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

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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)[1] 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 isoform numbering) 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 ASP1 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

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[1] 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; *i*APPSwβ, 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; PBS/T, phosphate-buffered saline plus Tween 20; ER, endoplasmic reticulum; BFA, brefeldin A.

blocked Aβ peptide generation by directly inhibiting β-secretase activity (11). There is little information published about other potential specific inhibitors of β-secretase. Several studies have shown that peptide aldehyde protease inhibitors affect the secretion of Aβ peptides (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 (APPSwβ; see Fig. 1*A*), 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. 1*A* as 22C11 and BIOSOURCE were obtained from Roche Molecular Biochemicals and BIOSOURCE International (monoclonal antibody P2-1), respectively. The mouse monoclonal antibody 6E10, which recognizes an epitope in the first 17 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 Schenk (Elan Pharmaceuticals). The rabbit polyclonal antibody, 945, was raised against a synthetic peptide corresponding to the last 19 amino acid residues of the carboxyl terminus of APP (CMQQNGYENPTYKFFEQMQN) 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 19 amino acid residues (CRPGSGLTNIKTEEISEVNL) just amino-terminal to the β-secretase cleavage site of APPSw that was similarly cross-linked to keyhole limpet hemocyanin via an amino-terminal cysteine.

The peptide aldehyde, MG132, was dissolved in dimethyl sulfoxide (Me_2SO) at a concentration of 10 mM (Peptides International). The proteasomal inhibitor, *clasto*-lactacystin β-lactone (Calbiochem), was also dissolved in Me_2SO at 2 mM. Me_2SO was used in all experiments as a vehicle control. The serine protease inhibitor AEBSF (Sigma) was dissolved in sterile water at 0.2 M.

Cell Culture and Transfection—The Chinese hamster ovary cell line, CHOK1, and the HEK293 cell line used for transient transfections were obtained from the American Type Culture Collection. The cell lines stably expressing the 695 isoforms of either APPSw (CHOAPPSw) or APP wild type were a generous gift from Taraneh Haske (Pfizer Pharmaceuticals, Ann Arbor, MI). All CHO cell lines were grown in DMEM supplemented with 10% heat-inactivated fetal calf serum, glutamine, nonessential amino acids, and penicillin/streptomycin/fungizone as described (19). Experiments involving transient expression of APPSw or APP were conducted using cDNAs encoding for the 751 isoforms that were cloned into the pCDNA3 mammalian expression vector (Invitrogen, Inc.). Transfections of HEK293 cells grown on 60-mm plates were conducted using LipofectAMINE as described by the Life Technologies, Inc. Forty hours following transfection, cells were metabolically labeled or lysed for immunoblot analysis as described below.

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 [^{35}S]methionine and [^{35}S]cysteine (Tran^{35}S-label; ICN Pharmaceuticals) at 50 μCi/ml for 1 h. In pulse-chase studies, cells were preincubated in methionine/cysteine-free medium for 15 min and then pulsed for 12 min with Tran^{35}S-label (100 μCi/ml). Cells were then washed and incubated in complete medium containing excess methionine and cysteine for the chase times shown. After 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 using 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 (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 amino-terminal end of APP molecules from cell lysates or conditioned medium. Membranes were alternatively incubated with 6E10 to detect APPSwα 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 APPSwβ Sandwich ELISAs—The 945 and 931 antisera were used as capture antibodies for the full-length APP and APPSwβ sandwich ELISAs, respectively. Each was first affinity-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 (Sterogene). 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 (PBS/T) 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 the cell lysates were aliquoted 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

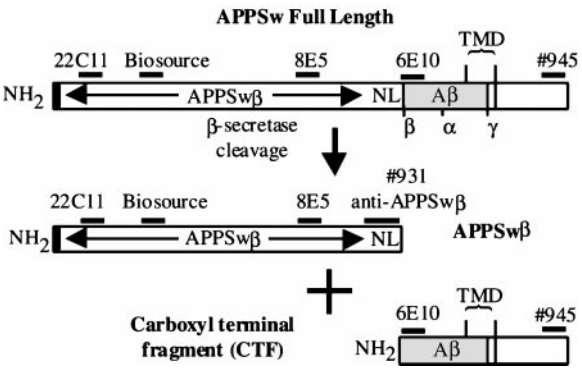

FIG. 1. **A schematic illustration showing full-length APPSw and products of β-secretase cleavage.** The region of APP that gives rise to Aβ peptide is shown in *gray*, and the region to become the soluble APPSwβ fragment is indicated by *arrows*. The sites of α-, β-, and γ-secretase cleavages are shown on the full-length protein. Also shown are the relative locations of epitopes for antibodies used in this study. *TMD* refers to the transmembrane domain.

used in the ELISA were diluted using a solution of 10% SuperBlock and 90% PBS/T. The detector antibody was incubated with each sample for 4 h with constant shaking. The wells were again washed four times with PBS/T and subsequently incubated for 1 h with rabbit anti-mouse IgG conjugated to horseradish peroxidase (diluted 1:4000; Southern Biotechnology Associates, Inc.). Following three washes with PBS/T and two washes with PBS alone, 100 μl of 3,3′,5,5′-tetramethylbenzidine (Pierce) solution was added to each well. The reaction was stopped by the addition of an equal volume of 2 M sulfuric acid. The relative amount of full-length APP in the sample was then quantified colorimetrically at 450 nm. The levels of secreted APPSwβ were assayed in the exact same way except that plates coated with affinity-purified 931 antibody were used to capture the β-secretase-cleaved protein and just 25 μl of conditioned medium was loaded per well. Each experiment was repeated at least three times, and the indicated values are averages of triplicate measurements ± S.D.

RESULTS

Characterization of Polyclonal Antibodies to APP and APPSwβ—As shown in Fig. 1, β-secretase cleavage of APPSw generates a large soluble amino-terminal fragment (APPSwβ) that is secreted into the medium and a CTF that is subsequently cleaved by γ-secretase to derive the Aβ 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 antisera (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. 2*A*. CHO cells stably expressing APPSw (CHOAPPSw) were metabolically labeled for 1 h with Tran^{35}S-label. Following cell lysis, equal amounts of supernatant were incubated with 1, 2, or 4 μl of 945 antiserum or with 4 μl 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. The second antisera specifically recognizes only the carboxyl terminus of the soluble amino-terminal fragment (APPSwβ) created when β-secretase cleaves APPSw just COOH-terminal to Leu652 (751 numbering). Results shown in Fig. 2*B* demonstrate that antisera raised in two rabbits (931 and 932) against the 20-amino acid sequence just amino-terminal to the β-secretase cleavage site are capable of immunoprecipitating a soluble APPSw fragment from the conditioned medium of CHOAPPSw cells. Culture medium conditioned for 24 h by CHOAPPSw cells was divided into equal aliquots and incubated with preimmune sera from rabbit 931, 931 antisera, or 932 antisera. The mouse monoclonal antibody, 8E5, which recognizes an epitope in the lumenal region of APP (Fig. 1), served as a positive control. The immunoprecipitates were resolved by SDS-PAGE and the APPSw amino-terminal fragments were identified by Western blot analysis using the mouse monoclonal antibody 22C11. Both 931 and 932 antisera, but not preimmune sera, immunoprecipitated a specific APP soluble fragment from the conditioned media. The 931 antisera appeared to have a higher titer than 932. Thus, all subsequent experiments utilized only 931.

As others have demonstrated, the α- and β-cleaved soluble fragments are difficult to electrophoretically resolve from one another (20, 21). Therefore, it was unclear from this Western blot analysis of conditioned medium whether the soluble APPSw fragment immunoprecipitated by 931 was limited to secreted APPSwβ or also included α-secretase-cleaved APPSw (APPSwα). Furthermore, it was not known if 931 could immunoprecipitate wild type APPβ and APPα. Consequently, HEK293 cells were transiently transfected with either pCDAPP or pCDAPPSw. Medium that had been conditioned for 36 h by these transiently transfected cells was collected and soluble APP fragments were immunoprecipitated with 931 or the mouse monoclonal antibody 8E5. Since the epitope recognized by 8E5 is amino-terminal to the β-secretase cleavage site, it is capable of immunoprecipitating APPα, APPβ, APPSwα, and APPSwβ. Immunoprecipitates were resolved by SDS-PAGE and immunoblotted using the mouse monoclonal antibody, 22C11 to detect all forms of soluble APP and APPSw (see Fig. 1). As expected, 8E5 was able to immunoprecipitate both secreted APP and APPSw from the conditioned media (Fig. 2*C*, *panel 1*). However, 931 only immunoprecipitated soluble APPSw amino-terminal fragments from the conditioned medium of cells transiently transfected with pCDAPPSw and did not recognize wild type secreted APP fragments. A set of immunoprecipitations from conditioned medium identical to that conducted in Fig. 2*C* (*panel 1*) was immunoblotted with the mouse monoclonal antibody 6E10, which detects only secreted APP and APPSw soluble fragments that have been cleaved by α-secretase. Although both APPα and APPSwα were detected in the 8E5 immunoprecipitates, neither form was immunoprecipitated using the 931 antibody (Fig. 2*C*, *panel 2*). Taken together, these results demonstrate that the 931 antibody specifically recognizes only the neoepitope derived with β-secretase cleavage of APPSw. It does not cross-react with full-length APPSw (compare Fig. 4, *A* and *C*, chase at 0 min), soluble APPα, APPβ, or APPSwα.

Characterization of ELISAs for Detection of Full-length APP and the Soluble β-Secretase Cleaved APPSw Amino-terminal Fragment—Since 931 specifically recognized the neoepitope of β-secretase-cleaved APPSw in conditioned medium, it was used to create an enzyme-linked immunosorbent assay (ELISA) to quantitatively measure the amount of secreted APPSwβ. 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 μg/ml), BIOSOURCE (0.25 μg/ml), or LN27 (0.5 μg/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. 2*D*). Full-length APPSw was specifically detected in CHOAPPSw lysates with 8E5 and the BIOSOURCE mouse monoclonal antibodies showing the

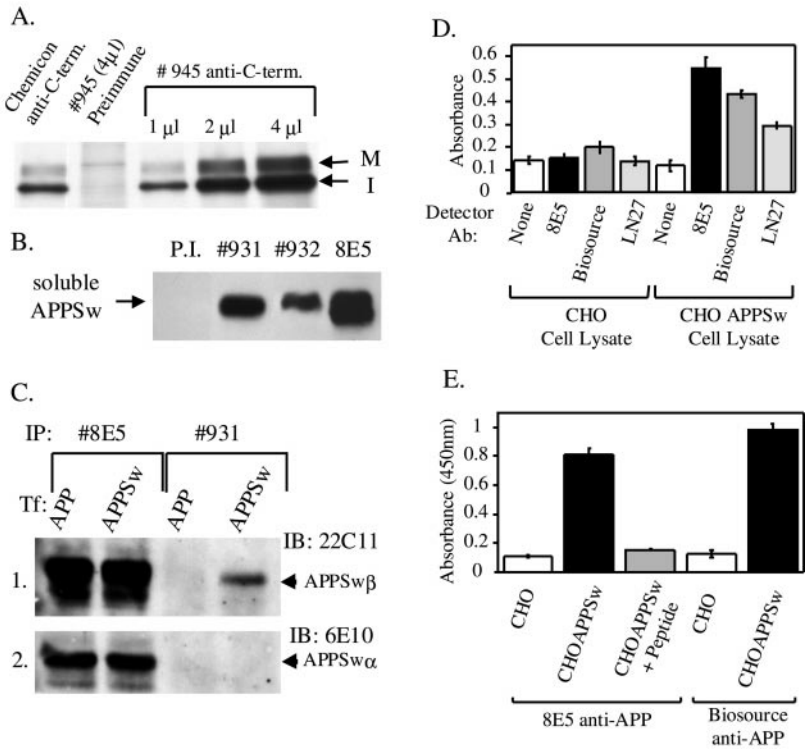

FIG. 2. **Characterization of novel polyclonal antibodies.** *A*, the ability of 945 to immunoprecipitate full-length APPSw was compared with the Chemicon rabbit polyclonal antibody to the carboxyl terminus of APP. CHOAPPSw cells were metabolically labeled with [^{35}S]cysteine and [^{35}S]methionine 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 APPSwα and APPSwβ 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 APPα, APPSwα, APPβ, and APPSwβ. *Panel 2*, a duplicate set of immunoprecipitated samples was immunoblotted for APPα and APPSwα 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 APPSw 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 931 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.

greatest sensitivity. A total protein concentration of 10 μg of cell lysate was found to be optimal to detect full-length APPSw (data not shown).

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. 2*E*). 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 APPSwα 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. 3*A*, *panel 2*). This nonspecific ^{35}S-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 neoepitope. CHOAPPSw cells were pulse-labeled with Tran^{35}S-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 immunoisolated using 931 from conditioned media and cell lysates, respectively. Full-length APPSw was subsequently immunoprecipitated from the same cell lysates using 945. Full-length APPSw was still immunoprecipitated in the presence of the neoepitope peptide, indicating that the peptide was not causing a general block in the immunoisolation of proteins (Fig. 3*A*, *panel 1*). However, the synthetic neoepitope peptide blocked immunoprecipitation of secreted APPSwβ (Fig. 3*A*, *panel 2*) and intracellular APPSwβ (Fig. 3*A*, *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

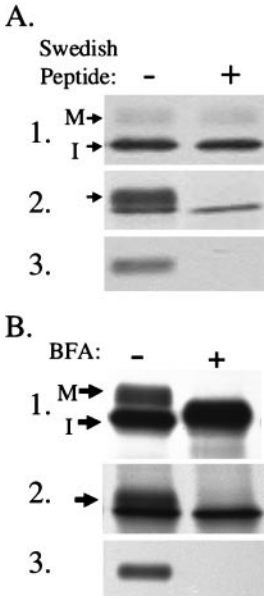

FIG. 3. **Immunoprecipitation of radiolabeled intracellular and secreted APPSwβ is blocked by a synthetic peptide of the Swedish mutant and brefeldin A treatment.** *A*, CHOAPPSw cells were pulse labeled as described and chased for 45 min in complete DMEM. Conditioned media and cell lysates were isolated 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). *1*, the excess peptide did not interfere with immunoprecipitation of full-length APPSw with 945. *2*, the CEIESVNL peptide blocked immunoprecipitation of *i*APPSwβ (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 Tran^{35}S-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 antisera (*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 antisera.

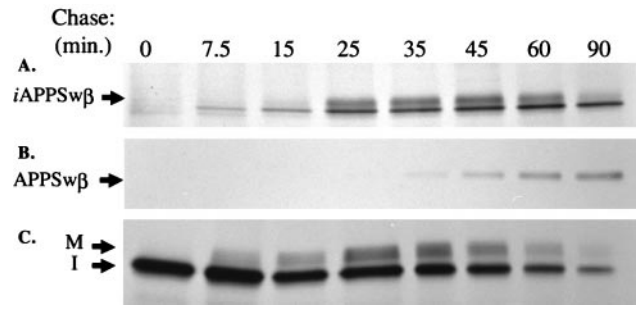

FIG. 4. **Pulse-chase analysis of intracellular APPSwβ production and secretion from CHOAPPSw cells.** Cells were pulse-labeled for 12 min with Tran^{35}S-label and chased for the indicated times in minutes in complete DMEM with excess unlabeled methionine and cysteine as described under "Experimental Procedures." *A*, *i*APPSwβ appears in the cell lysate between 15 and 25 min of chase. By 90 min, little *i*APPSwβ is still detectable. *B*, secreted APPSwβ is not detected in the conditioned medium until after 35–45 min of chase. *C*, the appearance and disappearance of *i*APPSwβ coincides with that observed for the mature form of full-length APPSw in cell lysates.

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 Tran^{35}S-label for 1 h in the presence or absence of 20 μg/ml brefeldin A. 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 immunoisolated from cell lysates using the 945 antibody. As expected, BFA treatment blocked the appearance of APPSwβ in the conditioned medium (Fig. 3*B*, *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. 3*B*, *panel 2*). Thus, blocking maturation of APPSw prevents it from being cleaved by β-secretase.

Taken together, the results described above indicated that *i*APPSwβ 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 Tran^{35}S-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 immunoisolated from the supernatants using 945. Secreted APPSwβ was 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. 4*C*) 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. 4*A* shows that *i*APPSwβ was readily detectable after chasing for about 25 min and corresponded with the appearance of the completely glycosylated, mature APPSw. The level of *i*APPSwβ reached a maximum at 35–45 min and was barely detectable after 90 min of chase. Thus, as expected, the appearance of *i*APPSwβ preceded the secretion of APPSwβ into the medium (Fig. 4*B*). Only a trace of intracellular APPSwβ was detected after 35 min, while secreted APPSwβ continued to accumulate after chasing for 90 min.

The APPSwβ ELISA Is a Specific and Sensitive Detector of Inhibitors of β-Secretase Cleavage—The serine protease inhibitor AEBSF, inhibits Aβ-peptide secretion in a concentration-dependent manner in both neuronal and non-neuronal cell lines stably expressing APP or APPSw by presumably blocking β-secretase cleavage (11). The APPSwβ ELISA enabled us to compare the concentration dependence of the AEBSF-induced inhibition of β-secretase activity to that reported for inhibition of Aβ peptide secretion. CHOAPPSw cells plated the previous day were rinsed in PBS and incubated for 5 h with fresh medium containing increasing concentrations of AEBSF (0.1–1.0 mM). The conditioned medium was then collected, and APPSwβ levels were quantified using the 931 ELISA. AEBSF produced a concentration-dependent inhibition of APPSwβ secreted into the medium (Fig. 5*A*). CHOAPPSw cells treated with 1 mM AEBSF had secreted only 43% of the APPSwβ detected in conditioned medium from untreated cells. This supports the observation that AEBSF inhibits β-secretase cleavage of APPSw by a direct or indirect mechanism.

The highly specific proteasomal inhibitor, lactacystin, in-

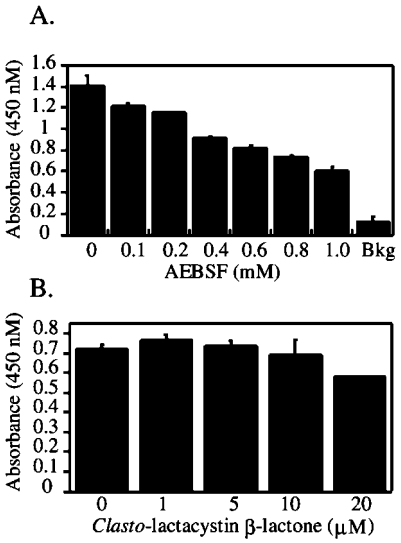

FIG. 5. **The effects of protease inhibitors on APPSwβ secretion in CHOAPPSw cells.** *A*, CHOAPPSw cells were incubated for 5 h in complete DMEM containing either vehicle or 0.1–1.0 mM AEBSF, a serine protease inhibitor. Conditioned medium was collected and analyzed by ELISA for APPSwβ secretion. *B*, 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.

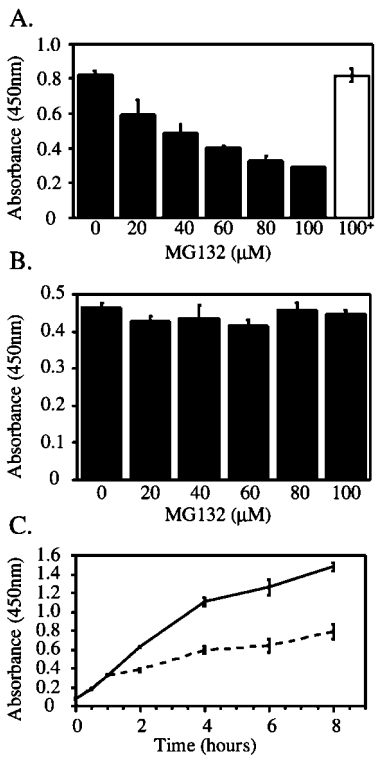

FIG. 6. **The peptide aldehyde, MG132, inhibits β-secretase cleavage of APPSw in stably transfected CHO cells.** *A*, CHOAPPSw cells were incubated for 6 h with Me_2SO vehicle or 20–100 μM MG132. Conditioned medium was collected and analyzed for secreted APPSwβ by ELISA. To control for any interference 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 Me_2SO 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.

creases APPα and intracellular and secreted Aβ42 (18, 23, 24). Other investigators reported that lactacystin increased secretion of both Aβ40 and Aβ42 from SH-SY5Y 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 IC_{50} 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. 5*B*). 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 Aβ 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. 6*A*). 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. No significant difference in full-length APPSw levels was detected with MG132 treatment (Fig. 6*B*). Thus, MG132 treatment caused a concentration-dependent decrease in the amount of secreted APPSwβ. To examine the time course of this block in APPSwβ production, CHOAPPSw cells were incubated for increasing time with or without 80 μM MG132. Conditioned medium was collected at the times indicated and the amount of secreted APPSwβ determined by ELISA (Fig. 6*C*). Our results show that inhibition of APPSwβ secretion could not be detected until 2 h following addition of the peptide aldehyde inhibitor. Following this incubation period, APPSwβ continued to be secreted into the media by MG132-treated cells but at a much slower rate compared with untreated controls.

The continued increase in APPSwβ secretion 30 min and 1 h after incubation with MG132 could be explained in two ways. Either this was the time required for MG132 to penetrate the cell and block β-secretase cleavage or inhibition was immediate but was not detectable by this assay because of the lag time required for secretion of APPSwβ generated prior to the block.

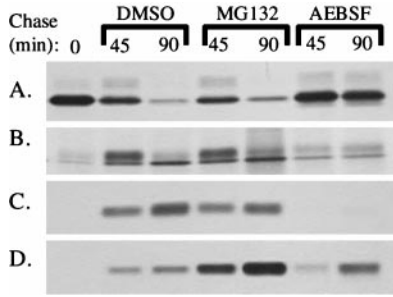

FIG. 7. **A comparison of AEBSF and MG132 incubation on intracellular production of APPSwβ.** CHOAPPSw cells were pulsed for 12 min with Tran^{35}S-label. The cells were washed and then chased in complete DMEM containing Me_2SO, 80 μM MG132, or 1 mM AEBSF for either 45 or 90 min as described under "Experimental Procedures." Intracellular APPSwβ indicated by an *arrow* (*panel B*) and full-length APPSw (*panel A*) were isolated from cell lysates by sequential immunoprecipitation with 931 and 945 antisera, respectively. Conditioned medium was isolated and analyzed for secreted APPSwβ (*panel C*) and APPSwα (*panel D*) by immunoprecipitation using the 931 and 6E10 antibodies, respectively.

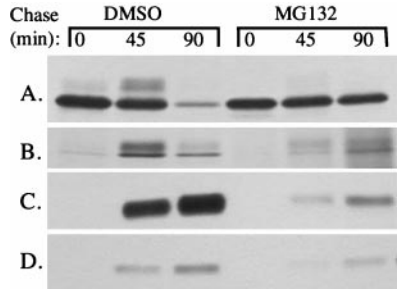

FIG. 8. **A 2-h preincubation with MG132 blocks intracellular production of APPSwβ in CHOAPPSw cells.** CHOAPPSw cells were preincubated for 2 h with either Me_2SO control or 80 μM MG132. The cells were subsequently starved of methionine and cysteine, pulsed for 12 min with Tran^{35}S-label and chased all in the presence of either Me_2SO or 80 μM MG132 for either 45 or 90 min as described under "Experimental Procedures." Intracellular APPSwβ indicated by an *arrow* (*panel B*) and full-length APPSw (*panel A*) were isolated from cell lysates by sequential immunoprecipitation with 931 and 945 antisera, respectively. Conditioned medium was isolated and analyzed for secreted APPSwβ (*panel C*) and APPSwα (*panel D*) by immunoprecipitation using the 931 and 6E10 antibodies, respectively.

It was also of interest to determine whether the block in APPSwβ production by MG132 was similar in its immediacy and specificity to that observed for AEBSF. Therefore, CHOAPPSw cells were pulsed for 12 min with Tran^{35}S-label and then chased for 0, 45, or 90 min in complete medium containing Me_2SO, 80 μM MG132, or 1 mM AEBSF. The resulting cell lysates were immunoprecipitated first with 931 followed by 945. Equal aliquots of conditioned medium were incubated with 931 or 6E10 to immunoprecipitate secreted APPSwβ and APPSwα, respectively. When 80 μM MG132 was present only during the chase period, it had little effect on full-length APPSw maturation, intracellular APPSwβ production, or APPSwβ secretion compared with control. MG132 treatment, however, produced a large increase in the amount of secreted APPSwα (Fig. 7*D*). In contrast to this is the effect of chasing in the presence of 1 mM AEBSF, which immediately blocked the appearance of APPSwβ in the medium (Fig. 7*C*). The amount of *i*APPSwβ was markedly reduced after chasing for 45 min compared to control and was still reduced after 90 min (Fig. 7*B*). AEBSF treatment also stabilized the immature form of full-length APPSw, which was nearly unchanged through 90 min of chase in the presence of the inhibitor (Fig. 7*A*). Although AEBSF nearly completely blocked APPSwβ secretion, secreted levels of APPSwα were slightly increased after 90 min of chase (Fig. 7*D*). The lack of an effect of MG132 during this chase period indicates that it does not produce an immediate block in cleavage as observed with AEBSF. These results suggest that, although both AEBSF and MG132 induced a block in APPSwβ production, they differ in their molecular mechanisms.

MG132 had little effect on secretion of APPSwβ in the first 90 min of incubation (see Figs. 6 and 7). Therefore, an additional pulse-chase experiment was conducted in which cells were preincubated for 2 h with 80 μM MG132 or Me_2SO since a block in APPSwβ production was detected after 2 h of MG132 treatment (Fig. 6*C*). After the pretreatment, cells were pulsed and then chased in the presence of vehicle or MG132. Conditioned media and cell lysates were isolated and analyzed as above for full-length APPSw, *i*APPSwβ, APPSwβ, and APPSwα. A profound effect was observed on APPSw processing after 2 h of MG132 preincubation (Fig. 8). After a 45-min chase, mature APPSw was readily detectable in CHOAPPSw cells treated with Me_2SO, while the MG132-treated cells showed only a trace of the mature form (Fig. 8*A*). After 90 min of chase, very little radiolabeled immature or mature APPSw remained in the control sample, whereas in the MG132-treated cells a large portion of the radiolabeled immature APPSw detected at time zero still remained. Corresponding with this impaired maturation of APPSw with MG132 treatment was a decrease in the levels of *i*APPSwβ (Fig. 8*B*), secreted APPSwβ (Fig. 8*C*), and secreted APPSwα (Fig. 8*D*). This suggests that treatment of cells with high concentrations of peptide aldehydes may cause a more general impairment of the secretory pathway. This impairment blocks transport and processing of APPSw through the secretory pathway, thus explaining the decrease in Aβ peptide secretion observed by others (12–14).

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 APPSwα 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 APPSwα and APPSwβ, 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 *i*APPSwβ, may better represent the effects of peptide aldehydes on Aβ peptide secretion over the long incubation period used by these and other investigators (13–18).

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 C83 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 APPα. 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-cleaved fragments associating with the cell membrane.

Although BACE is an aspartic protease, 1 mM 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 mM 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 APPSwα 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 *i*APPSwβ 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), 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 APPSwβ 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 (13–18), Aβ peptide levels were measured after 3–16 h of incubation with peptide aldehyde. Thus, the MG132-induced block in β-secretase cleavage that we observed here could account for the decrease in Aβ peptide secretion. This observation indicates that the inhibitory effect by peptide aldehydes such as MG132 is not limited to inhibition of γ-secretase activity. In addition to inhibiting cysteine proteases, MG132 is known to inhibit proteasomal cleavage with an IC_{50} of a few micromolar (29). 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 IC_{50} 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-APPSwβ antibody, 931, may prove even more useful in analyzing APPSw processing in neuronal cell lines where greater levels of *i*APPSwβ 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.

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