data not shown). This indicated that 8E5 and BIOSOURCE monoclonal antibodies that recognize epitopes in the lumenal region of human APP were screened as detector antibodies. Lysates of CHO cells expressing only endogenous hamster APP served as controls for specificity.

Two newly derived rabbit polyclonal antisera (931 and 932) were used to immunoprecipitate APP soluble fragments from conditioned medium collected from CHOAPPSw cells. This was compared with APP fragments immunoprecipitated by the 8E5 antibody by immunoblotting with 22C11, which recognizes both APPSw and APPβ fragments. C, immunoblot analysis indicates that the rabbit polyclonal antisera 931 specifically immunoprecipitates only APPSwβ from conditioned media. Conditioned media collected from HEK293 cells transiently transfected with either pCDAPP or pCDAPPSw were incubated with either 8E5 or 931 and protein-antibody complexes immunoabsorbed using protein G- or protein A-agarose, respectively. Duplicate samples of immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose.

Fig. 2. Characterization of novel polyclonal antibodies. A, the ability of 945 to immunoprecipitate full-length APPSw was compared with the Chemicon rabbit polyclonal antibody to the carboxyl terminus of APP. CHOAPPSw cells were metabolically labeled with [35S]cysteine and [35S]methionine for 1 h. Cells were lysed and equal aliquots of cleared supernatant incubated with the indicated amounts of polyclonal antisera or with 4 μl of preimmune serum followed by protein A-Sepharose. M and I, respectively, refer to the mature, fully glycosylated and immature, N-glycosylated forms of APPSw. B, immunoblot analysis of proteins specifically immunoprecipitated from conditioned medium of CHOAPPSw cells. Two newly derived rabbit polyclonal antisera (931 and 932) were used to immunoprecipitate APP soluble fragments from conditioned medium collected from CHOAPPSw cells. This was compared with APP fragments immunoprecipitated by the 8E5 antibody by immunoblotting with 22C11, which recognizes both APPSw and APPβ fragments. C, immunoblot analysis indicates that the rabbit polyclonal antisera 931 specifically immunoprecipitates only APPSwβ from conditioned media. Conditioned media collected from HEK293 cells transiently transfected with either pCDAPP or pCDAPPSw were incubated with either 8E5 or 931 and protein-antibody complexes immunoabsorbed using protein G- or protein A-agarose, respectively. Duplicate samples of immunoprecipitated proteins were resolved by SDS-PAGE and transferred to nitrocellulose. Panel 1, one set of samples was immunoblotted with the 22C11 mouse monoclonal antibody to detect secreted APPβs, APPSwβ, and APPSwβ. Panel 2, a duplicate set of immunoprecipitated samples was immunoblotted for APPβs and APPSwβ using the mouse monoclonal antibody, 6E10. D, two ELISAs described under “Experimental Procedures” were derived to measure APPSwβ in conditioned media and full-length APP or APPSwβ in cell lysates. Affinity-purified 945 antibody was used to capture full-length APPSw molecules from the lysate of CHOAPPSw cells. Three different mouse monoclonal antibodies that recognize epitopes in the lumenal region of human APP were screened as detector antibodies. Lysates of CHO cells expressing only endogenous hamster APP served as controls for specificity.

E, affinity-purified 945 antibody was used to capture secreted APPSwβ molecules from the conditioned media of CHOAPPSw cells. CHO cell conditioned medium served as a negative control. Both of the anti-APP lumenal domain antibodies, 8E5 and BIOSOURCE, were effective detector antibodies. Addition of the CEIESVNL peptide to the CHOAPPSw conditioned medium prevented detection of secreted APPSwβ.

The 8E5 and BIOSOURCE mouse monoclonal antibodies were also evaluated as detector antibodies in the 931 ELISA to measure secreted APPSwβ. Both 8E5 and BIOSOURCE antibodies were sensitive detectors of APPSwβ captured in wells coated with affinity-purified 931 (Fig. 2E). The addition of the eight-amino acid synthetic peptide corresponding to the carboxyl terminus of APPSwβ to the conditioned medium from CHOAPPSw cells blocked detection of the β-cleaved product. When using 6E10 as the detector antibody, no signal above background was observed (data not shown). This indicated that no APPSw was captured in the ELISA by the affinity-purified 931. Together, these results demonstrate the high specificity of this novel ELISA for APPSwβ.

Intracellular APPSwβ Is Detected by Pulse-Chase Analysis—Experiments were next conducted to determine whether 931 recognized intracellular APPSwβ. Intracellular APPSwβ was detectable in the cell lysate as a slightly diffuse band migrating just above a nonspecific band (Fig. 3A, panel 2). This nonspecific 35S-labeled band was detected in both CHOAPP and CHOAPPSw cell lysates (data not shown). Therefore, an additional experiment was conducted to verify that this protein was not recognized by antibodies specific for the APPSwβ neo-epitope. CHOAPPSw cells were pulse-labeled with Tran35S-label for 12 min and chased for 45 min as described under “Experimental Procedures.” Conditioned media and cell lysates were isolated and divided in half. An excess of a synthetic peptide with a sequence corresponding to the last eight amino acids of the neoepitope (EISEVKNL) in APPSwβ was added to one set of samples. Secreted and intracellular APPSwβ were then immunoprecipitated using 931 from conditioned media and cell lysates, respectively. Full-length APPSwβ was subsequently immunoprecipitated from the same cell lysates using 945. Full-length APPSwβ was still immunoprecipitated in the presence of the neoepitope peptide, indicating that the peptide was not causing a general block in the immunoprecipitation of proteins (Fig. 3A, panel 1). However, the synthetic neoepitope peptide blocked immunoprecipitation of secreted APPSwβ (Fig. 3A, panel 2) and intracellular APPSwβ (Fig. 3A, panel 3) using 931 antibody. In contrast, the nonspecific band was still detectable in the presence of synthetic peptide. Brefeldin A (BFA) treatment of HEK293 cells to block transport of proteins beyond the
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Fig. 3. Immunoprecipitation of radiolabeled intracellular and secreted APPSwβ is blocked by a synthetic peptide of the Swedish mutant and brefeldin A treatment. A, CHOAPPSw cells were pulse labeled as described and chased for 45 min in complete DMEM. Conditioned media and cell lysates were isolated and divided in half. 931 was used to immunoprecipitate secreted and intracellular APPSwβ in the presence or absence of excess synthetic peptide corresponding in sequence to the carboxyl terminus of APPSwβ (CEIESVNL). The excess peptide did not interfere with immunoprecipitation of full-length APPSw with 945; 2, the CEIESVNL peptide blocked immunoprecipitation of iAPPβ (indicated by an arrow), but not the nonspecific protein just below it. 3, secreted APPSwβ was not immunoprecipitated by 931 when the CEIESVNL peptide was added to the conditioned medium. B, CHOAPPSw cells were metabolically labeled for 1 h with Tran35S-label in the presence (+) or absence (−) of 20 μg/ml brefeldin A (BFA). Conditioned medium was collected and cell lysates isolated as described. Full-length APPSw was immunoprecipitated with 945 antibodies (panel 1). Intracellular (indicated by arrow in panel 2) and secreted (panel 3) APPSwβ were immunoprecipitated from cell lysate and conditioned media, respectively, with 931 antibodies.

Golgi apparatus inhibited β-secretase cleavage of APPSw (20, 22). Thus, a block in maturation of APPSw by BFA does not permit access to BACE in the late Golgi. We sought to confirm these findings using the 931 antisera. CHOAPPSw cells were metabolically labeled with Tran35S-label for 1 h in the presence or absence of 20 μg/ml brefeldin A (BFA). Labeled conditioned medium was collected and cells lysed as above. Intracellular and secreted APPSwβ were isolated by immunoprecipitation using the 931 antisera from cell lysates and conditioned media, respectively. Full-length APPSw was subsequently immunoprecipitated from cell lysates using the 945 antibody. As expected, BFA treatment blocked the appearance of APPSwβ in the conditioned medium (Fig. 3B, panel 3). Completely glycosylated, mature full-length APPSw was not detected in BFA-treated cell lysates indicating that maturation was blocked (panel 1). Similar to earlier reports (20, 22), our studies found that intracellular APPSwβ was not detected in CHOAPPSw cells treated with BFA, yet the nonspecific band was still detectable (Fig. 3B, panel 2). Thus, blocking maturation of APPSw prevents it from being cleaved by β-secretase.

Taken together, the results described above indicated that iAPPβ is detectable in the lysates of radiolabeled CHOAPPSw cells. Consequently, a pulse-chase study was conducted to evaluate the time course of APPSwβ production and secretion by CHOAPPSw cells. Cells were pulsed for 12 min with Tran35S-label and subsequently chased in complete medium containing excess methionine and cysteine for 0–90 min. At each indicated chase time, conditioned medium was collected and the PBS-washed cells were lysed. Intracellular APPSwβ was immunoprecipitated from the cleared supernatants using 931. Full-length APPSw was subsequently immunoprecipitated from the conditioned media with 931. These radiolabeled proteins were resolved by SDS-PAGE, and the resulting autoradiographs are shown in Fig. 4. A 12-min pulse was sufficient to label N-glycosylated APPSw (I in Fig. 4C) but not fully glycosylated, mature APPSw (M). Mature APPSw was detectable after 7.5 min of chase and peaked after 25–35 min. After a 90-min chase, no mature APPSw was detectable and only a trace of immature protein was still present. Note that a large portion of APPSw fails to chase into the fully mature form over the chase period, but instead appears to remain as incompletely glycosylated APPSw. This may represent misfolded protein that is retained in the ER for eventual degradation by the proteasome in these APPSw-overexpressing cells. Fig. 4A shows that iAPPβ was readily detectable after chasing for about 25 min and corresponded with the appearance of the completely glycosylated, mature APPSw. The level of iAPPβ reached a maximum at 35–45 min and was barely detectable after 90 min of chase. Thus, as expected, the appearance of iAPPβ coincides with that observed for the mature form of full-length APPSw in cell lysates.
creases APPα and intracellular and secreted Aβ42 (18, 23, 24). Other investigators reported that lactacystin increased secretion of both Aβ40 and Aβ42 from SH-SYSY cells (14). The proposed explanation for these increases is that inhibition of the proteasome prevents degradation of both full-length and COOH-terminal fragments of APP making more of each available for cleavage by the α- and γ-secretases, respectively (18). We reasoned that, if lactacystin disrupted ER-associated degradation of APPSw in a manner similar to other transmembrane proteins (25–28), then more full-length protein would also be available for cleavage by β-secretase. To test this hypothesis, CHOAPPSw cells were incubated for 6 h with increasing concentrations of the active form of lactacystin, clasto-lactacystin β-lactone (β-lactone). This cell-permeable, irreversible inhibitor of the proteasome has a 5–10 fold greater potency than lactacystin with an IC50 in intact cells of 1 μM (29). ELISA measurements of secreted APPSwβ revealed that a concentration of 10 times the IC50 (i.e. 10 μM) still had no effect on the levels of secreted APPSwβ (Fig. 5B). Only at a concentration of 20 μM β-lactone was a decrease in β-secretase-cleaved APPSw detectable (about 85% of control levels). Thus, proteasomal inhibition has little influence on β-secretase cleavage of APPSw.

Intriguingly, peptide aldehyde protease inhibitors, which are capable of inhibiting both the proteasome and cysteine proteases, increase Aβ peptide secretion at low concentrations and inhibit β-secretion at high concentrations (13–17). MG132 was one of the most potent peptide aldehyde inhibitors of Aβ peptide secretion (15, 18). We hypothesized that MG132 was inhibiting Aβ peptide secretion at high concentrations by interfering with β-secretase cleavage of APP. To test this hypothesis, CHOAPPSw cells were incubated for 6 h with MG132 at concentrations demonstrated to inhibit Aβ peptide secretion (20–100 μM). The 931 ELISA was used to quantify the amount of APPSwβ secreted by MG132-treated cells and compared with untreated controls. MG132 treatment produced a concentration-dependent decrease in the levels of APPSwβ detected in the conditioned media (Fig. 6A). Cells incubated with 100 μM MG132 secreted APPSwβ at 35% the level secreted by untreated cells. As a control, MG132 was added to a final concentration of 100 μM following collection of media that had been conditioned for 6 h by untreated cells (100+). The level of APPSwβ was identical to untreated samples, indicating that MG132 was not interfering with the ELISA itself. To determine whether MG132 was inhibiting APPSwβ levels by decreasing the levels of full-length APPSw, the 945 ELISA was performed on equal amounts of lysates of the treated cells. MG132 inhibits secretion of APPSwβ in a time-dependent manner. CHOAPPSw cells were incubated with either MeSO vehicle (solid line) or 80 μM MG132 (dashed line) for 0, 0.5, 1, 2, 4, 6, or 8 h. Conditioned medium was collected and the levels of secreted APPSwβ determined by ELISA.

![FIG. 5. The effects of protease inhibitors on APPSwβ secretion in CHOAPPSw cells.](image)

A. CHOAPPSw cells were incubated for 5 h in complete DMEM containing either vehicle or 0.1–1.0 mM AEBSF, a serine protease inhibitor. Conditioned medium was collected and analyzed by ELISA for APPSwβ secretion. B. CHOAPPSw cells were incubated for 6 h with vehicle or 1–20 μM clasto-lactacystin β-lactone, a specific inhibitor of the proteasome. Secreted APPSwβ levels were measured in triplicate by ELISA.

![FIG. 6. The peptide aldehyde, MG132, inhibits β-secretase cleavage of APPSw in stably transfected CHO cells.](image)

A. CHOAPPSw cells were incubated for 6 h with MeSO vehicle or 20–100 μM MG132. Conditioned medium was collected and analyzed for secreted APPSwβ by ELISA. To control for any interference with the ELISA, MG132 was added to a final concentration of 100 μM after CHOAPPSw cells had conditioned medium for 6 h (white bar labeled 100+). B. An ELISA was also conducted on 10-μg aliquots of CHOAPPSw cell lysates to determine whether MG132 treatment altered levels of APPSw in the cells. C. MG132 inhibits secretion of APPSwβ in a time-dependent manner. CHOAPPSw cells were incubated with either MeSO vehicle (solid line) or 80 μM MG132 (dashed line) for 0, 0.5, 1, 2, 4, 6, or 8 h. Conditioned medium was collected and the levels of secreted APPSwβ determined by ELISA.
MG132 Block in Maturation Prevents APPSw β-Secretase Cleavage

**DISCUSSION**

In previous studies, peptide aldehydes such as MG132 were thought to inhibit Aβ and p3 peptide secretion by blocking γ-secretase cleavage of the COOH-terminal fragment of APP (12, 13, 17, 18). This conclusion was based on the observation that CTFs were detectable in cells incubated with peptide aldehydes. Our data suggest that peptide aldehydes, such as MG132, have a more widespread effect on APP maturation, processing and secretion. First and foremost, β-secretase cleavage of APPSw was blocked at concentrations of MG132 reported to impair Aβ peptide secretion (15, 18). This inhibition in β-secretase cleavage is sufficient to account for the reported decrease in Aβ peptide secretion. Second, we demonstrated that, after 2 h of incubation with MG132, secretion of APPSw was also inhibited. Finally, in pulse-chase studies on CHOAPPSw cells preincubated with MG132, fully mature APPSw was barely detectable in the cell lysates. Instead, the immature, N-glycosylated form that is located in the ER was stabilized, suggesting MG132 incubation has multiple effects on APPSw processing. Like Skovronsky et al. (18), we were unable to detect stabilization of mature APPSw and inhibition of secreted APPSwa and APPSβ, when cells were only incubated with MG132 during the chase period. However, our studies, in which a 2-h preincubation with MG132 blocked production of iAPPSβ, may better represent the effects of peptide aldehydes on Aβ peptide secretion over the long incubation period used by these and other investigators (12–18).

Our results on MG132-induced inhibition of β-secretase cleavage along with results of others describing the stabilization of CTFs in cell lysates by such peptide aldehydes can be best explained if treatment with these compounds is somehow impeding progression of APPSw through the secretory pathway. Haass et al. (30) have shown that impairing maturation of APPSw by treating cells with BFA prevents production of in-
tracellular APPSwβ because transport to the site of β-secretase cleavage is blocked. We obtained the same results using the 931 antisera described here. Other investigators achieved similar results by blocking movement of APP from the endoplasmic reticulum (ER). In those studies, a dilysine ER retrieval motif introduced into the cytoplasmic tail of APP strongly impaired cleavage by β-secretase (31). We, again, have obtained equivalent results expressing APPSw bearing a dilysine ER retrieval motif in CHO cells.2 Like BFA treatment or introduction of a dilysine retrieval motif, a 2-h MG132 treatment of CHO cells stably expressing APPSw blocked the maturation of the full-length molecule in experiments described here. This, in turn, blocked production of intracellular APPSwβ and its subsequent appearance in the media. We suggest that the increase in the CS3 and C99 CTFs observed by others with MG132 treatment results from a similar block in access of these α- and β-secretase-cleaved fragments to γ-secretase. Regardless of the mechanism, our results showing that MG132 blocks β-secretase cleavage of APPSw call into question the utility of such peptide aldehydes as γ-secretase inhibitors.

The 931 antibody, described here for the first time, specifically recognized APPSwβ and did not cross-react with full-length APPSw, APPSwα, APPβ, or APPo. This enabled us to detect intracellular APPSwβ in stably transfected CHOAPPSw cells. The results observed in our pulse-chase studies agree closely with previous findings of APPSw processing in HEK293 cells (22). Intracellular APPSwβ was detectable within 25 min in pulse-chase experiments and coincided with the appearance of mature APPSw. Furthermore, we clearly detected APPSwβ intracellularly prior to its appearance in the medium, eliminating the possibility that the APPSwβ detected in cell lysates was due to already secreted β-secreted fragments associated with the cell membrane.

Although BACE is an aspartic protease, 1 μm AEBSF (a serine protease inhibitor) reportedly blocks secretion of Aβ peptide by inhibiting β-secretase activity to about 44% of untreated cells (11). Our results measuring the secretion of APPSwβ using the 931 ELISA showed that 1 μm AEBSF reduced APPSwβ secretion to a remarkably similar 43% of control levels. Our results also agreed with theirs in that AEBSF treatment caused a slight increase in APPSwβ secretion and stabilized full-length APPSw. Furthermore, we extended these observations by showing that this inhibition of β-secretase cleavage had a concentration dependence similar to that observed for Aβ peptide secretion (11). Only a small amount of iAPPSwβ was detected in AEBSF-treated cells. This showed that AEBSF inhibition was not due to accumulation of APPSwβ intracellularly because of a block in secretion, but due to a block in β-secretase cleavage of APPSw. Knowing now that BACE is an aspartic protease, it is unclear how a serine protease inhibitor such as AEBSF may block β-secretase cleavage, but it would argue against a direct inhibition of BACE. However, the discovery of such an inhibitor illustrates the importance of an in vivo screening method for compounds that block β- and γ-secretase cleavage of APP.

The reported effects of peptide aldehyde protease inhibitors on Aβ peptide secretion have been complex and conflicting. Nevertheless, results have repeatedly shown that Aβ and p3 peptide secretion is inhibited by the following peptide aldehydes: MDL23170 (12, 32), ALLN (13, 15, 17), calpeptin (14, 15, 18), and MG132 (15, 18) with the latter proving to be one of the most potent. Because increased levels of CTFs were detected in cells incubated with peptide aldehydes, the impairment in subsequent Aβ peptide secretion has been attributed to an inhibition of γ-secretase activity by peptide aldehydes (12). Our results using both an APPSw ELISA and pulse-chase studies revealed that β-secretase cleavage of APPSw was also impeded by MG132. The concentration dependence of this β-secretase cleavage inhibition closely corresponds to that reported for the decrease in Aβ40, Aβ42, and p3 peptide secretion (15, 18). Inhibition of β-secretase cleavage was detectable after as little as 2 h of exposure to MG132. In studies by others (15–18), Aβ peptide levels were measured after 3–16 h of incubation with peptide aldehyde. Thus, the MG132-induced block in β-secretase cleavage of APPSw call into question the utility of such peptide aldehydes as γ-secretase inhibitors. Nevertheless, results have repeatedly shown that Aβ peptide secretion is inhibited by the following peptide aldehydes: MDL23170 (12, 32), ALLN (13, 15, 17), calpeptin (14, 15, 18), and MG132 (15, 18) with the latter proving to be one of the most potent. Because increased levels of CTFs were detected in cells incubated with peptide aldehydes, the impairment in subsequent Aβ peptide secretion has been attributed to an inhibition of γ-secretase activity by peptide aldehydes (12). Our results using both an APPSw ELISA and pulse-chase studies revealed that β-secretase cleavage of APPSw was also impeded by MG132. The concentration dependence of this β-secretase cleavage inhibition closely corresponds to that reported for the decrease in Aβ40, Aβ42, and p3 peptide secretion (15, 18). Inhibition of β-secretase cleavage was detectable after as little as 2 h of exposure to MG132. In studies by others (15–18), Aβ peptide levels were measured after 3–16 h of incubation with peptide aldehyde. Thus, the MG132-induced block in β-secretase cleavage of APPSw call into question the utility of such peptide aldehydes as γ-secretase inhibitors. Nevertheless, results have repeatedly shown that Aβ peptide secretion is inhibited by the following peptide aldehydes: MDL23170 (12, 32), ALLN (13, 15, 17), calpeptin (14, 15, 18), and MG132 (15, 18) with the latter proving to be one of the most potent. Because increased levels of CTFs were detected in cells incubated with peptide aldehydes, the impairment in subsequent Aβ peptide secretion has been attributed to an inhibition of γ-secretase activity by peptide aldehydes (12). Our results using both an APPSw ELISA and pulse-chase studies revealed that β-secretase cleavage of APPSw was also impeded by MG132. The concentration dependence of this β-secretase cleavage inhibition closely corresponds to that reported for the decrease in Aβ40, Aβ42, and p3 peptide secretion (15, 18). However, the potent and highly specific proteasomal inhibitor, clasto-lactacystin β-lactone, only caused a partial inhibition in APPSwβ secretion in our studies at 20 times its IC50 for proteasomal inhibition. Therefore, we conclude that the decrease in β-secretase cleavage of APPSw is not due to MG132 inhibition of proteasomal activity. In fact, lactacystin and low concentrations of peptide aldehydes are reported to increase Aβ peptide secretion (14). We detected no increase in β-secretase cleavage of APPSw with clasto-lactacystin β-lactone or low concentrations of MG132 (data not shown). Thus, we suggest that low concentrations of peptide aldehydes increase Aβ peptide secretion because they inhibit proteasomal degradation of β and α-secretase-cleaved CTFs. This, in turn, makes more CTFs available for γ-secretase cleavage. We further hypothesize that, at higher concentrations, peptide aldehydes inhibit Aβ and p3 peptide secretion by blocking cysteine proteases that have roles in protein processing and trafficking in the secretory pathway. This would explain the biphasic effect of peptide aldehydes observed on Aβ peptide secretion (14, 15, 17).

Although BACE activity in CHO cells was the highest of all non-neuronal cells, it was still significantly less than that observed for neuronal cell cultures (7). Therefore, the anti-APPβ antibody, 931, may prove even more useful in analyzing APPSw processing in neuronal cell lines where greater levels of iAPPβ may be expected. In particular, using affinity-purified 931 in the APPSwβ sandwich ELISA described here will be immensely useful for exploring the cellular and molecular mechanisms that regulate β-secretase cleavage.

Acknowledgments—We gratefully thank Dr. Dale Schenk for the generous gift of the mouse monoclonal antibody, 8E5. We also thank Taranah Haske for providing the two CHO cell lines stably expressing APPSw and APP wild type.

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J. Biol. Chem. 2001, 276:4476-4484.
doi: 10.1074/jbc.M008793200 originally published online November 17, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008793200

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