Cleavage of Alzheimer's Amyloid Precursor Protein by α-Secretase Occurs at the Surface of Neuronal Cells†

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Received March 23, 1999; Revised Manuscript Received May 20, 1999

ABSTRACT: The amyloid precursor protein (APP) is proteolytically processed predominantly by α-secretase to release the ectodomain (sAPPα). In this study, we have addressed the cellular location of the constitutive α-secretase cleavage of endogenous APP in a neuronal cell line. Incubation of the neuroblastoma cell line IMR32 at 20 °C prevented the secretion into the medium of soluble wild-type APP cleaved by α-secretase as revealed by both immunoelectrophoretic blot analysis with a site-specific antibody and immunoprecipitation following metabolic labeling of the cells. No sAPPα was detected in the cell lysates following incubation of the cells at 20 °C, indicating that α-secretase does not cleave APP in the secretory pathway prior to or within the *trans*-Golgi network. Parallel studies using an antibody that recognizes specifically the neoepitope revealed on soluble APP cleaved by *β*-secretase indicated that this enzyme was acting intracellularly. α-Secretase is a zinc metalloproteinase susceptible to inhibition by hydroxamate-based compounds such as batimastat [Parvathy, S., et al. (1998) *Biochemistry 37*, 1680−1685]. Incubation of the cells with a cell-impermeant, biotinylated hydroxamate inhibitor inhibited the release of sAPPα by >92%, indicating that α-secretase is cleaving APP almost exclusively at the cell surface. The observation that α-secretase cleaves APP at the cell surface, while *β*-secretase can act earlier in the secretory pathway within the neuronal cell line indicates that there must be strict control mechanisms in place to ensure that APP is normally cleaved primarily by α-secretase in the nonamyloidogenic pathway to produce the neuroprotective sAPPα.

Alzheimer's disease is a neurodegenerative disorder characterized by the progressive deposition of the 4 kDa *β*-amyloid peptide (A*β*)[1] in extracellular senile plaques. A*β* is a 40−43 amino acid peptide derived by proteolytic cleavage of the integral membrane *β*-amyloid precursor protein (APP) (*1*, *2*). Cleavage of APP at the N-terminus of the A*β* peptide by *β*-secretase and at the C-terminus by one or more *γ*-secretases constitutes the amyloidogenic pathway for processing of APP (Figure 1b). *β*-Secretase acts on APP both within the endocytic pathway following reinternalization of cell-surface APP (*3*−*5*) and in the secretory pathway within the endoplasmic reticulum and Golgi (*6*, *7*). In the nonamyloidogenic pathway, α-secretase cleaves APP within the A*β* domain between Lys16 and Leu17 (*8*), precluding the formation of the A*β* peptide (Figure 1a). The action of α-secretase releases the large ectodomain of APP (sAPPα) which has neuroprotective properties (*9*, *10*).

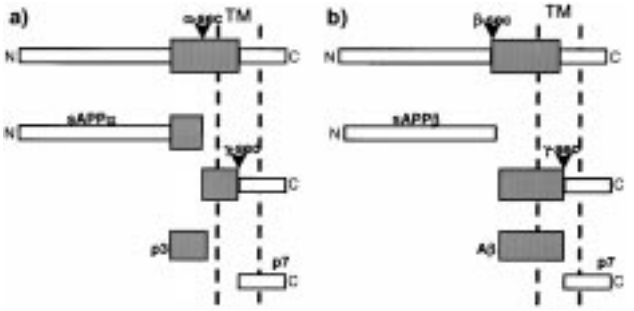

FIGURE 1: Schematic representation of APP and its processing by α-, *β*- and *γ*-secretases. APP (open bar), which is attached to the cell surface by a transmembrane region (TM), is processed via either (a) the nonamyloidogenic or (b) amyloidogenic pathways. In the nonamyloidogenic pathway α-secretase (α-sec) cleaves APP within the A*β* peptide releasing a large soluble fragment of APP (sAPPα). In the amyloidogenic pathway, *β*-secretase (*β*-sec) and *γ*-secretase (*γ*-sec) generate the A*β* peptide (grey box), and release the soluble sAPP*β*.

α-Secretase is an integral membrane protein (*11*, *12*) that is inhibited by hydroxamic-acid-based zinc metalloproteinase inhibitors such as batimastat, marimastat, and BB2116 (*13*). In addition, α-secretase has many properties in common with the secretase that cleaves angiotensin converting enzyme from the membrane (*14*), including a similar structure−activity relationship with a number of hydroxamic-acid-based inhibitors (*13*). However, the cellular location of the α-secretase cleavage of APP has been the subject of much debate. There is conflicting data as to whether α-secretase cleaves

† We thank the Medical Research Council (U.K.) for financial support of this work. I.H. was in receipt of a Science and Engineering Research Council CASE studentship in association with SmithKline Beecham (Harlow, U.K.). S.P. was in receipt of an Emma and Leslie Reid studentship from the University of Leeds.

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[1] Abbreviations: APP, amyloid precursor protein; A*β*, *β*-amyloid peptide; MDCK, Madin Darby Canine Kidney; PVDF, poly(vinylidene) difluoride; sAPPα, APP released by α-secretase; sAPP*β*, APP released by *β*-secretase; TGN, *trans*-Golgi network.

10.1021/bi9906827 CCC: $18.00
Published on Web 07/01/1999

APP at the cell surface, within an intracellular compartment, or at both locations (discussed in refs *1*, *15*, and *16*). For example, the lack of effect of exogenously added proteinase inhibitors and the intracellular presence of cleaved APP following disruption of the secretory pathway has been taken to indicate that APP is cleaved within the *trans*-Golgi network (TGN) and/or exocytic vesicles (*17*−*20*). In contrast, following surface labeling with either ^{125}I or biotin at 4 °C, the detection of labeled APP in the medium upon subsequent incubation of the cells at 37 °C suggested that APP is cleaved at the cell surface (*4*, *11*, *21*−*23*). However, none of these studies distinguished between sAPPα and other forms of APP released by the cells, and therefore, it is possible that some of the releasing activity was due to *β*-secretase or other secretases in addition to, or instead of, α-secretase. Although an antibody specific for residues 1−16 of A*β* was used to distinguish between sAPPα and the large ectodomain of APP released by *β*-secretase (sAPP*β*) in a cell-free system using a reporter construct of APP (*12*), a crude membrane preparation was used which included both the plasma membrane and internal membranes. A site-specific antibody was also used to immunoisolate biotinylated sAPPα from the conditioned medium of surface biotinylated mouse N2a neuroblastoma cells expressing wild-type APP (*24*). However, in both of these studies, the relative contribution of surface to internal α-secretase action was not determined. Another study, again using site-specific antibodies, revealed that surface biotinylated APP was secreted from the apical and basolateral faces of the nonneuronal Madin Darby Canine Kidney (MDCK) cells stably transfected with APP (*25*), implicating that α-secretase acts at the cell surface. However, in a separate study, again using stably transfected MDCK cells, the same authors observed that sAPPα was generated intracellularly, leading them to conclude that the majority of sAPPα is generated within the secretory pathway, with a minor proportion being cleaved after its insertion into the plasma membrane (*26*). Another study indicated that, in human embryonic kidney cells transfected with mutant APP, cleavage by α-secretase occurs during transport of APP through Golgi compartments where O-glycosylation takes place or in compartments after the *trans*-Golgi (*27*).

The α-secretase cleavage of APP is known to be upregulated by phorbol esters and other activators of protein kinase C (*1*). In a previous study, we showed that batimastat blocks both the constitutive and regulated secretion of sAPPα (*13*), and thus in the present study, we examined only the constitutive release of sAPPα. To address the question of whether α-secretase cleaves APP in neuronal cells at the cell surface or within an intracellular compartment within the late secretory pathway, we have compared directly the constitutive production of sAPPα and sAPP*β* from endogenous wild-type APP in a neuronal cell line (IMR32) following disruption of the secretory pathway. In addition, we have exploited our recent observation that α-secretase is inhibited by hydroxamic-acid-based zinc metalloproteinase inhibitors (*13*) to determine the relative contribution of cell surface to intracellular α-secretase activity by using a cell-impermeant biotinylated derivative of one such inhibitor. These results clearly show that the constitutive α-secretase cleavage of APP occurs almost completely at the cell surface.

EXPERIMENTAL SECTION

Cell Culture. The neuronal cell line IMR32 was cultured in Dulbecco's modified Eagle's medium/Ham's F12 supplemented with 10% foetal bovine serum, penicillin (50 units/mL), streptomycin (50 *μ*g/mL), and 2 mM glutamate (Gibco BRL, Paisley, U.K.). Cells were maintained at 37 °C in 5% CO_2 in air. When the cells were 70−80% confluent, the medium was changed to Opti-MEM and the cells incubated in the absence or presence of inhibitor [AMG 2380 or its biotinylated derivative AMG110552 (Amgen Inc., Boulder, CO)] for 7 h at 37 °C, or overnight at 20 or 37 °C. The medium was then harvested, concentrated approximately 50-fold using Vivaspin 15 Concentrators (Vivascience Ltd., Lincoln, U.K.) and subjected to immunoelectrophoretic blot analysis. For analysis of cell-associated APP, cells were washed with phosphate-buffered saline (20 mM Na_2HPO_4, 2 mM NaH_2PO_4, and 150 mM NaCl, pH 7.4) and scraped from the flasks into the same buffer. Following centrifugation at 500*g* for 10 min, the pelleted cells were lysed in lysis buffer [0.1 M Tris/HCl, 5 mM EDTA, and 1% Triton X-100, pH 7.4 containing leupeptin (1 *μ*g/mL) and dichloroisocoumarin (10 *μ*M)].

SDS−PAGE and Immunoelectrophoretic Blot Analysis. Samples (medium or cell lysates) were resolved on SDS−polyacrylamide gels and blotted onto poly(vinylidene) difluoride (PVDF) membranes (Immobilon P, Millipore) as described previously (*28*). Membranes were probed with antibodies to APP (Ab1−25 at a dilution of 1:4000; Ab1A9 at a dilution of 1:3000; Ab54 at a dilution of 1:20 000) followed by a secondary horseradish peroxidase-conjugated antibody. Membranes probed with Ab1A9 were subjected to an additional incubation with a peroxidase anti-peroxidase antibody. Bound antibody was detected with the enhanced chemiluminescent detection system (Amersham, Slough, U.K.). The specificities of the antibodies used have been previously reported (*13*). Ab1−25 recognizes the N-terminal sequence of the *β*A4 peptide and thus detects sAPPα. Ab1A9 recognizes the neoepitope formed at the C-terminus of the large ectodomain of APP following *β*-secretase cleavage (*29*). As this neoepitope is cryptic in both full-length APP and sAPPα, Ab1A9 recognizes only sAPP*β*. Ab54 recognizes the C-terminal cytosolic domain of APP and was used to detect full-length protein. Protein concentrations were determined using the bicinchoninic acid method (*30*) with bovine serum albumin as standard.

Metabolic Labeling and Immunoprecipitation. Cells were grown in 25 cm^2 flasks and, once confluent, were used for labeling. The growth medium was replaced with 8 mL of methionine-free Minimal Essential Medium supplemented with 10% foetal bovine serum, penicillin (50 units/mL) and streptomycin (50 *μ*g/mL). Following a 2 h incubation at 37 °C the medium was replaced with fresh methionine-free medium containing 100 *μ*Ci of [^{35}S]methionine in the absence or presence of biotinylated inhibitor (20 *μ*M), and the cells labeled continuously for 7 h either at 37 °C or at 20 °C. The medium was then removed, the cells washed in phosphate-buffered saline, harvested, and lysed in 1 mL of lysis buffer. Medium and cell lysate samples were precleared by adding 20 *μ*L of protein A-Sepharose that was previously made into a 1:1 solution with phosphate-buffered saline. After a 2 h incubation at 4 °C with continuous shaking, the

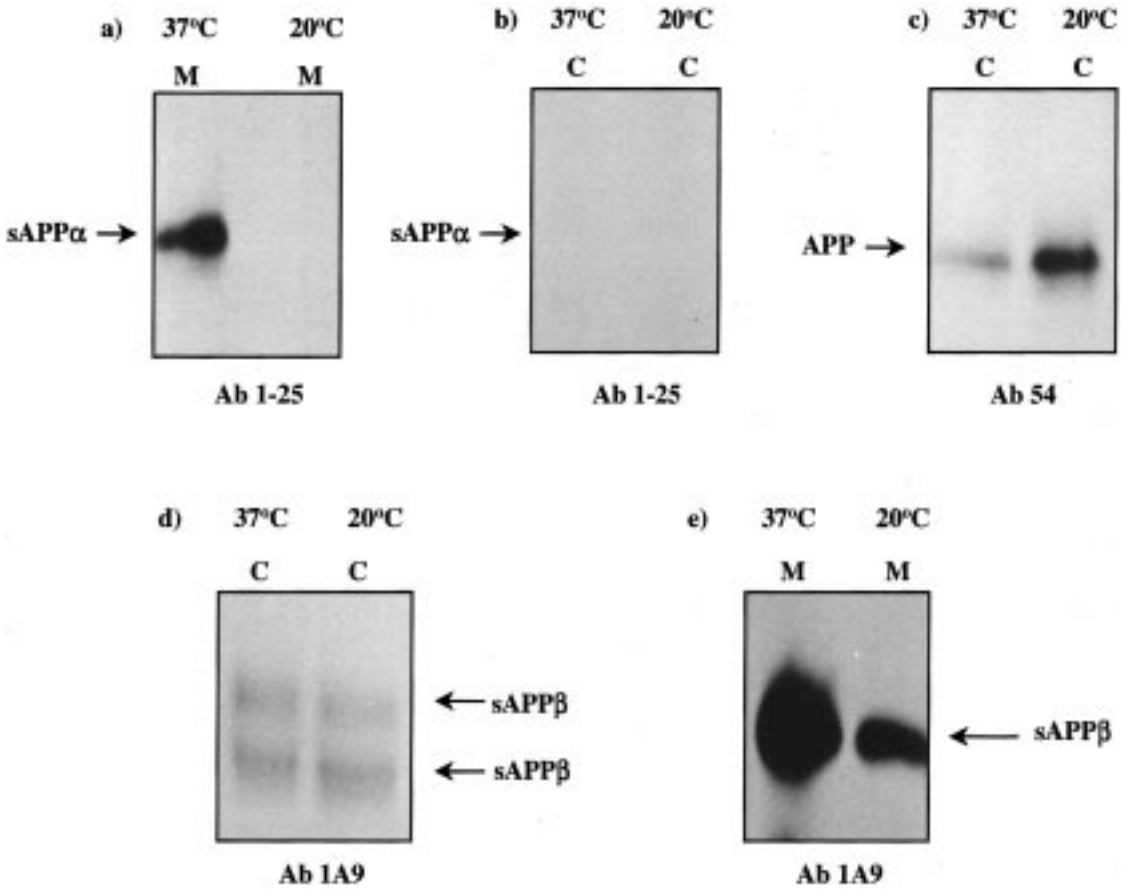

FIGURE 2: Effect of temperature block on the processing of APP. Concentrated conditioned medium samples (M; 25 μg of protein) or cell lysates (C; 30 μg of protein) were prepared from IMR32 cells incubated overnight either at 37 °C or at 20 °C as described in the Experimental Section. Samples were then resolved on 10% polyacrylamide SDS gels, electroblotted onto PVDF membranes and probed for (a and b) sAPPα with Ab1−25, (c) full-length APP with Ab54, (d and e) sAPPβ with Ab1A9.

Sepharose was removed by centrifugation. Antibodies were added at 1 in 1000 dilution to the supernatants and incubated overnight at 4 °C. Antibody−antigen complexes were precipitated by the addition of 30 μL of protein A-Sepharose. Following a 4 h incubation at 4 °C with continuous shaking, the Sepharose was pelleted by centrifugation and washed with phosphate-buffered saline containing 0.5% Triton X-100, 0.05% sodium deoxycholate, pH 8.0, followed by three more washes with 500 mM NaCl, 125 mM Tris/HCl, 0.5% Triton X-100, and 10 mM EDTA, pH 8.0. Finally, the Sepharose was boiled for 5 min with dissociation buffer [140 mM Tris/HCl, 80 mM dithiothreitol, 10% (w/v) SDS, 20% (v/v) glycerol, and 0.005% bromophenol blue, pH 6.8]. After removing the Sepharose by centrifugation, the supernatant was electrophoresed on a 10% polyacrylamide gel. The gels were dried and analyzed using a Fuji Film BAS1000 Bio-Imaging Analyzer.

RESULTS

α-Secretase Does Not Cleave APP Intracellularly in the Secretory Pathway. To investigate whether α-secretase cleaves APP intracellularly in a neuronal cell line, we disrupted the secretory pathway by incubation of the IMR32 cells at 20 °C, which causes proteins that are normally transported to the cell surface to accumulate in the TGN (*31*). IMR32 cells endogenously expressing APP were incubated either at 37 °C or at 20 °C and the conditioned medium and cell lysates subjected to immunoelectrophoretic blot analysis with site-specific antibodies. Following growth of the cells at 37 °C, sAPPα was clearly detected in the conditioned medium (Figure 2a) as reported previously (*13*). However, following growth of the cells at 20 °C, no sAPPα could be detected in the medium (Figure 2a), indicating that secretion of sAPPα was blocked. If α-secretase acts in a compartment of the secretory pathway prior to, or within, the TGN, treatment at 20 °C would be expected to increase the amount of sAPPα detected within the cells. No sAPPα was detected in the cell lysates following incubation of the cells either at 37 °C or at 20 °C (Figure 2b), indicating that sAPPα is not produced intracellularly even when transport beyond the TGN is blocked. Compared with growth of the cells at 37 °C, growth at 20 °C caused an accumulation of full-length APP within the cells (Figure 2c) consistent with an accumulation of APP in the TGN. In case the immunodetection system employed was not sufficiently sensitive, we also metabolically labeled the cells and used immunoprecipitation in an attempt to detect lower amounts of sAPPα. Although sAPPα could clearly be immunoprecipitated from the conditioned medium of cells grown at 37 °C (results not shown), no sAPPα was immunoprecipitated from the conditioned medium of cells grown at 20 °C. Again, immunoprecipitation failed to detect sAPPα in the cell lysates from cells grown either at 37 °C or at 20 °C (results not shown).

We used the neoepitope antibody 1A9 to detect sAPPβ, the product of β-secretase cleavage of APP. Following incubation of the IMR32 cells either at 37 °C or at 20 °C,

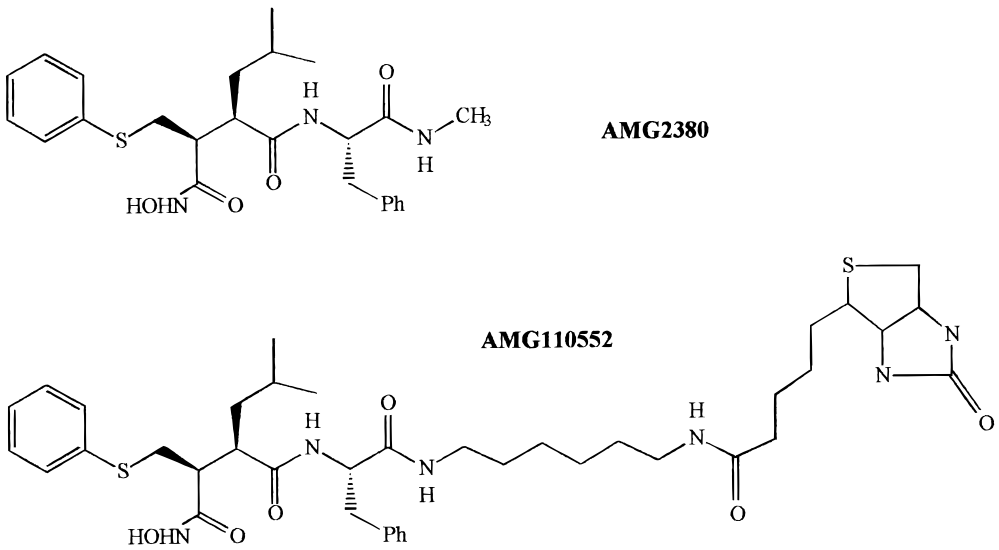

FIGURE 3: Structures of AMG2380 and its biotinylated derivative AMG 110552.

Ab1A9 clearly detected two polypeptide bands in the cell lysates (Figure 2d). The upper of the two polypeptide bands recognized by Ab1A9 corresponds in size to sAPPβ detected in the medium of the cells (Figure 2e), which is derived from the mature form of APP, while the lower polypeptide band corresponds in size to sAPPβ derived from the immature form of APP. This was confirmed by digestion with endoglycosidase-H, which revealed that the N-linked glycans on the lower polypeptide band were still in the high mannose form (result not shown), indicating that the protein had not undergone the further posttranslational modifications (sialylation, sulfation, and phosphorylation) that are present in the mature form (*32*, *33*). Interestingly, there was no observable difference in the levels of intracellular sAPPβ following incubation of the cells at 20 °C as compared with incubation of the cells at 37 °C, suggesting that accumulation of APP in the TGN does not lead to an increase in its cleavage by β-secretase. The release of sAPPβ into the medium was reduced, but not completely blocked, following growth of the cells at 20 °C (Figure 2e), indicating that a proportion of secreted sAPPβ is not dependent on transport through the TGN. Thus, even though we could clearly detect sAPPβ intracellularly, using two different detection methods we were unable to detect sAPPα intracellularly, indicating that α-secretase does not cleave APP significantly within compartments of the secretory pathway up to and including the TGN.

α-*Secretase Cleaves APP at the Cell Surface.* Although sAPPα was clearly not being produced in either the TGN or in an earlier compartment of the secretory pathway, the temperature block experiment does not indicate whether α-secretase is cleaving APP in a post-TGN intracellular compartment (such as exocytic vesicles) or at the cell surface. The inability to detect sAPPα even in the cell lysate from cells grown at 37 °C (Figure 2) would appear to indicate that sAPPα is not being produced significantly in a post-TGN intracellular compartment. To investigate this further, we used a biotinylated derivative of the hydroxamic-acid-based zinc metalloproteinase inhibitor AMG2380 (Figure 3) to inhibit α-secretase at the cell surface. Incubation of the IMR32 cells with the biotinylated inhibitor AMG110552 caused a dose-dependent inhibition of the release of sAPPα (Figure 4a), with an I_{50} value (determined by quantitative densitometric analysis of the immunoblots) of 0.55 μM (n = 2) (Figure 4d). This is comparable to the I_{50} values determined for the structurally related hydroxamate-based compounds batimastat and marimastat (*13*), indicating that modification of the inhibitor with biotin has no significant effect on its ability to inhibit α-secretase. The biotinylated inhibitor did not significantly affect the level of full-length APP present in the cells as assessed by densitometric analysis of the immunoblots (Figure 4b), indicating that it does not have a general effect on the expression level of APP. At a concentration of 20 μM, AMG110552 appeared to inhibit completely the release of sAPPα (Figure 4a). This was examined further by metabolically labeling the cells and immunoprecipitating sAPPα following incubation of the cells in the absence or presence of AMG110552 (Figure 4c). Quantitative analysis of the autoradiographs revealed that at a concentration of 20 μM AMG110552 inhibited the production of sAPPα by 92.1% (n = 2). Thus, the virtually complete block of sAPPα production by the biotinylated inhibitor indicates that α-secretase is cleaving APP almost entirely at the cell surface.

DISCUSSION

There is conflicting data as to whether α-secretase cleaves APP at the cell surface, within an intracellular compartment, or at both locations because previous studies have failed either to pinpoint the site of action of α-secretase and/or to quantitate the amount of cell surface to intracellular activity (*1*, *15*, *16*). In the present study, we have investigated the cellular site of action of α-secretase in a neuronal cell line by disrupting the secretory pathway at a late stage with a temperature block and by the novel approach of inhibiting α-secretase activity at the cell surface with a biotinylated inhibitor. Initially we determined whether cleavage of endogenous APP by α-secretase occurs within the TGN or an earlier compartment of the secretory pathway by incubating the cells at 20 °C, at which temperature proteins that are normally transported to the cell surface accumulate within the TGN (*31*). Under these conditions, the secretion of sAPPα, which is a direct measure of α-secretase activity, was completely blocked and no sAPPα was detected inside

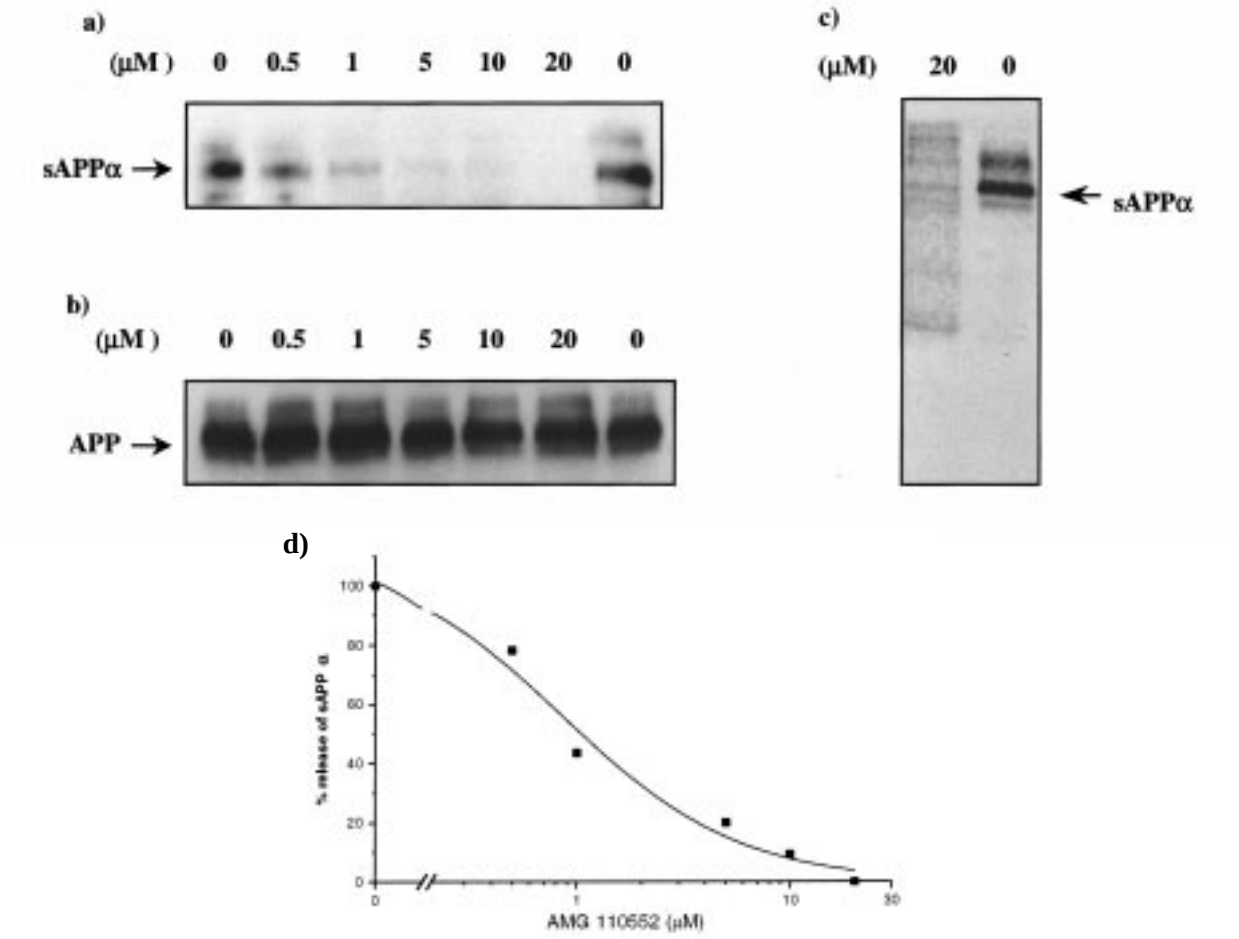

FIGURE 4: Inhibition of α-secretase by a biotinylated inhibitor. IMR32 cells were incubated in the presence of the indicated concentration of the biotinylated inhibitor AMG110552 as described in the Experimental Section. (a) The medium was harvested, concentrated and 45 μg of protein subjected to immunoelectrophoretic blot analysis for sAPPα with Ab1−25. (b) Full-length APP in the cell lysates (112 μg of protein) was subjected to immunoelectrophoretic blot analysis with Ab54. (c) sAPPα was immunoprecipitated from the medium of metabolically labeled cells incubated in the presence or absence of AMG110552. Following electrophoresis on 10% polyacrylamide gels sAPPα was detected by autoradiography. (d) Inhibition curve constructed from densitometric analysis of the immunoblots in (a). The curve is the best fit to the experimental points using the program Origin with Sigmoidal Best Fit.

the cells, indicating that APP is not cleaved by α-secretase in the TGN or in an earlier compartment of the secretory pathway. An alternative interpretation of these data is that α-secretase, or some enzyme or factor critical to its action, is temperature dependent. However, most mammalian enzymes are still partially active at 20 °C.

Direct evidence for α-secretase acting at the cell surface was obtained by using a biotinylated inhibitor. Previously, we have shown that batimastat, marimastat, and related hydroxamic-acid-based zinc metalloproteinase inhibitors can inhibit the release of α-secretase from a variety of cell types (IMR32, SH−SY5Y, HUVEC) with I_{50} values in the low micromolar range (*13*). These hydroxamic-acid-based compounds have no significant effect on the levels of either sAPPβ (*13*) or Aβ (*34*). All of these compounds are relatively hydrophobic (*28*), possessing bulky hydrophobic groups, and therefore the possibility that they may penetrate the cell, thus inhibiting α-secretase both at the cell surface and in intracellular compartments, cannot be excluded. As biotin is cell impermeant, it has been used extensively to label selectively components on the surface of cells (*35*, *36*). Thus, we reasoned that a biotinylated derivative of one of the hydroxamic-acid-based inhibitors would not penetrate the cell and would inhibit α-secretase solely at the cell surface. The observation that almost a complete block of α-secretase action was obtained with the biotinylated inhibitor AMG110552 indicates that α-secretase is acting almost exclusively at the cell surface and not in an intracellular compartment such as the TGN or exocytic vesicles.

The constitutive α-secretase has many properties in common with the secretase that releases angiotensin converting enzyme from the cell surface. Proteolysis mediated by both enzymes is stimulated by phorbol esters, both cleave their respective substrates between a basic and a hydrophobic residue, and both are integral membrane proteins with a distinctive detergent solubilization profile (*8*, *12*, *14*, *37*). In addition, we have recently shown that both α-secretase and the angiotensin converting enzyme secretase display a markedly similar inhibition profile with a range of hydroxamic-acid-based zinc metalloproteinase inhibitors (*13*). Subcellular fractionation, pulse-chase, and surface labeling experiments have established that the angiotensin-converting enzyme secretase is localized at the plasma membrane (*38*−*41*). The localization of α-secretase to the plasma membrane would thus be consistent with these two activities being the same, or closely related, cell-surface zinc metalloproteinases. α-Secretase and the angiotensin-converting enzyme secretase are members of the family of membrane protein zinc

metalloproteinases involved in the proteolytic release of integral membrane proteins (*42*). One of these membrane protein secretases, tumor necrosis factor-α convertase, has been cloned and identified as a member of the adamalysin family of zinc metalloproteinases (*43*, *44*).

Recently, it has been shown that the regulated, phorbol ester-induced release of sAPPα is deficient in tumor necrosis factor-α convertase knockout nonneuronal cells, although the uninduced, constitutive release of sAPPα was unaffected (*34*, *45*). Although Buxbaum et al. (*34*) showed that tumor necrosis factor-α convertase could cleave a synthetic peptide spanning the α-secretase cleavage site in APP, the kinetics of this cleavage were not reported, and it was not shown whether tumor necrosis factor-α convertase can cleave full-length, membrane-bound APP. Thus, although tumor necrosis factor-α convertase appears to be involved in the regulated α-secretase cleavage of APP in nonneuronal cells, it is not possible to conclude as yet whether tumor necrosis factor-α convertase directly cleaves APP and, thus, that there are two separate α-secretases, one being responsible for the regulated cleavage and the other for the constitutive cleavage of APP, or whether tumor necrosis factor-α convertase is acting either directly or indirectly on a separate α-secretase, which is responsible for both the constitutive and regulated cleavage of APP. In support of this latter hypothesis, we have shown that tumor necrosis factor-α convertase displays a different inhibitor profile to both α-secretase and the angiotensin converting enzyme secretase and is unable to cleave angiotensin-converting enzyme (*46*). Thus, although α-secretase exhibits a number of properties in common with tumor necrosis factor-α convertase and has the characteristics of an adamalysin metalloproteinase, it is likely to be distinct from tumor necrosis factor-α convertase itself.

To reside at the cell surface, α-secretase is almost certainly trafficked through the secretory pathway. The lack of intracellular sAPPα shows that α-secretase does not cleave APP on route to the cell surface. This therefore raises the question as to how α-secretase only acts on APP at the cell surface and not in the secretory pathway? One possibility is that α-secretase and APP are segregated in different subcompartments or membrane microdomains within the secretory pathway (*47*) and only physically come into contact at the plasma membrane, possibly in response to external stimuli. Alternatively, α-secretase may be synthesized initially as a catalytically inactive proenzyme which is only activated either at the cell surface or in a late compartment of the secretory pathway (e.g., exocytic vesicles). Activation could occur through proteolysis [as with the matrix metalloproteinases and the adamalysin tumor necrosis factor-α convertase (*42*, *43*, *48*)], removal of an associated inhibitor or upon contact with some other membrane or extracellular component. The former mechanism is consistent with the presence of a "cysteine switch" in the matrix metalloproteinases and adamalysins which is removed by proteolysis at a furin-like cleavage site. Recently, it has been shown that another adamalysin proteinase, ADAM 10, is involved in the constitutive and regulated α-secretase cleavage of APP (*49*). Consistent with the results of the present study, the proteolytically activated form of ADAM 10 was localized by cell-surface biotinylation in the plasma membrane, while the enzymically inactive proenzyme was found in the Golgi.

During this study, we compared the cellular site of action of β-secretase with that of α-secretase. The action of β-secretase was monitored directly by following the production of sAPPβ using a neoepitope antibody that only recognizes the cleaved C-terminus of this large ectodomain fragment (*13*, *29*). sAPPβ derived from both mature and immature forms of APP was detected in the cell lysates from cells grown either at 37 °C or at 20 °C. Thus, blockade of forward secretory transport from the TGN does not appear to affect the cleavage of APP by β-secretase. The Gly-Tyr residues upstream of the tetrapeptide internalization signal Asn-Pro-Thr-Tyr in the cytoplasmic domain of APP have been proposed as a putative sorting signal directing proteins directly from the TGN to endocytic compartments (*18*), where processing of the mature form of APP by β-secretase can take place (*16*, *23*). As there is evidence suggesting that growth at 20 °C has no effect on the recycling of proteins from the endosomes to the cell surface (*50*), sAPPβ could be secreted from the cells by this route, thus accounting for the presence of sAPPβ in the medium of cells grown at 20 °C. The observation that within cells, grown either at 37 °C or at 20 °C, the immature form of APP was identified as having been cleaved by β-secretase would indicate that this enzyme is acting in a pre-TGN compartment. This result is consistent with reports of β-secretase acting in the endoplasmic reticulum/Golgi (*6*, *7*, *51*). If β-secretase can potentially act on APP in the secretory pathway before it reaches the cell surface where α-secretase acts, there must be additional, as yet unidentified, control mechanisms in place under normal physiological conditions to ensure that APP is processed primarily by the nonamyloidogenic pathway to produce the neuroprotective sAPPα. A fuller understanding of the mechanisms whereby APP is directed into the nonamyloidogenic rather than the amyloidogenic processing pathway may provide new therapeutic targets for Alzheimer's disease.

ACKNOWLEDGMENT

We thank Dr. Mary Rosendahl and Dr. David Thompson (Amgen Inc., Boulder, Colorado, USA) for provision of AMG 2380 and AMG110552, and Dr. Carol Gray (SmithKline Beecham, Harlow, U.K.) for provision of antibodies Ab1-25, Ab1A9, and Ab54.

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BI9906827