The Transmembrane Domain of the Alzheimer’s β-Secretase (BACE1) Determines Its Late Golgi Localization and Access to β-Amyloid Precursor Protein (APP) Substrate

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Release of Aβ peptides from β-amyloid precursor protein (APP) requires sequential cleavage by two endopeptidases, β- and γ-secretases. β-Secretase was recently identified as a novel membrane-bound aspartyl protease, named BACE1, Asp2, or memapsin 2. Employing confocal microscopy and subcellular fractionation, we have found that BACE1 is largely situated in the distal Golgi membrane with a minor presence in the endoplasmic reticulum, endosomes, and plasma membrane in human neuroblastoma SHEP cells and in mouse Neuro-2a cell lines expressing either endogenous mouse BACE1 or additional exogenous human BACE1. The major cellular β-secretase activity is located in the late Golgi apparatus, consistent with its cellular localization. Furthermore, we demonstrate that the single transmembrane domain of BACE1 alone determines the retention of BACE1 to the Golgi compartments, through examination of recombinant proteins of various BACE1 fragments fused to a reporter green fluorescence protein. In addition, we show that the transmembrane domain of BACE1 is required for the access of BACE1 enzymatic activity to the cellular APP substrate and hence for the optimal generation of the C-terminal fragment of APP (CTF99). The results suggest a molecular and cell biological mechanism for the regulation of β-secretase activity in vivo.

The pathological hallmarks of Alzheimer’s disease are neuritic plaques and neurofibrillary tangles (see reviews in Refs. 1–3). The neuritic plaques, also known as senile plaques, are predominantly composed of Aβ, a cluster of aggregated and heterogeneous peptides of 39–43 amino acids (4). The most pathogenic Aβ peptide is the less soluble 42-amino acid peptide (Aβ42), although the concentration of Aβ42 is generally much higher than Aβ42. Excision of Aβ peptides from their amyloid precursor protein requires sequential proteolytic cleavages by the β- and γ-secretases, respectively. β-Secretase cleaves APP1

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The abbreviations used are: APP, β-amyloid precursor protein; AD, Alzheimer’s disease; Asp2, aspartyl protease 2; BACE, beta-site APP cleaving enzyme; TGN, trans-Golgi network; ER, endoplasmic reticulum; EEA1, early endosome antigen 1; HA, a peptide epitope from influenza hemagglutinin protein; GFP, green fluorescence protein; TM, transmembrane; CTF99, β-C-fragment; CTF83, α-C-fragment; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; BFA, brefeldin A.

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domain of BACE1 disrupts the optimal processing of APP substrate at the β-site and thus inhibits CTF99 formation. The indispensable role of the transmembrane domain is further strengthened by the observation that BACE1, lacking its transmembrane domain, remains inactive even when it is retained in the ER by addition of an ER retention signal.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Expression Constructs**—Antibodies against γ-adaptin, EEA1, GM130, and syntaxin 6 were purchased from Transduction Laboratory (Lexington, KY); an antibody against p58 was purchased from Sigma Chemical Co. (St. Louis, MO); antibodies 22C11 and anti-HA (clone 3F10) were purchased from Roche Molecular Biochemicals (Indianapolis, IN); and antibodies 4G8 and 6E10 were purchased from Senetek PLC (St. Louis, MO). DNA expression constructs were generated by standard polymerase chain reaction amplification methods, and the engineered plasmid DNA was confirmed by sequencing. For expressing GFP chimeric proteins, polymerase chain reaction-amplified products were inserted in-frame between BanHI and EcoRI sites within the vector pEGFP-N3 (CLONTECH, Palo Alto, CA). For the other expression constructs, the mammalian expression pCDNA3.1 vector (Invitrogen, Carlsbad, CA) was used.

**Production of Antibodies against BACE1**—By using the GCG PepPlot program, four peptides were selected and synthesized for producing peptide antibodies. The peptide B278, which corresponds to the sequence YLRVPEDVATQSD from 366 to 378; B279, which corresponds to the sequence LRLPKVFTVSIK from 295 to 310; B280, which corresponds to the sequence DDSLEPFFDSLVKQTHV from 191 to 207; and B690, which corresponds to the sequence LRLPQACRQDGFAD from 378 to 392, were individually conjugated with keyhole limpet hemocyanin via a cysteine residue according to the instructions provided (Pierce, Rockford, IL). The protocol for generating the peptide antibody and injection of conjugated peptides into rabbits was conducted by Covance (Denver, PA).

**Immunofluorescence Confocal Microscopy**—Immunofluorescence on whole cells (SHEP cells, Neuro-2a cells, or HEK-293 cells) was performed using standard methods. Cells were first grown to 50–80% confluence in chambers (Lab-Tek, Naperville, IL) and then transfected with an individual construct using either the calcium phosphate method or LipofectAMINE (Invitrogen, Carlsbad, CA). For expressing GFP chimeric proteins, polymerase chain reaction-amplified products were inserted in-frame between BamHI and EcoRI sites within the vector pEGFP-N3 (CLONTECH, Palo Alto, CA). For the other expression constructs, the mammalian expression pCDNA3.1 vector (Invitrogen, Carlsbad, CA) was used.

**Sucrose Density Gradient Fractionation**—To separate and enrich TGN and ER membranes, Neuro-2a cells stably transfected with human APP695 and BACE1 were labeled with [35S]methionine (500 μCi/ml) at 37 °C for 10 min and chased for 2 h at 20 °C in complete growth medium to accumulate labeled proteins in TGN. To restore vesicle trafficking from TGN, cells were transferred from 20 °C to 37 °C for 0, 10, 20, 30, 45 min, 1, 2, and 4 h. At the end of each time point, cells were incubated at 4 °C for biotinylation of cell surface proteins.

Cell surface biotinylation was performed by washing plates three times with freshly prepared PBS-CM buffer (phosphate-buffered saline containing 1.0 mM MgCl₂ and 1.3 mM CaCl₂) followed by incubating cells with biotin labeling buffer (0.5 mg/ml sulfo-N-hydroxysuccinimido-biotin in PBS-CM buffer) on ice for 15 min with gentle shaking. The biotinylation reaction was terminated by removing the labeling solution followed by the addition of 1 ml of quenching buffer (50 mM NH₄Cl in PBS-CM) to each plate of cells. After washing plates twice with ice-cold PBS-CM buffer, cells were harvested and pelleted by spinning at 1000 × g for 3 min in a Brinkmann centrifuge.

To detect the surface BACE1 protein, biotinylated cells were lysed in 100 μl of PBS buffer containing 0.5% deoxycholic acid and 0.5% Nonidet P-40 at 4 °C. Cell lysates were collected by spinning at 14,000 rpm for 3 min at 4 °C in a Brinkmann centrifuge. The resultant supernatants were diluted with 1 ml of immunoprecipitation buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.8), 6 mM EDTA, 2.5% Triton X-100, 5 mM mithionine, and 1 mg/ml bovine serum albumin. Biotin-labeled surface proteins and non-biotinylated intracellular proteins were separated by neutravidin beads (Pierce, Rockford, IL). Biotinylated proteins were eluted from the beads by heating at 95 °C for 5 min in 100 μl of 0.5% SDS-PBS buffer. Samples of both biotinylated and non-biotinylated BACE1 were immunoprecipitated using B690 antibody. Immunoreactive materials were analyzed by SDS-PAGE using a 12% Tris-glycine gel and visualized by autoradiography.

In addition, Neuro-2a cells stably expressing human BACE1 proteins were grown to 70–80% confluence and subjected to proteinase K treatment at a concentration of 25 μg/ml for 15 min at 4 °C. After the treatment, medium was removed by aspiration, and the cells were collected from the plates by washing with PBS buffer containing protease inhibitors. Cells were then centrifuged at 800 × g for 5 min and followed by washing twice with a large volume of PBS containing protease inhibitors to remove and inactivate the proteinase K. After washing, equal volumes of cell lysates were prepared in 1× SDS sample buffer and analyzed by 12% SDS-PAGE and Western blot using antibody B690 to detect the BACE1 proteins.

**Immunoprecipitation and Western Blot Analysis**—Cell extracts were prepared as previously described (9). Equal amounts of cell extracts were used for Western analysis to detect APP with antibody C8 or 369; both recognize the APP C-terminal fifteen amino acids. Alternatively, equal amounts of cell extracts were immunoprecipitated with monoclonal antibody 4G8 followed by Western analysis with antibody C8 (9) or 369 (23) to visualize BACE1 cleavage product CTF99. The protein levels of BACE1 were measured with antibody B278 as indicated. For detecting secreted BACE1 variants, the con-
The TM Domain of BACE1 Determines Its Localization and Access

**Localization of Endogenous BACE1 by Double Immunofluorescence Confocal Microscopy**—To examine the cellular localization of endogenous BACE1, we first developed several lines of antibodies against different regions of BACE1. The specificity of the antibodies has been validated in immunofluorescence confocal microscopy studies. Human neuroblastoma SHEP cells were transfected with a low dose (0.01 μg of DNA per 10^6 cells; panels A–C) or a high dose (0.2 μg of DNA per 10^6 cells; panel D) of human BACE1 cDNA with HA tag, or with vector alone (panels E and F). BACE1 was visualized by incubation with the indicated primary antibody followed by Texas red-conjugated (red) secondary antibody. Localization of BACE1-HA was visualized by incubation with primary antibody followed by Alexa 488-conjugated (green) secondary antibody 3 F10 (panel B). Overlaps (panel C) represent digitally merged images. Yellow fluorescence indicates co-localization of BACE1 and HA immunoreactivity (bar, 10 μm).

**RESULTS**

**Kinetics of BACE1 Trafficking from the TGN to the Plasma Membrane**—Recent reports indicate that trafficking of BACE1 to the plasma membrane and the endocytic pathway occurs. The presence of BACE1 at the plasma membrane may be overlooked by immunofluorescence microscopic approaches due to a predominant level of BACRE1 in the Golgi apparatus. Therefore, we assessed the amounts of BACE1 that exits from the TGN to the plasma membrane using a pulse-chase method in combination with cell surface biotinylation. Neuro-2a cells were pulse-labeled for 10 min with [35S]methionine at 37 °C and chased for 2 h at 20 °C. Under this low temperature condition, transport of BACE1 from the ER to the TGN occurs, but budding from TGN is prevented. Subsequent incubation at 37 °C for various times allowed trafficking of TGN BACE1 to the plasma membrane. Newly arrived cell surface BACE1 molecules containing [35S]methionine were then labeled with biotin at 4 °C for 15 min and separated from TGN-associated BACE1 by neutravidin bead precipitation. As shown in Fig. 3, most nascent BACE1 that accumulated in the TGN during the

**Fig. 1. Cellular localization of BACE1 and characterization of anti-BACE1 antibodies.** Human neuroblastoma SHEP cells were transfected with a low dose (0.01 μg of DNA per 10^6 cells; panels A–C) or a high dose (0.2 μg of DNA per 10^6 cells; panel D) of human BACE1 cDNA with HA tag, or with vector alone (panels E and F). BACE1 was visualized by incubation with the indicated primary antibody followed by Texas red-conjugated (red) secondary antibody. Localization of BACE1-HA was visualized by incubation with primary antibody followed by Alexa 488-conjugated (green) secondary antibody 3 F10 (panel B). Overlaps (panel C) represent digitally merged images. Yellow fluorescence indicates co-localization of BACE1 and HA immunoreactivity (bar, 10 μm).

To further ascertain that the late Golgi/TGN is the major cellular site for BACE1, we treated either Neuro-2a cells or SHEP cells with brefeldin A (BFA), a fungal metabolite that inhibits secretory vesicles exit from the ER and leads to dissolution and redistribution of the Golgi apparatus. BFA has relatively little effect on the integrity of the TGN of treated cells. Treatment of Neuro-2a or SHEP cells with BFA resulted in a disruption of the Golgi apparatus, illuminated by a partial redistribution of GM130 immunofluorescence (Fig. 2E) but did not affect the staining of γ-adaptin, an adaptor protein highly enriched in the TGN membrane (24) (Fig. 2D). The immunofluorescence of BACE1 was only marginally affected, suggesting that the majority of BACE1 is in BFA-insensitive vesicles.

It has been demonstrated that treatment of cells with okadaic acid disrupts the entire Golgi complex, including TGN (25). We then treated SHEP cells with okadaic acid to induce a disruption of the Golgi stack. The BACE1 fluorescence signal became dispersed (Fig. 2E, right panel), reinforcing our conclusion that the majority of endogenous BACE1 is in the later Golgi/TGN.

To evaluate a possible localization of BACE1 within endocytic compartments, we compared immunoreactivity of BACE1 with that of EEA1, a protein marker for endocytic endosomes. A punctate and typical endosomal staining pattern was observed for EEA1, distinct from that observed for BACE1 (Fig. 2C), suggesting that the majority of endogenous BACE1 is not stored in the endosomes. These findings, however, do not rule out an important role of the endocytic pathway in Aβ generation, which has been previously implicated (26). BACE1 has been shown to travel to the plasma membrane and endosomes, although most BACE1 molecules remain in the TGN, and this trafficking event is regulated by protein phosphorylation of BACE. Furthermore, two-way trafficking pathways have been described between the plasma membrane, recycling endocytic compartments and the TGN, which may contribute to a dynamic transport of a protein between these compartments.

Most studies on BACE1 localization used cell lines overexpressing various levels of exogenous BACE1, a condition that may account for the discrepancies in BACE1 localization reported by these studies. We transiently transfected SHEP cells with different amounts of BACE1 expression construct and examined the localization of BACE1 in cells expressing a high (2 μg of DNA per 10^6 cells), low (0.1 μg of DNA per 10^6 cells), or endogenous level of BACE1 using immunofluorescence confocal microscopy. The high expression cells exhibited a broader pattern, including ER, Golgi, and endosomal-like staining (Fig. 1D) whereas cells expressing low levels of BACE1 (as shown in Fig. 1, A–C) or endogenous BACE1 (Fig. 1, E and F) showed a more stringent Golgi-like pattern.

We next examined the co-localization of endogenous BACE1 with several intracellular vesicle markers using non-transfected SHEP and mouse neuroblastoma Neuro-2a cells. BACE1 immunoreactivity is confined to an area of the cells that corresponds to the localization of a TGN protein, syntaxin 6 (Fig. 2A). The immunoreactivity of BACE1 showed a distinct pattern that differed from that of p58, a marker for the cis-Golgi compartments (Fig. 2B), although these two signals were near each other. A similar result was obtained when comparing the immunostaining patterns of BACE1 to GM130, another marker for early Golgi compartments (data not shown). These results suggest that the majority of BACE1 is transported beyond ER and early Golgi compartments to late Golgi or TGN after its synthesis in the ER.
20 °C incubation remains as an immature/non-cleaved form. BACE1 gradually matures in the TGN after the temperature is shifted back to 37 °C. These results suggest that the maturation and/or cleavage of pro-BACE1 is temperature-dependent, because many endoproteolysis reactions such as cleavages of APP and prohormones have been known to be temperature-dependent (27, 28). It is evident that the majority (over 90%) of mature BACE1 stably resides intracellularly, likely in the TGN, even after 4-h chase at 37 °C. Only a small portion of BACE1 (mostly in a mature form) leaves the TGN and travels slowly to the cell surface, with a maximal appearance of BACE1 at the surface after a 2-h 37 °C chase. Our results suggested that BACE1 molecules targeted to the endocytic compartments from the plasma membrane only account for a small fraction of the total intracellular pool.

To further assess the amount of cell surface BACE1 under steady state, proteinase K treatment was applied to the Neuro-2a cells stably expressing human BACE1 proteins. After a 15-min treatment with proteinase K at 4 °C, a small portion of BACE1 protein (~10%, based on the results using densitometer scanning) was sensitive whereas the majority of BACE1 remained resistant to proteinase K digestion. When 0.25% Triton X-100 was added during proteinase K treatment, all BACE1 molecules were degraded, indicating that the proteinase K penetrates into the cells under this condition and all the cellular BACE1 is labile to proteinase K.

FIG. 2. Localization of endogenous BACE1 in human and mouse neuroblastoma cells by double immunofluorescence confocal microscopy. SHEP cells, human neuroblastoma cells (panels A, B, and E) and Neuro-2a, mouse neuroblastoma cells (panels C and D) were used in this study. BACE1 colocalizes with TGN protein syntaxin 6 (A), but not with p58 (B), a marker protein for the cis-Golgi/intermediate compartments, or EEA1 (C) a marker protein for early endosomes. The TGN-like localization pattern of BACE1 and γ-adaptin, another TGN marker, was not altered after treatment with 5 mM BFA (D) while the Golgi-like pattern of GM130 was redistributed (E, left and middle panels). Okadaic acid treatment of SHEP cells disrupted the TGN-like staining pattern of BACE1 (E, right panel) (bar, 10 μm).

FIG. 3. Biotinylation of newly arrived cell surface BACE. A, Neuro-2a cells doubly expressing human APP695 and BACE1 were labeled with [35S]methionine at 37 °C for 10 min and chased at 20 °C for 2 h to accumulate labeled BACE1 in the TGN. Cells were then incubated at 37 °C for various times to allow transport of BACE1 to the plasma membrane. Cell surface proteins were biotinylated at 4 °C for 15 min. Biotinylated proteins were separated from non-biotinylated proteins using neutravidin beads. Both biotinylated and non-biotinylated BACE1 were immunoprecipitated with BACE1 antibody B690 and analyzed by SDS-PAGE and radiography. Arrows indicate mature BACE1 and immature/pro-BACE1.

B, cells were treated with proteinase K in the absence or presence of detergent Triton X-100 followed by Western analysis of BACE1. BACE1 from the untreated cells was used for comparison.
digested (Fig. 3B). In agreement with the result obtained from cell surface biotinylation, this confirms that cell surface BACE1 represents a small population of BACE1 proteins in Neuro-2a cells.

Cleavage Activity of BACE1 Correlates with Its Subcellular Localization—The major cellular residence of BACE1 may not necessarily correlate with the site of its enzymatic activity. We attempted to locate the cellular site(s) involved in the generation of CTF99, an APP product of BACE1 cleavage, using a well-defined sucrose gradient fractionation method. A stable Neuro-2a cell line doubly expressing human BACE1 and APP was homogenized, and a post-nuclear supernatant was subjected to a stepwise sucrose gradient that was previously characterized for the enrichment of various vesicle proteins (29). Proteins present in each subcellular fraction were analyzed by Western blot. Consistent with our immunofluorescence data, the majority of mature BACE1 was found in the TGN-enriched fractions whereas two forms of immature BACE1 (likely representing non-glycosylated and glycosylated pro-BACE1) were the majority of mature BACE1 was found in the TGN-enriched fractions. Proteins present in each fraction were separated by 12% (for BACE) or 4% (for APP and CTFs) SDS-PAGE, followed by immunoblotting using antibodies against BACE1 (antibody BE90), APP+CTF99 (6E10), and CTFs (36B). Arrows indicate mature BACE1, pro-BACE1, immature-BACE1, APP, CTF99, and CTFs. The enrichment of protein markers for various organelles across the gradient is indicated.

Expression of a full-length BACE1 with a GFP tag at the C terminus resulted in a TGN-like intracellular localization, mimicking the localization of HA-tagged BACE1 (shown in Fig. 5B). This indicates that addition of GFP to the BACE1 C terminus does not compromise its trafficking within the secretory pathway. However, the signal sequence of BACE1 alone was unable to target the SP-GFP correctly in the secretory pathway as shown by a diffuse staining pattern (Fig. 5B). Interestingly, removal of the entire C-terminal domain of BACE1 (BACE1-(1-477)-GFP) did not affect TGN-like localization; precise co-localization with syntaxin 6 still occurs (Fig. 5C). In another set of experiments expression of BACE1-(1-497), a construct lacking the di-leucine motif, also shows a TGN-like localization (data not shown). The data suggest that the entire cytoplasmic tail of BACE1 is not required for its TGN localization.

We next tested whether the transmembrane domain alone is able to direct localization of the recombinant protein to the Golgi. We generated a construct, SP-TM-GFP, that ligated the signal peptide sequence of BACE-(1-21) to the TM (BACE1-(454-477)-GFP). A TGN-like localization that is superimposable with syntaxin 6 immunoreactivity was observed upon expression of this construct (Fig. 5D). In addition, expression of another construct containing the transmembrane domain, SH-TM-GFP, which includes an additional helical region, also resulted in a similar TGN-like localization (data not shown). Taken together, our results indicate that the transmembrane domain, rather than the cytoplasmic tail of BACE1, is necessary and sufficient for its TGN localization.

BACE1 Activity in Vivo Requires the Presence of the Transmembrane Domain—Having demonstrated that the transmembrane domain of BACE1 determines its cellular localization, we next investigated whether this transmembrane domain is also required for BACE1 activity within cells. BACE1 constructs with various modifications/deletions as shown in Fig. 6 were expressed in SHEP cells. Deletion of the entire cytoplasmic tail (BACE1-ΔC) resulted in no significant changes in its intracellular localization, although its maturation may be less efficient, as reported previously (14). The β-secretase activity of BACE1-ΔC was not affected as judged by the formation of CTF99, compared with the wild-type BACE1 (Fig. 6). Further deletions that remove (partly or entirely) the transmembrane domain (BACE1-Δ36 or BACE1-Δ48) cause a quick release of non-membrane-anchored BACE1 into the medium and a loss of BACE1 activity. Addition of the cytoplasmic domain to a construct lacking the transmembrane domain (BACE1-ΔT 77) does not recover its intracellular localization and activity (Fig. 6).

To exclude the possibility that intracellular localization of BACE1 rather than the presence of the transmembrane domain per se is essential for in vivo activity, we generated a construct that is retained in the ER, a compartment where APP

The Transmembrane Domain Rather Than the Cytoplasmic Tail of BACE1 Determines Its TGN Localization—Despite a lack of consensus sequences among TGN membrane proteins, TGN retention signals often reside in the cytoplasmic and/or transmembrane domains. Signal(s) for Golgi/TGN retention have also been shown to function as endocytic targeting signals, although this has been controversial. For example, the NPXY (31) or the di-leucine (32) motifs in the cytoplasmic tails of several proteins have been implicated as TGN retention signals, although many reports suggest they may target proteins to endocytic pathways. To elucidate the domain(s) that determine(s) BACE1 localization to the TGN, we generated various chimeric reporter constructs in which a portion of BACE1 was ligated to GFP and expressed these constructs in SHEP cells (Fig. 5A). All constructs contained the BACE1 signal peptide sequence to ensure proper targeting to the secretory pathway.

Expression of a full-length BACE1 with a GFP tag at the C terminus resulted in a TGN-like intracellular localization, mimicking the localization of HA-tagged BACE1 (shown in Fig. 5B). This indicates that addition of GFP to the BACE1 C terminus does not compromise its trafficking within the secretory pathway. However, the signal sequence of BACE1 alone was unable to target the SP-GFP correctly in the secretory pathway as shown by a diffuse staining pattern (Fig. 5B). Interestingly, removal of the entire C-terminal domain of BACE1 (BACE1-(1-477)-GFP) did not affect TGN-like localization; precise co-localization with syntaxin 6 still occurs (Fig. 5C). In another set of experiments expression of BACE1-(1-497), a construct lacking the di-leucine motif, also shows a TGN-like localization (data not shown). The data suggest that the entire cytoplasmic tail of BACE1 is not required for its TGN localization.

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To exclude the possibility that intracellular localization of BACE1 rather than the presence of the transmembrane domain per se is essential for in vivo activity, we generated a construct that is retained in the ER, a compartment where APP...
is present and BACE1 is also active (17). Addition of a KDEL motif to a BACE1 construct lacking both the transmembrane domain and the cytoplasmic tail leads to an accumulation of this modified BACE1 variant in the ER (Fig. 7B), unlike that of the secreted BACE1-HA9004TM. However, compared with the action by wild type BACE1, there is no increase of CFT99 production when this construct is expressed in cells producing high levels of APP. In addition, we did not observe a reduction of sAPPα (Fig. 7C), a secreted α-secretase-cleaved APP fragment, a phenomenon that was often observed when wild type BACE1 was overexpressed. Furthermore, increased presence of APP in the ER compartments after brefeldin A treatment did not increase the interaction between membrane-bound APP with the soluble form of BACE1 (BACE1-ΔTM-C-KDEL), because no increased CTF99 production was observed (Fig. 7D), whereas wild type BACE1 efficiently caused the accumulation of CTF99. All of these data support our hypothesis that co-localization of BACE1 and its substrate within the same subcellular compartment is not sufficient for APP cleavage to occur. The presence of the membrane anchor is necessary to ensure enzymatic activity.

The fact that BACE-GFP was also able to process APP and to produce CTF99 to the same extent as wild type BACE1 (data not shown) ruled out the possibility that the GFP tag used in the localization studies could affect BACE1 activity.

**DISCUSSION**

BACE1 is a potentially important therapeutic target in AD. BACE1 knockout mice fail to produce any Aβ due to a lack of β-secretase activity but otherwise appear normal, without apparent deleterious effects (33, 34). Hence, inhibitors of BACE1 may be more specific than γ-secretase inhibitors, which may affect Notch and other signaling pathways. Recent reports concerning BACE1 localization have been controversial, and this may be a consequence of the BACE1 expression levels in each system. In the present study, we have demonstrated that en-
endogenous BACE1 predominantly localizes to the TGN, using morphological and biochemical approaches. Furthermore, using a combination of pulse-chase and biotinylation methods, we showed that a small portion of BACE1 is delivered to the plasma membrane from the TGN, from which it recycles to endocytic compartments. It is also likely that a small fraction of endogenous BACE1 may go to endosomes directly from the TGN.

The mechanisms for regulating retention of membrane proteins in specific organelles remain unclear. These may include numerous factors such as specific targeting/retention signals, membrane thickness in particular organelles (35), and oligomerization of proteins within the lipid bilayer (36). We found that the BACE1 transmembrane domain alone is able to selectively locate GFP to the TGN rather than produce ubiquitous membrane localization. Recently, we showed that BACE2 is not restricted to TGN localization. However, a chimeric protein with only the BACE2 TM domain being replaced by the BACE1 transmembrane domain is predominantly resided in the TGN, mimicking the localization pattern of BACE1 (37). In addition, deletion of the entire BACE1 cytoplasmic tail does not disrupt its predominant accumulation in the TGN. Thus far, it is still unclear whether an additional motif coordinates its TGN localization. Deletion of the transmembrane domain alone or its partial disruption (Fig. 7) causes the truncated proteins to be quickly secreted into the culture medium. Thus, these data suggest that the TM domain contains targeting signals and/or may be important for membrane partitioning and oligomerization.

Recently Huse et al. (16) reported that a di-leucine motif near the C terminus of BACE1 is required for transporting BACE1 from the TGN to later endosomes. It is possible that the transmembrane domain of BACE1 serves to target the majority of

![Fig. 6. Transmembrane domain of BACE1 is necessary for the generation of intracellular CTF99.](image-url)
BACE1 to the TGN while the di-leucine motif directs retrieval of
only a fraction of BACE1 from the TGN to other organelles,
including the late endosomes and plasma membrane. Indeed,
several examples demonstrate that a small proportion of major
TGN proteins leaves the TGN and recycles between TGN,
plasma membrane, and endocytic compartments. Those in-
clude syntaxin 6 (38), furin (39), and perhaps BACE1 as well.

Using sucrose density gradient fractionation, we showed
that the majority of BACE1 and its cleavage product, CTF99,
are enriched in the Golgi/TGN fractions, consistent with the
observation that Aβ is largely produced in the TGN (28). Two
forms of BACE1, containing pro regions, were detected along
with a small fraction of CTF99 in the ER-enriched fractions,
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