γ-Secretase Cleavage Is Distinct from Endoplasmic Reticulum Degradation of the Transmembrane Domain of the Amyloid Precursor Protein*

(Received for publication, November 11, 1997, and in revised form, September 9, 1998)

William L. Bunnell, Huan V. Pham, and Charles G. Glabe‡

From the Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92697-3900

One of the critical cleavage events that generates Alzheimer’s amyloid Aβ peptide occurs within the transmembrane domain (TMD) of the amyloid precursor protein (APP) and is carried out by a poorly understood enzyme activity known as γ-secretase. To investigate this processing, a probe molecule, H26–57C, was constructed containing the TMD of APP flanked immediately on each side by unique epitope tags. H26–57C-transfected cells secrete a ~2.9-kDa fragment, indicating that the luminal and cytosolic domains of APP are not required for γ-secretase processing. Pulse-chase experiments indicate that the probe turns over with a half-life of 8 min. No degradation intermediates are detected during the chase period, indicating that TMD turnover is a highly processive mechanism. The protease inhibitors, ALLN and MG132, cause a dramatic (50-fold) increase in the steady-state amount of the probe. All of the inhibitors that prevent degradation of the probe in the rough endoplasmic reticulum increase the amount of the ~2.9-kDa fragment that is secreted into the media and also causes a similar increase in the secretion of 4 kDa Aβ from APP-transfected cells. These results indicate that the system responsible for the degradation of the probe in the rough endoplasmic reticulum and the intramembrane cleavage by γ-secretase that produces soluble, secreted Aβ are distinct and opposing processes.

Intracellular protein degradation is an integral part of cellular homeostasis that regulates the level of normal proteins and removes abnormal proteins from the cell. Newly synthesized secretory and membrane proteins are translocated into the endoplasmic reticulum (ER), folded, assembled, and transported to a cellular destination, secreted from the cell (1). Incorrectly folded proteins, unassembled subunits of multisubunit complexes, and mutated proteins are rapidly eliminated from the cell, often in the ER by a process referred to as quality control. Examples of transmembrane proteins degraded in the ER due to this process include the asialoglycoprotein receptor subunit H2a, the T cell receptor α subunit, and the cystic fibrosis transmembrane conductance regulator, respectively (2–8). Normal proteins may also be degraded in the ER and in the case of the multiple membrane spanning protein 3-hydroxy-3-methylglutaryl-CoA reductase, its rate of degradation in the ER is accelerated in response to regulatory signals (9).

Processing and degradation of the membrane-spanning segments of proteins presents some interesting mechanistic and experimental challenges because of the hydrophobic environment in which they reside. The details of these mechanisms are only beginning to emerge. One mechanism of ER degradation involves reverse translocation of the protein through the ER membrane into the cytoplasm. The MHC class I heavy chain is co-translationally inserted into the ER membrane and glycosylated. In cytomegalovirus-infected cells, it is then transported back into the cytoplasm, deglycosylated, and degraded by the proteasome (10, 11). The same mechanism operates for misfolded MHC class I heavy chains (12). A soluble, ubiquitinated species appears to be an intermediate in this pathway. Two other secretory proteins, secreted α1-antitrypsin Z and the yeast protein carboxypeptidase yscY, are degraded in the cytosol by the proteasome (13, 14). On the other hand, 3-hydroxy-3-methylglutaryl-CoA reductase degradation in the ER occurs while associated with the membrane (15). The luminal contents are not required for degradation and no proteolytic intermediates are detected during this membrane associated degradation. This appears to be a distinct pathway with a mechanism that has yet to be elucidated.

Processing of the TMD of type I transmembrane proteins is particularly important with respect to Alzheimer’s disease. In this devastating disease, insoluble extracellular deposits of a 42-amino acid peptide called Aβ accumulate in the brains of affected individuals. It is believed that this peptide is responsible for the neuronal pathogenesis observed in the brains of Alzheimer’s disease patients (16). Cleavage within the transmembrane domain of the APP creates the carboxyl terminus of the Aβ peptide. This proteolysis is accomplished by one or more enzymes, termed γ-secretase, that remain to be identified. The precise amino acid cleavage site by γ-secretase is critical for Alzheimer’s disease pathogenesis because it determines whether the product is the longer Aβ1–42 form of the peptide that aggregates rapidly (17, 18) and is resistant to degradation leading to its accumulation (19, 20).

Although γ-secretase cleavage may occur within several different subcellular compartments (21–25), attention has recently focused on the ER. In autosomal dominant forms of Alzheimer’s disease caused by mutations within the transmembrane domain of APP or in the presenilin 1 or 2 genes, the mutation is associated with enhanced production of Aβ1–42 (16). The presenilin genes encode ER-resident transmembrane

* This work was supported by National Institutes of Health Grants NS-31230 and AG00538 (to C. 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.

‡ To whom correspondence should be addressed; Dept. of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, CA 92697-3900. Tel.: 714-824-6081; Fax: 714-824-8551; E-mail: cglabe@uci.edu.

1 The abbreviations used are: ER, endoplasmic reticulum; Aβ, amyloid β-peptide; APP, amyloid precursor protein; TMD, transmembrane domain; ALLN, N-acetyl-leucyl-leucyl-norleucinal; MHC, myosin heavy chain; BFA, brefeldin A; PBS, phosphate-buffered saline; HA, hemagglutinin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; PAGE, polyacrylamide gel electrophoresis.

Printed in U.S.A.

THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 273, No. 48, Issue of November 27, pp. 31947–31955, 1998

© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

This paper is available on line at http://www.jbc.org

31947
Degradation and Processing of the APP Transmembrane Domain

proteins that span the bilayer seven times (26–28). Recently, it has been demonstrated that Aβ is produced intracellularly (29). At least some of this Aβ may be produced in the ER, because the production of intracellular Aβ is not inhibited by BFA (30, 31), especially peptides ending at residue 42 (30). The potential association of this cleavage event with the protein degradation systems in the ER has not yet been explored. This may be due at least in part, to technical difficulties in detecting intracellular Aβ by immunoprecipitation (32) and unambiguously examining the cleavage and degradation of the APP TMD per se. This is not surprising because very little is known about the proteolysis and degradation of the membrane spanning domains of transmembrane proteins. Some of the ambiguity arises from relying on a single epitope for studying transmembrane proteins and the location of the epitope at distances relatively far away from the segment to be examined.

These studies describe a novel probe molecule containing two epitope tags immediately flanking the APP TMD that is designed to focus on cleavage events within the TMD. Any single cleavage event should give rise to fragments that are immunoprecipitable by at least one of the antibodies. The probe is efficiently incorporated into the ER membrane and it is rapidly degraded in a highly processive fashion, such that no intermediates are detected by either antibody in the absence of protease inhibitors. Additionally, no soluble cytosolic or ubiquitinated intermediates are detected, suggesting that the major pathway is distinct from the proteasome-dependent pathway recently identified for the MHC class I molecules. The results also indicate that the ER degradative pathway is distinct from the pathway that produces soluble, secreted Aβ.

**EXPERIMENTAL PROCEDURES**

**Construction of Expression Plasmid and Cell Culture**—The HindIII-KpnI signal sequence fragment of APP571 was ligated to 11 pre-annealed oligonucleotides spanning the sequence: (gtaccctattagttcagtttactggttcagttttacaatgtagctaggctgctttgaagctgctttggtggtgtgtctgctctagtga). The resulting HindIII-XbaI fragment was ligated into the polylinker of the pGEM-3z plasmid (Promega) for in vitro transcription/translation. The sequence was confirmed by DNA sequencing using the Pharmacia A.L.F. DNA sequence. For transfection, the HindIII-XbaI fragment was subcloned into the polylinker of a pCB6 expression vector containing a cytomegalovirus promoter and the neomycin selectable marker. Human embryonic kidney cells (293 cells) were stably transduced with 20 μg of plasmid DNA by the CaPO4 method (33).

**Individual colonies were selected and maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and G418.**

**Antibody Production and Purification**—Rabbits were immunized with a synthetic peptide, CKKKQYEQKLISEEDL, that contains the c-Myc epitope tag, QYEQKLISEEDL, an additional 3 lysine residues with a synthetic peptide, CKKKQYEQKLISEEDL, that contains the [9-(2-fluorenylethoxycarbonyl)-(9-fluorenyl)maleimidobenzoyl]-maleimidoester (Pierce) and emulsified in Freund's complete adjuvant for the first immunization. After two immunizations, serum samples were collected and either lysed in RIPA buffer for time 0, incubated in complete medium containing an excess of cold methionine for 1, 2, 4, or 6 h, or incubated in complete medium with ALLN for 6 h (6 + ALLN). The inhibitors were E64 (250 μg/ml), leupeptin (200 μg/ml), pepstatin A (75 μg/ml), BFA (35 μg/ml), ALLN (135 μg/ml), lactacytin (20 μg/ml), and MG 132 (50 μg/ml), chloroquine (50 μg/ml), phosphoramidon (20–60 μg/ml), and NH4Cl (30 mM). Cells were viable under the conditions employed as determined by trypan blue exclusion.

**Peptide Synthesis**—The peptides were synthesized by N-((9-fluorenylethoxycarbonyl)-methoxycarbonyl chemistry using a continuous flow semiautomatic instrument, purified by reverse-phase high performance liquid chromatography, and the expected structure was verified by sequencing and electrospray mass spectrometry as described previously (19). Estimates of the fraction of expected product range from 85 to 90%, and no single failure product represents more than 1% of the total, which is typical of synthetic peptides of this size (19).

**Immunoprecipitations and Gel Electrophoresis**—Media was collected, centrifuged at 13,000 × g for 10 min and the supernatant added to an equal volume of 2 × RIPA buffer containing protease inhibitors for immunoprecipitation. Cells were lysed in RIPA buffer and the soluble cell lysate and media were precleared with protein A- or protein G-Sepharose Fast Flow beads (Pharmacia) for the HA and c-Myc antibodies, respectively, and then primary antibody was added. After 2 h with the primary antibody, protein A- or protein G-Sepharase Fast Flow beads were added and incubated overnight at 4 °C on a rocker. Protein concentration was determined using the Pierce BCA protein assay kit and the same amount of protein was immunoprecipitated in each experiment. The c-Myc antibody was used at 1:300 and the HA antibody used at 4 μg/ml for immunoprecipitations. The monoclonal antibody 4G8, that is specific to residues 17–24 of Aβ, was used at 1:300. The immunoprecipitates were collected at 2500 rpm for 5 min, rinsed 3 times with RIPA buffer and resuspended in 2 × sample buffer (0.125 M Tris, pH 6.8, 4% SDS, 20% glycerol, 1% β-mercaptoethanol). After a 5-min incubation at 100 °C, the samples were subjected to either 7% Tris glycine PAGE (34), 16.5% Tris-Tricine PAGE (35), or 10–20% Tris-Tricine gradient PAGE (Bio-Rad). The gel was treated with ENHANCE (NEN Life Science Products Inc.), dried, and exposed to x-ray film.

**Metabolic Labeling**—Rabbits were immunized with methionine-deficient Dulbecco's modified Eagle's medium for 40 min prior to labeling. Cells were then incubated with 500 μCi/ml [35S]methionine in the presence or absence of inhibitors for the steady-state experiments. After removal of the media, cells were immediately lysed in RIPA buffer. Pulse-chase experiments were pulsed with 1 μCi/ml [35S]methionine for 5 min, rinsed with PBS, and chased in methionine containing media (0.3 mg/ml) for the indicated time. For degradation rate determinations, the inhibitor was present during the 40-min incubation in methionine minus media, during the 5-min pulse, and during the chase period. Quantitation of pulse-chase experiments was performed on a Molecular Dynamics PhosphorImager. It should be noted that experiments that used a chase period lacking [35S]methionine were rinsed with PBS after the labeling period and then incubated in the chase media, whereas steady-state experiments were immediately lysed after removal of [35S]methionine containing media. For the ALLN washout experiment, cells were incubated overnight with [35S]methionine and ALLN. The cells were then rinsed in PBS and either lysed in RIPA buffer for time 0, incubated in complete media containing an excess of cold methionine for 1, 2, 4, or 6 h, or incubated in complete media with ALLN for 6 h (6 + ALLN). The inhibitors were E64 (250 μg/ml), leupeptin (200 μg/ml), pepstatin A (75 μg/ml), BFA (35 μg/ml), ALLN (135 μg/ml), lactacytin (20 μg/ml), and MG 132 (50 μg/ml), chloroquine (50 μg/ml), phosphoramidon (20–60 μg/ml), and NH4Cl (30 mM). Cells were viable under the conditions employed as determined by trypan blue exclusion.

**In Vitro Transcription/Translation—**Coupled in vitro transcription/translation was performed using a pGEM plasmid in Promega's TNT-coupled reticulocyte lysate using the manufacturer's protocol. Dog pancreas microsomal membranes were also from Promega. Proteinase K was added to the reaction to a final concentration of 0.5 mg/ml and the reaction was renatured and reisolated with the primary antibody which phenylmethylsulfonyl fluoride was added to inhibit the enzyme. When included, Triton X-100 was used at 0.1% to solubilize the membranes. 10 μl of in vitro translation mixture was added to 1 ml of RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxocholate, 0.1% SDS in PBS buffer containing the following inhibitors added fresh: 0.3 μM aprotinin, 1 μM phenylmethylsulfonyl fluoride, 17 μg/ml ALLN, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM EDTA) and immunoprecipitated with each antibody and immunoprecipitates run on a 16.5% Tris-Tricine PAGE.

**Limited Degradation**—When included, Triton X-100 was added to the reaction mixture to a final concentration of 0.1% to solubilize the membranes. 10 μl of in vitro translation mixture was added to 1 ml of RIPA buffer (1% Nonidet P-40, 0.5% sodium deoxocholate, 0.1% SDS in PBS buffer containing the following inhibitors added fresh: 0.3 μM aprotinin, 1 μM phenylmethylsulfonyl fluoride, 17 μg/ml ALLN, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 5 mM EDTA) and immunoprecipitated with each antibody and immunoprecipitates run on a 16.5% Tris-Tricine PAGE.
then both potential fragments will be labeled. The small size of peaks shown are interpreted as M intensity of the most abundant peak were selected for assignment. All PAWS software (Rockefeller University) using the default mass accuracy of 2000–4000 Da at the Beckman Research Institute of the City of Hope (Duarte, California). Peak assignment was made using the software.

FIG. 1. Schematic diagram of H26–57C. The expected molecular mass of the probe is 6066 Da after signal sequence cleavage at the predicted location (arrowhead). Residues 26–57 are derived from human APP. The specific location of the cleavage sites that produce Aβ peptides ending at residues 40 and 42 are indicated by the arrows below the amino acid sequence. The expected product containing only the HA epitope that results from γ-secretase cleavage within the transmembrane domain is shown at the bottom of the diagram.

and added to an equal volume of 2× RIPA buffer for immunoprecipitation with the c-Myc or HA antibody. The pellet (sodium carbonate-extracted membrane pellet) was resuspended in 1× RIPA buffer, centrifuged at 13,000 rpm for 10 min, and the soluble fraction was immunoprecipitated with the c-Myc or HA antibody.

Mass Spectrometric Analysis—Two ml of culture media from a 16-h incubation of a 10-cm dish of H26–56C transfected cells was mixed with an equal volume of 2× RIPA buffer containing a mixture of protease inhibitors and immunoprecipitated with the anti-HA monoclonal antibody as described above. The washed protein G-Sepharose beads were eluted with 60 μl of 0.1 M glycine, pH 3.0, and the eluate dialyzed for 16 h against distilled water at 4°C using a 10,000 Da molecular mass cut-off membrane. The dialyzed sample was infused into a Finnigan LCQ electrospray mass spectrometer and data collected in the mass range of 1000–4000 Da at the Beckman Research Institute of the City of Hope (Duarte, California). Peak assignment was made using the PAWS software (Rockefeller University) using the default mass accuracy of 1000 parts per million. Only peaks greater than 1% of the intensity of the most abundant peak were selected for assignment. All peaks shown are interpreted as M + 2H.

RESULTS

Characterization of H26–57C—To study the turnover of the TMD of APP, the probe H26–57C was constructed containing the TMD of APP flanked immediately on both sides by unique epitope tags as diagrammed in Fig. 1. The name of the probe is an acronym with the numbers referring to the TMD of APP from residue 1 of Aβ with the first letter of the epitope tag at the ends. The signal sequence from human APP is situated at the amino terminus of the probe. The rationale for the design is to detect proteolytic events localized to the luminal side of the membrane, within the membrane, or on the cytoplasmic side of the membrane. If one epitope is degraded from one side of the membrane, the resulting protein fragment should be detected with an antibody to the epitope on the other side of the membrane and any single cleavage event should give rise to a larger fragment. Cleavage within the TMD, such as the secretase type cleavage known to cleave full-length APP, may release two fragments that are potentially detectable with antibodies to each of the epitopes. Naturally occurring methionine residues at positions 35 and 52 of the Aβ sequence flank the expected sites of γ-secretase cleavage. If γ-secretase cleavage occurs between residues 35 and 52, then both potential fragments will be labeled. The small size of the probe should facilitate a high resolution analysis of cleavage events with respect to the lumen and cytoplasmic ends, the highest resolution being achieved by placing the epitopes adjacent to the transmembrane domain.

Initial characterization of the probe was performed using an in vitro coupled, transcription/translation system with dog pancreatic microsomes to verify the fidelity of ER translocation and signal sequence removal. In the absence of microsomes, a translation product of the appropriate size of ~7.9 kDa is detected with both antibodies, indicating that the efficiency of immunoprecipitation is the same from both epitopes (Fig. 2A, lanes 1 and 4). In the presence of microsomal membranes, virtually all of the translation product migrates with an increased mobility (Fig. 2A, lanes 2 and 5), indicating that the probe is efficiently inserted into the membrane and the signal peptide removed. This small decrease in size correlates with the loss of the signal peptide. Also shown are the results of experiments aimed at identifying the orientation of insertion of H26–57C into the membrane. In vitro transcription/translation of H26–57C in the presence of microsomal membranes were subsequently incubated with protease K (Fig. 2A, lanes 3 and 6). The orientation of the probe within the membrane

FIG. 2. H26–57C is efficiently inserted into the membrane in the correct orientation and processed by signal peptidase. A, H26–57C was translated in vitro using a rabbit reticulocyte lysate in the absence (lanes 1 and 4) or presence (lanes 2 and 5) of dog pancreatic microsomal membranes. Translations in the presence of microsomal membranes were subsequently treated with proteinase K (lanes 3 and 6). The c-Myc epitope is susceptible to proteinase K digestion and the HA epitope is protected within the lumen of the microsome. Each of the reactions was immunoprecipitated with the c-Myc or HA antibodies as indicated. Differences in band intensity may be due to varying efficiency of translation. B, H26–57C was translated in vitro using a rabbit reticulocyte lysate in the absence (lanes 1) or presence (lanes 2) of dog pancreatic microsomal membranes and compared with cell-associated H26–57C metabolically labeled in stably-transfected 293 cells (lane 3). H26–57C was immunoprecipitated with the HA antibody. C, the media from H26–57C transfected cells was immunoprecipitated with each antibody. The arrow points to a 2.9-kDa band that is immunoprecipitated by the HA antibody but not the c-Myc antibody, corresponding to the expected product of γ-secretase cleavage. D, synthetic peptides spanning the region from the HA epitope to residue 40 (HA-40) and 42 (HA-42) of the probe were run on a 16.5% Tris-Tricine SDS-PAGE and stained in Coomassie Blue. Although the same amount of synthetic peptide was loaded in D, the HA-40 peptide does not stain well with Coomassie Blue. Both synthetic peptides run at the approximate molecular mass of the secreted 2.9-kDa fragment.
Degradation and Processing of the APP Transmembrane Domain

was examined by subjecting the translations performed in the presence of microsomes to proteinase K digestion. After digestion with proteinase K, the probe is no longer immunoprecipitable with the c-Myc antibody (Fig. 2A, lane 3), but with the HA antibody a smaller fragment is observed (Fig. 2A, lane 6). These results imply that the c-Myc epitope is exposed to the cytoplasmic (outside) surface of the microsomal membranes and is cleaved by proteinase K, while the HA epitope is within the lumen of the microsomal membranes, the correct orientation. When Triton X-100 is included during the proteinase K digestion of intact microsomes and is labeled with [35S]Met indicates that probe is cleaved within the transmembrane domain (Fig. 1). In addition, the expected carboxyl-terminal fragment resulting from γ-secretase cleavage with the TMD that contains the c-Myc epitope is not detected in the media, nor is full-length H26–57C. Immunoprecipitation of the media with the c-Myc and HA antibodies reveals that the cells secrete a 2.9-kDa fragment that is detected with the HA antibody and not the c-Myc antibody (Fig. 2C), consistent with the expected product of γ-secretase cleavage (Fig. 1). In addition, the expected carboxyl-terminal fragment resulting from γ-secretase cleavage with the TMD that contains the c-Myc epitope is not detected in the media, nor is full-length H26–57C. The secreted 2.9-kDa fragment comigrates at the approximate size of synthetic peptides spanning the region from the HA epitope to residue 40 (HA-40) or residue 42 (HA-42) within the transmembrane domain (Fig. 2D). The fact that the secreted fragment is smaller than the one obtained from proteinase K digestion of intact microsomes and is labeled with [35S]Met indicates that probe is cleaved within the transmembrane domain between residues 35 and the cytosolic end of the TMD at approximately residue 52.

The structures of the secreted fragments were further characterized by mass spectrometry after immunoprecipitation with the HA antibody (Fig. 3). Eleven different fragments are observed in the mass range of 2184.5 to 2880, indicating that considerable amino- and carboxyl-terminal heterogeneity is present in the secreted products. The amino termini of the fragments begin at residues 12 and 14–18 (according to the numbering scheme for human Aβ), consistent with the predicted site of signal peptidase cleavage between residues 11 and 12 and the expectation that the fragments should contain all or most of the HA epitope tag (residues 17–25). Fragments with carboxyl termini ending at residues 35 and 37–42 were identified (Table I). This is similar to the distribution of secreted Aβ products reported for human neuroblastoma cells (53) and transfected mouse neuroblastoma cells (54). Comparisons of the relative peak intensities in terms of fragment concentrations are inappropriate, since the ionization efficiency of the different species can vary dramatically (54). It is not clear whether this carboxyl-terminal heterogeneity represents a broad site specificity of γ-secretase-type processing or arise by subsequent proteolysis or peptidase processing. These results suggest that the probe is a substrate for the same type of γ-secretase processing that produces secreted Aβ and demonstrate that the TMD is sufficient for recognition by the enzymes responsible for γ-secretase activity.

Turnover of H26–57C—The turnover of H26–57C in stably transfected cells was examined by pulse-chase analysis to determine the half-life of the probe and whether any intermediates associated with degradation could be detected. This experiment was performed both in the presence (Fig. 4A, top panels) or absence (Fig. 4A, bottom panels) of BFA, which blocks ER to Golgi transit, to determine whether egress from the ER is necessary for turnover. Fig. 4A shows the products immunoprecipitated from the RIPA soluble lysate that were quantitated to generate the data in Fig. 4B. Fig. 4B reveals that the turnover is very rapid and indistinguishable using antibodies.
Degradation and Processing of the APP Transmembrane Domain

TABLE I

| Observed mass | Segment | Expected mass | Sequence |
|---------------|---------|---------------|----------|
| 2184.5        | Pro16-Met35 | 2184.6 | PYPYDPVDASYNKGAIIGLMVGG |
| 2233.4        | Pro15-Val38 | 2236.6 | PYPYDPVDASYNKGAIIGLVMVG |
| 2302.0        | Tyr17-Gly38 | 2300.6 | PYPYDPVDASYNKGAIIGLVMG |
| 2337.0        | Pro16-Val39 | 2335.7 | EYPYDPVDASYNKGAIIGLVMG |
| 2411.6        | Glu14-Met35 | 2412.7 | PYPYDPVDASYNKGAIIGLVMG |
| 2498.0        | Pro16-Val39 | 2496.9 | PYPYDPVDASYNKGAIIGLVMG |
| 2596.8        | Pro16-Val40 | 2596.0 | PYPYDPVDASYNKGAIIGLVMG |
| 2690.0        | Tyr17-Ile41 | 2611.4 | PYPYDPVDASYNKGAIIGLVMG |
| 2753.0        | Ala12-Gly37 | 2753.2 | PYPYDPVDASYNKGAIIGLVMG |
| 2781.0        | Pro16-Ala42 | 2780.2 | PYPYDPVDASYNKGAIIGLVMG |
| 2880.2        | Val15-Ala42 | 2879.4 | PYPYDPVDASYNKGAIIGLVMG |

**Fig. 4.** The turnover of H26–57C is very rapid in the presence and absence of BFA. Cells were incubated in methionine-deficient media for 40 min, pulsed for 5 min with [35S]methionine, rinsed in PBS, and chased in medium containing an excess of unlabeled methionine (0.3 mg/ml). At the indicated times, the media was removed and the cells lysed. The soluble cell lysate was immunoprecipitated with the c-Myc or HA antibody, A, autoradiograph of the soluble cell lysate immunoprecipitates. Top panels were performed in the presence of BFA, and bottom panels were performed in the absence of BFA. No higher molecular weight intermediates or breakdown products are detected during the turnover. B, quantitation of the bands from A using a Molecular Dynamics PhosphorImager. 100% is the amount of H26–57C at the end of the pulse (0 min).

to the lumenal and cytosolic domains of H26–57C. The kinetics of degradation are very similar in the presence and absence of BFA. Most of the probe is degraded with a half-life of approximately 8 min, well within the range of reported half-lives for proteins degraded in the ER (10, 41). No intermediates associated with the turnover of H26–57C were detected in the RIPA soluble (Fig. 4A) or RIPA insoluble lysate (data not shown), even with much longer exposures of the PhosphorImager plate. These results indicate that the vast majority of the probe is degraded in the ER by a highly processive mechanism. Once a probe molecule is selected for degradation, turnover is so rapid that no intermediates are detected at a limit of detection of about 1% of the initial amount of the probe.

**Effects of Inhibitors on the Turnover of H26–57C and Secretion of the 2.9-kDa Fragment—**H26–57C-transfected cells were treated with several protease and metabolic inhibitors to identify potential proteases involved in the degradation of H26–57C and to stabilize potential degradation intermediates. Leupeptin, E64, and pepstatin treatment have little or no effect on the steady-state levels of H26–57C or on the secretion of the 2.9-kDa fragment and no intermediates are detected (data not shown). Similarly, chloroquine, ammonium chloride, and phosphoramidon have no effect. Treatment of cells with BFA has only a slight effect in decreasing the steady-state levels of the probe (Fig. 5A), but it inhibits secretion of the 2.9-kDa fragment (Fig. 5B). The 2.9-kDa fragment is not detected in the cell lysate of BFA-treated cells, indicating that this product does not accumulate in the ER when ER-Golgi trafficking is inhibited. ALLN has been shown to inhibit calpains, the proteasome, and the lysosomal cysteine proteases cathepsin B and cathepsin L (42–44). Different concentrations of ALLN (calpain I inhibitor) and ALLM (calpain II inhibitor) were evaluated to determine the consequence on the steady-state level of H26–57C (Fig. 6A) and secretion of the 2.9-kDa fragment (Fig. 6B). Both ALLN and ALLM cause a dramatic increase in the steady-state amount of H26–57C, with ALLN being more effective at lower concentrations. Concentrations of ALLN greater than 135 μM produce no further increase in the accumulation of the probe. Control cells treated with vehicle only (MeSO) have no effect on the degradation of the probe or secretion of the 2.9-kDa fragment. Quantitation of the gel images indicates that the steady-state concentration is increased by 50-fold in the presence of 135 μM ALLN. We also examined whether any ubiquitinated derivatives of the probe accumulate in the presence of ALLN. No higher molecular weight species that might represent ubiquitinated derivatives of H26–57C are immunoprecipitated with either antibody and Western blots of the immunoprecipitated probe from ALLN-treated cells using anti-ubiquitin antibodies do not detect any ubiquitinated species (data not shown). Secretion of the 2.9-kDa fragment is also increased up to 50-fold when cells are treated with the ALLN and ALLM (Fig. 6B). 135 μM ALLN is the most effective concentration at increasing the secretion of the 2.9-kDa fragment. To examine whether similar effects are observed with the secretion of Aβ from APP, APP751 transfected 293 cells were metabolically labeled overnight in the presence of ALLN and the media was immunoprecipitated with an antibody specific to Aβ (Fig. 7). ALLN treatment causes a 3-fold increase in the secretion of Aβ. The p3 fragment of Aβ is also increased in the presence of ALLN, but the failure to observe a distinct band in
the untreated controls precludes any quantitation of the increase in this fragment. The simplest explanation for increased secretion of the 2.9-kDa fragment is that stabilization of H29–57C by ALLN makes more substrate available to g-secretase. Accordingly, the smaller magnitude effect of ALLN on Aβ-secretase cleavage, and P3, the product of α- and γ-secretase cleavage.

during this chase period does not affect degradation (data not shown). In addition, a smaller molecular weight fragment is observed with the HA antibody that is not observed with the c-Myc antibody, indicating that the cytoplasmic epitope tag has been removed. This fragment is approximately the same size as the proteinase K digestion product seen in the in vitro translation experiments (Fig. 2A). The fragment appears rapidly at time 0 after ALLN is removed and cells are rinsed with PBS (~4 min), and the amount of this smaller fragment remains constant as the amount of the full-length probe decreases. It is not detected in steady-state experiments because cells are immediately lysed after removing the media. Once formed, the intermediate may be eliminated by an ALLN insensitive pathway, because no intermediate is detectable when ALLN is added back to cells for 6 h after the PBS rinse (Fig. 8, 6 + ALLN). Since the cytoplasmic epitope tag has been cleaved from the probe, we determined whether the fragment remains associated with the membrane by subcellular fractionation and carbonate extraction (45). Virtually all of the intact probe and the soluble lysate was collected and immunoprecipitated with each of the antibodies. 6 + ALLN represents cells that were treated with [35S]methionine and ALLN overnight, rinsed in PBS, and chased in medium containing ALLN and an excess of unlabeled methionine for 6 h. The intermediate fragment appears rapidly after the PBS rinse at time 0 and its concentration remains constant between 1 and 6 h as the amount of the intact probe decays. The intermediate is not detectable in the lane labeled 6 + ALLN indicating that the small amount produced during the PBS rinse is degraded in the presence of ALLN.

FIG. 5. BFA blocks secretion of the 2.9-kDa fragment of H26–57C into the media. Cells were labeled with [35S]methionine in the presence or absence of BFA for 4 h, the media was collected, and the cells were lysed in RIPA buffer. A, the soluble cell lysate of untreated (Untr.) or BFA-treated (BFA) cells was immunoprecipitated with each antibody. No cell associated intermediates are detected. B, the media of untreated or BFA-treated cells was immunoprecipitated with the HA antibody. The 2.9-kDa fragment is not observed in the media of BFA-treated cells.
that focus on the proteolysis and turnover of the transmembrane domain of a protein is this detail. The probe offers several advantages for specifically focusing on the proteolysis of the transmembrane domain. The ability to immunoprecipitate the probe from the two sides of the membrane helps to overcome a serious ambiguity associated with using a single epitope. With a single epitope, you can only observe one of the products of a cleavage event and it may be difficult to distinguish between cleavage of the transmembrane domain and the destruction of the epitope. The small size of the probe and the location of the epitope tags at the extreme ends simplify the interpretation of the apparent molecular weight of fragments in terms of the actual site of cleavage on the probe. In view of the small size and non-natural structure of the probe, it is both fortuitous and remarkable that it is efficiently inserted into the rough endoplasmic reticulum as evidenced by the quantitative removal of the signal sequence, both in vitro and in transfected cells. Even though H26–57C is not a naturally occurring product, it appears to function as a substrate for γ-secretase processing. Secreted products with carboxyl-terminal ends at residues 35 and 37–42 were identified in the culture media by mass spectroscopy after immunoprecipitation. A similar spectrum of carboxyl-terminal products has been reported for secreted Aβ (53, 54). These results indicate that the H29–56C is subjected to the same type γ-secretase cleavage that produces secreted Aβ and suggest that the transmembrane sequence of APP alone is sufficient to make it a substrate for γ-secretase.

The analysis of the turnover of the probe indicates that most of the probe is rapidly degraded in the ER by a highly efficient and progressive mechanism. The destruction of the probe in the ER may be due to the fact that it is recognized as an aberrant protein by the quality control mechanisms of the ER. No smaller fragments or higher molecular weight intermediates are observed at a limit of detection of approximately 1% of the total initial amount of the probe. If proteolytic events on the cytoplasmic or lumenal side of the membrane were random and uncoupled, you would expect to find at least a few partially digested morsels. Rather, the results indicate that once degradation is initiated, the probe is rapidly depolymerized. Because the probe is so efficiently destroyed, inhibitors of degradation that might help identify intermediates and define the proteolytic enzyme systems operating on H26–57C were screened. Of all the inhibitors examined, ALLN and MG132 are the most effective in preventing its degradation, leading to a 50-fold increase in the steady-state concentration of the probe and a corresponding increase in its half-life. The probe is still degraded, albeit slowly in the presence of ALLN with a half-life of approximately 10 h and this degradation is not sensitive to lysosomal hydrolase inhibitors. Either ALLN does not completely inhibit its target proteases or the recently described ALLN-insensitive ER lumen pathway slowly degrades it (49). The ALLN-insensitive pathway may include γ-secretase, since inhibition of the ALLN-sensitive pathway increases the secretion of the 2.9-kDa fragment and Aβ. The finding that ALLN has opposite effects on the secretion of the 2.9-kDa extracellular fragment and the degradation of the probe in the ER indicates that these pathways are separate and distinct. The simplest interpretation for this inverse relationship is that inhibition of the destruction of the probe in the ER makes more substrate available to the γ-secretase pathway. Whether γ-secretase processing involves a single cleavage event within the transmembrane domain is still a mystery, because even in the presence of ALLN and lactacystin, the complementary fragment that contains the cytoplasmic epitope tag is not detectable. This cytoplasmic fragment of APP is also not detected in APP-transfected cells (39). These results do not rule out the

**DISCUSSION**

A novel fusion protein has been developed to probe the mechanisms and pathways of the degradation of the transmembrane domain of the APP. We are not aware of any previous studies
possibility that the ER degradation system plays a role in generating Aβ under other, presumably pathological, circumstances.

The fact that ALLN inhibits multiple proteases, including the proteasome, complicates the interpretation of which ALLN-sensitive proteases actually degrade the probe. Because the more specific proteasome inhibitor, lactacystin, significantly slows the turnover of H26–57C, this suggests that the proteasome plays a role in its catabolism. However, the fact that it is much less effective than ALLN argues that there is at least one additional pathway that is capable of degrading the probe. These findings are similar to the “two-step” mechanism recently proposed for the processing of the SREBP (50), but in this processing pathway, the first cleavage event occurs on the luminal side of membrane. When the degradation of H26–57C resumes after washing out the ALLN, the first cleavage event observed is the removal of the cytoplasmic epitope tag, producing a smaller fragment that is no longer immunoprecipitable with the e-Myc antibody. This fragment appears rapidly (within 4 min) after wash-out of the inhibitor with PBS and the amount of fragment stays constant as the full-length H26–57C decays, suggesting that its production is the rate-limiting step for the turnover of the probe under these conditions. It is interesting to note that this fragment is the same size as the in vitro protease K digestion product also detected with the HA antibody and the slow degradation of the fragment in cells may be related to the protease resistance of the fragment that we detected in vitro. The subsequent degradation of the fragment may occur via the ALLN-insensitive pathway, since the fragment disappears from cell cultures that are re-incubated in ALLN during the chase for 6 h (Fig. 8D, 6 + ALLN) following the PBS rinse. In this regard the degradation is similar to the two-site model proposed for apoB degradation in which the carboxyl terminus of partially-translocated apoB is cleaved by the proteasome and the luminal fragment is degraded by an ALLN-insensitive pathway (49).

These results also argue that calpains are not likely candidates for γ-secretase activity, since the calpain inhibitors ALLN and ALLM increase the amount of γ-secretase product secreted into the media from both H26–57C and APP. ALLN has been reported to reduce the secretion of total Aβ into the media (24). This group has reported that ALLN has opposite effects on the secretion of Aβ1–42 and Aβ1–40; inhibiting the secretion of Aβ1–40 and increasing the secretion of Aβ1–42. However, these experiments used a mutant variant of APP that already has increased levels of Aβ secreted compared with wild type (24). Another group has recently reported the similar increase in Aβ secretion that we observe (51). These discrepancies are not due to experimental variability, since the increased secretion of the γ-secretase product that we have observed has proven to be extremely reproducible in four separate experiments using H26–57C- and APP-transfected cells. One plausible explanation that would reconcile these seemingly opposite results would be if our APP-transfected cells secrete predominantly Aβ1–42, but this remains to be established by further experimentation.

The pathway that degrades the H26–57C probe is distinct from the proteasome pathway that has been described for the MHC class I heavy chain (10, 11) in at least two respects. In this system, the newly translocated MHC heavy chain is reverse translocated through the translocation pore into the cytosol, where it exists as a soluble, ubiquitinated intermediate. The turnover of the cystic fibrosis transmembrane conductance regulator and the yeast mutant carboxypeptidase y Y is also associated with the appearance of a ubiquitinated species (8, 13, 52). In the presence of ALLN and lactacystin, the levels of the cystic fibrosis transmembrane conductance regulator-ubiquitinated intermediate accumulate dramatically. For H26–57C, virtually all of the probe remains membrane associated and is resistant to carbonate extraction and no higher molecular weight potentially ubiquitinated adducts are observed in the presence of ALLN and lactacystin. In this regard, the turnover of H26–57C is more like 3-hydroxy-3-methylglutaryl-CoA reductase (9, 15), in which the protein appears to remain membrane associated and ubiquitinated species have not been observed. Under normal circumstances, both the proteasome and luminal proteolysis systems may cooperate to produce the highly processive degradation pattern observed for H26–57C in the absence of inhibitors. In summary, there appear to be multiple pathways for the degradation of the transmembrane domains of proteins that may cooperate to effect a selective, highly efficient and processive mechanism for removing them. Whether the failure of these systems to completely degrade or recycle proteins leads to the production of potentially pathogenic peptides, like Aβ1–42 in Alzheimer’s disease remains to be determined.

Acknowledgment—We thank Dr. Sam Sisodia for helpful discussions and suggestions.

REFERENCES

1. Hurtley, S. M., and Helenius, A. (1989) Annu. Rev. Cell Biol. 5, 277–307
2. Wang, C. L., and Koppito, B. (1994) J. Biol. Chem. 269, 25710–25718
3. Wikstrom, L., and Lodish, H. F. (1992) J. Biol. Chem. 267, 5–8
4. Wileman, T., Kane, L. P., and Terhorst, C. (1991) Cell Regul. 2, 753–765
5. Amara, J. F., Lederkremer, G., and Lodish, H. F. (1989) J. Cell Biol. 109, 3315–3324
6. Bonifacino, J. S., Suzuki, C. K., and Klausner, R. D. (1990) Science 247, 79–82
7. Bonifacino, J. S., Conson, P., Shah, N., and Klausner, R. D. (1991) EMBO J. 10, 2783–2793
8. Jensen, T. J., Loo, M. A., Pint, S., Williams, D. B., Goldberg, A. L., and Riordan, J. R. (1995) Cell 83, 129–135
9. Inoue, S., and Simoni, R. D. (1992) J. Biol. Chem. 267, 9080–9086
10. Wiertz, E. J., Jones, T. R., Rush, M., Lugo, M., Guzeu, J., and Hlpesto, H. L. (1996) Nature 384, 432–438
11. Hughes, E. A., Hammond, C., and Cresswell, P. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 1896–1901
12. Miller, M. M., Fingier, A., Schweiger, M., and Wolf, D. H. (1996) Science 273, 1725–1728
13. Qu, D., Teckman, J. H., Omura, S., and Perlmutter, D. H. (1996) J. Biol. Chem. 271, 22791–22795
14. McGeer, T. P., Cheng, H. H., Kumagai, H., Omura, S., and Simoni, R. D. (1996) J. Biol. Chem. 271, 25630–25638
15. Younkin, S. G. (1995) Ann. Neurol. 37, 287–288
16. Burdick, D., Soreghan, B., Kwon, M., Kasowski, J., Knauser, M., Henschen, A. Yates, J., Cotman, C., and Glabe, C. (1992) J. Biol. Chem. 267, 546–554
17. Hilbich, C., Kisters-Woike, B., Reed, J., Masters, C. L., and Beyreuther, K. (1991) J. Mol. Biol. 218, 149–163
18. Knauser, M. F., Soreghan, B., Kasowski, J., and Glabe, C. G. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7437–7441
19. Younkin, S. G. (1995) J. Biol. Chem. 270, 31853–31859
20. Koo, E. H., and Squazzo, S. L. (1994) J. Biol. Chem. 269, 17368–17389
21. Citron, M., Westaway, D., Xia, W., Carlson, G., Diehl, T., Levesque, G., Johnson, J., Wood, K., Davis, A., Sherrington, R., Perry, B., Yao, H., Strome, R., Lieberburg, I., Rommens, J., Kim, S., Schenk, D., Fraser, P., St George-Hyslop, P., and Selkoe, D. J. (1997) Nat. Med. 3, 67–72
22. Doan, A., Thiknakanar, G., Borchelt, D. R., Slunt, H. H., Ratovitskay, T. Podilinsky, M., Selkoe, D. J., Seeger, M., Gandy, S. E., Price, D. L., and Sisodia, S. S. (1996) Nature 383, 1023–1030
23. Sherrington, R., Fredrich, S., Campion, D., Chi, H., Rogavea, E. A., Levesque, G., Rogave, E. I., Lin, C., Liang, Y., Ibele, M., Mar, L., Brice, A., Agid, Y., Percy, M. E., Clerget-Darpoux, F., Picciomonti, S., Marcon, G., Norciais, B., Amaducci, L., Frebourg, T., Lanzafame, L., Rommens, J. M., and St George-Hyslop, P. H. (1995) Nature 375, 754–760
24. Turner, R. S., Suzuki, N., Ching, A. S. C., Younkin, S. G., and Lee, V. M. Y. (1996) J. Biol. Chem. 271, 8966–8970
25. Wild, B., C. Yamazaki, T. Capell, A., Leiner, U., Steiner, H., Ibara, Y., and Haase, C. (1997) J. Biol. Chem. 272, 16085–16088
26. Cook, D. F., Forman, M. S., Stung, J. C., Leight, S., Kolsen, D. L., Iwatsubo, T., Lee, V. M., and Dom, M. W. (1997) Nat. Med. 3, 1021–1025
Degradation and Processing of the APP Transmembrane Domain

32. Haass, C., Hung, A. Y., Schlossmacher, M. G., Teplow, D. B., and Selkoe, D. J. (1993) J. Biol. Chem. 268, 3021–3024
33. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1988) Current Protocols in Molecular Biology, Vol. I, Greene Publishing Associates and Wiley-Interscience, New York
34. Laemmli, U. K. (1970) Nature 227, 680–685
35. Schagger, H., and von Jagow, G. (1987) Anal. Biochem. 166, 368–379
36. Wilson, I. A., Nimn, H. L., Houghten, R. A., Cherenson, A. R., Connolly, M. L., and Lerner, R. A. (1984) Cell 37, 767–778
37. Egan, G. I., Lewis, G. K., Ramsay, G., and Bishop, J. M. (1985) Mol. Cell. Biol. 5, 3610–3616
38. Jarrett, J. T., Berger, E. P., and Lansbury, P. T., Jr. (1993) Biochemistry 32, 4693–4697
39. Haass, C., Schlossmacher, M. G., Hung, A. Y., Vigo-Pelfrey, C., Mellon, A., Ostaszewski, B. L., Lieberburg, I., Koo, E. H., Schenk, D., Teplow, D. B., and Selkow, D. J. (1992) Nature 359, 322–325
40. Tischer, E., and Cordell, B. (1996) J. Biol. Chem. 271, 21914–21919
41. Stafford, F. J., and Bonifacino, J. S. (1991) J. Cell Biol. 115, 1225–1236
42. Hiwasa, T., Sawada, T., and Sakiyama, S. (1990) Carcinogenesis 11, 75–80
43. Eick, K. L., Gramm, C., Rothstein, L., Clark, K., Stein, R., Dick, L., Hwang, D., and Goldberg, A. L. (1994) Cell 78, 761–771