PRESENILIN/γ-SECRETASE-MEDIATED CLEAVAGE OF THE VOLTAGE-GATED SODIUM CHANNEL β2 SUBUNIT REGULATES CELL ADHESION AND MIGRATION*

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Running title: Voltage-gated sodium channel β2 subunit is a γ-secretase substrate

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The voltage-gated sodium channel β2-subunit (β2) is a member of the IgCAM superfamily and serves as both an adhesion molecule and an auxiliary subunit of the voltage-gated sodium channel. Here we found that β2 undergoes ectodomain shedding followed by presenilin (PS)-dependent γ-secretase-mediated cleavage. 12-O-tetradecanoyl phorbol-13-acetate (TPA) treatment or expression of an α-secretase enzyme, ADAM10, resulted in ectodomain cleavage of β2 in CHO cells. Subsequent cleavage of the remaining 15 kDa C-terminal fragment (β2-CTF) was independently inhibited by three specific γ-secretase inhibitors, expression of the dominant negative form of PS1, and in PS1/PS2 knockout cells. γ-secretase inhibitor treatment also increased endogenous β2-CTF levels in neuroblastoma cells and mouse primary neuronal cultures. In a cell-free γ-secretase assay, we detected γ-secretase activity-dependent generation of a 12 kDa β2 intracellular domain (ICD), which was loosely associated with the membrane fraction. To assess the functional role of β2 processing by γ-secretase, we tested whether DAPT, a specific γ-secretase inhibitor, would alter β2-mediated cell adhesion and migration. We found that DAPT inhibited cell-cell aggregation and migration in a wound healing assay carried out with CHO cells expressing β2. DAPT also reduced migration of neuroblastoma cells in a modified Boyden chamber assay. Since DAPT treatment resulted in increased β2-CTF levels, we also tested whether β2-CTFs or β2-ICDs would directly affect cell migration by overexpressing recombinant proteins. Interestingly, elevated levels of β2-CTFs, but not ICDs, also blocked cell migration by 81 to 93%. Together, our findings show for the first time that β2 is a PS/γ-secretase substrate and γ-secretase-mediated cleavage of β2-CTF is required for cell-cell adhesion and migration of β2-expressing cells.

A major pathologic hallmark of Alzheimer’s disease (AD) is the deposition of amyloid β-peptide (Aβ) into senile plaques. Aβ is produced from β-amyloid precursor protein (APP) by sequential β- and γ-secretase-mediated cleavages. An alternative to the β-secretase pathway is a cleavage mediated by α-secretase, which cuts in the middle of the Aβ region of APP(1). APP has been shown to serially undergo either β-γ and α-γ-cleavages. Both Aβ and the β-secretase-mediated C-terminal cleavage product of APP, C99 or CTFβ, have been shown to contribute to neurodegeneration in AD(2,3).

γ-secretase is a multi-subunit aspartyl protease complex, harboring presenilin 1 (PS1) or 2 (PS2), nicastrin, aph-1, pen2, and likely additional factors(4). The presenilins are polytopic membrane proteins that constitute either catalytic subunits or necessary co-factors of the γ-secretase complex(5,6). Besides APP, PS/γ-secretase is required for the intramembranous cleavages of at least fifteen additional proteins, including Notch, nectin-1α, and recently NRADD(4,7-11). All the known substrates, with the exception of GluR3, are type I membrane proteins and require extracellular domain cleavage prior to subsequent γ-cleavage. Interestingly, many γ-secretase substrates (E-, N-cadherin, nectin-1, CD44, APP, ErbB4, p75NTR, APOER2, LRP, syndecan-3 and DCC) share
functions in cell adhesion and/or migration, implicating a possible role for PS/γ-secretase in these processes(11-16). For example, γ-secretase dependent cleavage of E-cadherin, a well-characterized cell adhesion molecule, promotes disassembly of the E-cadherin-catenin adhesion complex(17). This results in the release of β-catenin, a key mediator of the Wnt signaling pathway that controls cell migration and cell adhesion(18,19). Studies on CNS morphogenesis in PS1 knock-out mice also strongly support a function for PS/γ-secretase in neuronal cell migration and/or cell adhesion during brain development(20,21).

α-secretase is a generic name for several ectodomain-shedding proteases, primarily of the metalloprotease type, based on their sensitivity to generic metalloprotease inhibitors and inducible activation by phorbol ester treatment(22). ADAM-10 and ADAM-17 are among the best characterized α-secretases of APP(23,24). Ectodomain shedding of currently reported PS/γ-secretase substrates is likely to be mediated by α-secretase-like metalloproteases. However, the identity of the metalloproteases responsible for each γ-secretase substrate remains to be elucidated.

Voltage-gated sodium channels (VGSCs) consist of a single pore-forming α subunit and one or two β subunits(25). All β subunits, β1, 1A, 2, 3, and recently 4, serve as auxiliary subunits of the VGSC and have been known to be involved both in channel gating and cell surface expression of α subunits(25). Similarly to nectin-1 and DCC, both substrates for PS/γ-secretase mediated cleavages, the β subunits belong to the immunoglobulin superfamily of cell adhesion molecules (CAMs)(26). The β2 subunit, in particular, is enriched in the CNS, covalently linked to an α subunit, and well characterized as a cell-cell adhesion protein(25,27,28). β2 subunits interact homophilically, and also heterophilically with tenascin-R and β1 subunits via their N-CAM-like extracellular domains, mediating their function as CAMs(27,29,30).

Here we report the identification of β2 as a substrate for PS/γ-secretase mediated cleavage. TPA treatment or ADAM10 expression induce ectodomain cleavage of β2. The remaining 15 kDa membrane-anchored β2-CTF undergoes PS/γ-secretase mediated cleavage to generate a 12 kDa β2 intracellular domain (β2–ICD). Lack of γ-secretase cleavage of β2, resulting in accumulation of β2-CTFs, inhibits cell-cell adhesion and migration in CHO cells. These data suggest a functional role for PS/γ-secretase in cell adhesion and migration via processing of β2-CTFs.

Materials and Methods

Plasmids, transfection, and primary neuronal cultures – An expression construct encoding full-length human voltage-gated sodium channel β2 subunit (β2, GeneBank accession #, gi:21361089) containing a C-terminal V5/His tag (in pcDNA3.1/GS) was purchased from Invitrogen. An expression vector containing human ADAM10 was a kind gift of Dr. Lichtenthaler (Ludwig-Maximilians-Universität, Germany). Effectene (Qiagen) was routinely used for transfecting cell lines. We produced stably transfected CHO cells with β2, β2-CTF, β2–ICD, nectin, NE-CTF, NE-ICD, wild type PS1 and PS1 (D385A) expression constructs. Sequences of primers used to generate these constructs are available by request. Individual clones with similar expression levels were maintained in selection medium. The PS1/PS2 knockout ES cell line (BD1) and control wild-type ES cells (BD6) were a generous gift of Dr. Sisodia (University of Chicago). SH-SY5Y cells were routinely grown in DMEM medium (Cambrex) with 10% FBS and switched to RPMI 1640 medium (Cambrex) during migration assays. Mouse primary cortical neuronal cultures (DIV16) were prepared as described(8). Briefly, cortices were dissected from E16 mouse fetal brains and dissociated by repeated passages through a fire-polished Pasteur pipette. The dissociated cells were plated on L-polylysine/laminine coated six-well dishes and maintained in growth media consisting of Neurobasal Medium (Gibco-BRL) medium supplemented with B27 and 0.5 mM L-glutamine. Cultures were maintained at 37 °C in a humidified 5% CO₂ atmosphere for 16 days (DIV16).

Western blot analysis, antibodies, and inhibitors – For immunoblotting, cell extracts were prepared by directly extracting cells in a buffer containing 10 mM Tris-HCl pH 6.8, 1 mM EDTA, 150 mM NaCl, 0.25% Nonidet P-40, 1% Triton X-100 and a protease inhibitor cocktail (Roche Molecular Biochemicals, Indianapolis, IN), followed by a spin at 16,000 g. Protein levels were quantitated using the BCA protein assay kit (Pierce). 20-100 ug of protein were resolved on 4-12% gradient Bis/Tris gels or 12% Bis-Tris gels (Invitrogen).
Primary antibodies were used at the following dilutions: anti-V5 antibody (1:5000 dilution, Invitrogen), anti-sodium channel β2 subunit antibodies (Navβ2, 1:500 dilution, Chemicon and Navβ2, 1:500, US biological) anti-nectin-1 (1:1000 dilution, a kind gift from Dr. Federoff at the University of Rochester), anti-transferrin receptor (1:1000, BD Bioscience), anti-PARP (1:1000, Pharmingen), and anti-cleaved caspase 3 (1:2000, Cell signaling). The blots were visualized by enhanced chemiluminescence (ECL) using Lumiglo (KPL) or SuperSignal CL-HRP substrates (Pierce) according to the manufacturer’s instructions. The γ-secretase inhibitors DAPT and L-685,458 were obtained from Calbiochem and WPE31C was a kind gift of Dr. M. Wolfe (Brigham and Women’s Hospital). The α-secretase inhibitor TAPI-1 (TNF-α processing inhibitor-1, IC-2) was purchased from BIOMOL.

In vitro generation of β2-ICD – Membrane preparation and in vitro generation of β2-ICD were performed as described (8,31). The P2–P3 fractions were resuspended in Buffer H (20 mM HEPES, 150 mM NaCl, 10% glycerol, 5 mM EDTA, pH 7.4) with protease inhibitors. In vitro cleavage experiments were performed by incubating the membrane fractions at 37 °C for 1 h in the presence or absence of the indicated amounts of DAPT and L-685,458. After incubation, the soluble and membrane-associated fragments were separated by centrifugation of the reaction mixture at 120,000 g for 45 min. To dissociate ICD fragments from the membranes, 1 mM NaOH solution was added to the membrane fraction and centrifuged at 120,000 g for 45 min. Fluorescence confocal analysis – For immunostaining, cells were fixed with 4% paraformaldehyde, rinsed, permeabilized, and blocked by using 0.1% Triton X-100 and 1.5% goat IgG (Molecular Probes), respectively. Cells were then washed three times with PBS and incubated for three hours with an anti-V5 antibody (1:200, Invitrogen) in PBS containing 1.5% goat IgG. After washing three times with PBS, cells were incubated with secondary antibodies conjugated with Alexa fluore 488. Confocal fluorescence images were obtained using a LSM 5 Pascal Laser Scanning Microscope (Zeiss).

Cell-cell aggregation assay – In vitro cell-cell aggregation assays were performed as described by Miura et al. (32) with slight modifications. To induce β2-CTF accumulation, cells were pretreated with DAPT for 12 hrs. Cells were then incubated for 15 minutes at room temperature with 1 mM EDTA/HBSS containing 500 nM DAPT and dispersed by gentle pipetting. After washing three times, cells were resuspended in Ca²⁺/Mg²⁺ free-HBSS containing DAPT (500 nM), transferred into 35 mm polystyrene dishes pre-coated with BSA, and agitated (75 rpm) at room temperature for the indicated time intervals. The number of cell aggregates was counted three times in a hemocytometer. Aggregation was quantified by the index N(t)/N(0), where N(t) and N(0) are the total number of particles at incubation time at t and 0, respectively.

Wound healing assay – CHO cells expressing β2 were grown in monolayer, scraped with a P200 pipette tip, washed with PBS, and incubated for 18-21 hours in the presence and absence of 500 nM DAPT (33). For β2-CTF accumulation, cells were pretreated with DAPT for 12 hours. Migration of cells into the wound was examined by phase contrast microscopy. Photographic images were obtained immediately after scraping and after 21 hours in the same locations. Wound healing activity was quantified by measuring total area of the wound in each 10x field and the area covered by the migrating cells within the wound by using public image analysis software, NIH image software Version 1.61. At least three different fields were chosen randomly and the mean percentage of wound area covered by cells was calculated.

Cell migration assay with cell culture inserts – SH-SY5Y cell migration assays were performed as described by Pola et al. (34) with slight modifications. Briefly, cell culture inserts with polyethylene terephthalate membranes (8 μm pore, USA Scientific, Inc.) were coated with collagen type IV (Sigma) and placed in 6-well dishes. Confluent SH-SY5Y cells were preincubated with serum-free RPMI medium for 24 hours in the presence or absence of 500 nM DAPT. Cells were then dispersed with 0.02% EDTA solution in PBS. 4 X 10⁵ cells were replated into cell culture inserts placed in 6-well plates and incubated for 24 hours to allow for cell migration. PDGF-BB (20 ng/ml, Sigma) was added to the lower chamber as an attractant of SH-SY5Y cells. Cells on the upper surface of the inserts were removed by sterile cell scraper (Falcon), in order to leave the cells that had migrated across the membrane. Cells that had migrated and attached to the lower surface of the inserts, were stained with Hoechst33342 (Molecular Probes) and...
RESULTS

\textbf{β2 is sequentially cleaved by α-secretase and PS/γ-secretase activities.}

Common characteristics amongst currently reported PS/γ-secretase substrates include: 1. sequence homology with other PS/γ-secretase substrates at the membrane-cytosol interface(8,9); 2. ectodomain cleavage by α-secretase alone or both α- and β-secretase-like activities, which generate CTF derivatives; 3. accumulation of CTF derivatives with extracellular domains shorter than 300 amino acids in response to PS/γ-secretase inhibition(35); 4. detection of a PS/γ-secretase-dependent cleavage product, termed intracellular domain (ICD); 5. in many cases, modulation of nuclear transcriptional activity by the ICD that is released into the cytosol(36-38).

Although Aβ is released by γ-secretase cleavage of APP-CTFβ, a second intramembraneous clip (ε-cleavage) in APP is also PS-mediated, at a site homologous to the S3 cleavage of Notch(31,39-41). Similarly, all other PS/γ-secretase substrates harbor a loose consensus sequence at the membrane-cytosol interface, corresponding to the ε/S3-cleavage sites in APP/Notch(8,9,17). Using a BLAST search based on the homology between the ε/S3-cleavage sites, we found a large number of proteins containing homologous amino-acid sequences, including β2. We chose to characterize β2 because, similarly to nectin-1 and DCC, β2 is a cell adhesion protein belonging to the wide family of IgCAMs(25). Of the numerous members of the voltage gated sodium channel proteins, including α- and β-subunits, only β2 harbors sequence homology to the APP-ε and Notch-S3 cleavage sites (Fig. 1A). Interestingly, this putative cleavage site in β2 also harbors a lysine residue for potential monoubiquitination, which has recently been reported to control Notch-S3 cleavage(42).

Previously reported PS/γ-secretase substrates, such as APP, erbB4, LRP, and nectin-1 undergo phorbol ester-inducible ectodomain shedding to produce short CTFs, which are subsequently cleaved in a PS-dependent manner(8,43-45) (Fig. 1B). To assess whether β2 also yields shorter cleavage products in response to phorbol esters, we treated Chinese hamster ovary (CHO) cells expressing β2 with 12-O-tetradecanoylphorbol-13-acetate (TPA). Employing an antibody against the C-terminal V5 epitope-tag, Western blot analysis showed that full-length β2 migrated at ~47 kDa (Fig. 1C). Unlike nectin-1ε, but similarly to APP, untreated cells already processed β2 to yield a CTF of approximately 15 kDa. This indicates that β2 undergoes constitutive ectodomain shedding similar to APP, in addition to a phorbol ester inducible activity. The size of this fragment was consistent with the predicted molecular weight of the β2-CTF-V5/His cleavage product, approximately 14-15 kDa, if ectodomain shedding occurred near the plasma membrane. TPA treatment only slightly increased levels of the 15 kDa fragment, presumably because of immediate PS/γ-secretase processing of the protein. However, DAPT, a γ-secretase inhibitor, alone or in conjunction with TPA, further elevated cellular levels of the 15 kDa CTF (Fig. 1C). These data suggest that β2 undergoes TPA-inducible ectodomain shedding followed by γ-secretase activity-dependent processing of its CTF.

TPA-inducible cleavage as shown in Fig 1C is characteristic of metalloprotease or α-secretase-mediated protease activities(46). To test whether an α-secretase activity is involved in ectodomain shedding of β2, CHO cells expressing β2 were treated with 10 µM TAPI-1, a hydroxamic acid-based α-secretase inhibitor(47). DAPT treatment alone induced accumulation of the 15 kDa β2-CTF and this was blocked by the α-secretase inhibitor TAPI-1 (Fig. 1D). To further assess whether α-secretase was partially responsible for ectodomain shedding of β2, we directly tested a well-known α-secretase in our cells. For these experiments, we obtained a HA-tagged ADAM10 construct from Dr. Lichtenthaler (Ludwig-Maximilians-Universität, Germany). Overexpression of ADAM10 alone was sufficient to increase β2-CTF levels in CHO cells expressing β2 (Fig. 1E). Taken together, these data demonstrate that α-secretase-like proteases including ADAM10 partially mediate ectodomain cleavage of β2.

In Figure 1, we have shown that DAPT increased the levels of the 15 kDa β2-CTF in CHO cells overexpressing β2. This suggests that γ-secretase activity is responsible for the
The 15 kDa β2-CTF, generated from ectodomain shedding of β2. To confirm that this 15 kDa β2-CTF is a substrate for PS/γ-secretase activity, we treated β2-transfected CHO cells with two additional γ-secretase inhibitors, L-685,458 and WPE31C. These inhibitors independently elevated β2-CTF levels similarly to DAPT, suggesting that specific inhibition of γ-secretase activity is responsible for the increased β2-CTF levels (Fig. 2A). We also found that β2-CTF levels increased with increasing concentrations of DAPT (Fig. 2B). Moreover, β2-CTFs specifically accumulated in CHO cells stably transfected with a construct expressing the dominant negative PS1(D385A), and this was further enhanced by TPA treatment (Fig. 2C). Similarly, β2-CTF levels largely increased in PS1/PS2 knockout ES cells transfected with full-length β2 (Fig. 2D). These data show that the proteolytic processing of the 15 kDa β2-CTF is PS/γ-secretase-mediated.

In neuronal cells, β2 proteins are predominantly associated with voltage-gated sodium channel α subunits(48). To test whether β2 undergoes γ-secretase-mediated cleavage in neuronal cells, we generated rat neuroblastoma cell lines (B104) stably expressing β2-V5/His. We chose to use B104 cells because they have been reported to express functionally active voltage-gated sodium channels, consisting of α and β1 subunits(49,50). 8 hours of DAPT treatment, in the absence of TPA, induced the accumulation of the 15 kDa β2-CTF in B104 cells stably expressing β2, suggesting that β2 undergoes constitutive ectodomain shedding followed by γ-secretase cleavage in these cells (Fig. 2E). To test whether endogenous β2 in neurons also undergoes γ-secretase-mediated cleavage, cultured cortical neurons from E16 mouse embryos were also treated with DAPT for 8 hours. Western blot analysis using an antibody against the C-terminal sequence of β2 showed that the putative endogenous 10 kDa β2-CTF (lacking the V5/His tag) largely increased following DAPT treatment (Fig. 2F). This 10 kDa β2-CTF band was absent when the antibodies were pre-incubated with the same antigenic peptide used to generate the β2 antibody (data not shown). Full-length β2 was also detected in mouse cortical neuronal cultures (Fig. 2F). These data demonstrate that β2 is an endogenous substrate for PS/γ-secretase-mediated cleavage in neuronal cells.

The PS/γ-secretase-derived cleavage products (intracellular domains or ICDs) of previously identified substrates are rapidly degraded in intact cells(8,9,38,51). Therefore, we used a cell-free γ-secretase assay to detect β2-ICD(8). Membranes from β2-overexpressing CHO cells were incubated for one hour in the presence and absence of the γ-secretase inhibitor DAPT(8). Incubation at 37 °C resulted in the generation of a band at approximately 12 kDa, the expected molecular weight of the β2-ICD containing a V5/His tag (Fig. 3A). DAPT and another γ-secretase inhibitor, L-685,458, blocked the generation of this band (Fig. 3B). The 12 kDa fragment, as opposed to the 15 kDa β2-CTF, could be dissociated from the membranes when washed with 0.1 M sodium hydroxide (Fig. 3B). Therefore, the 12 kDa band likely represents the PS-dependent ICD fragment of β2 because it is not membrane-tethered, it is undetectable in intact cells, and its generation is inhibited by PS/γ-secretase inhibitors. Similar only to nectin-1α-ICD, β2-ICD is peripherally associated with membranes presumably via protein-protein interactions(8). However, while 0.1 M sodium carbonate was sufficient to dissociate nectin-1α-ICD, the same treatment did not dissociate β2-ICD from the membrane fraction, suggesting that the latter is more tightly associated with membranes than nectin-1α-ICD(8). Since β2- and nectin-ICDs remain associated with the membrane pool, it is unlikely that they would directly function in modulating transcription in the nucleus. However, it cannot be excluded that a fraction of these ICDs and/or selected adaptor proteins are released and enter the nucleus in intact cells, similar to β-catenin following PS/γ-secretase-like cleavage of cadherin(17).

PS/γ-secretase activity is required for β2-mediated cell-cell adhesion and cell migration.

Since β2 is involved in cell adhesion, we tested whether PS/γ-secretase activity modulates the cell adhesion function of β2. For these experiments, we used an in vitro cell aggregation model system in Ca2+/Mg2+-free conditions(32). CHO cells were pretreated with DAPT for 12 hours, dispersed into single cells by mechanical agitation in the presence of 1 mM EDTA, and...
incubated in Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-free Hank’s balanced salt solution (HBSS). 60 min incubation at room temperature induced cell-cell aggregation, enhanced by stable overexpression of β2 (Fig. 4A, bottom left panel). These data reconfirmed previous reports that β2 functions as a cell-cell adhesion molecule in non-neuronal cells (27). Quantification showed a two-fold increase in cell-cell aggregation in β2-expressing CHO cells as compared to parental CHO cells (Fig. 4B). Interestingly, β2-induced cell-cell aggregation was completely blocked by the γ-secretase inhibitor DAPT, while DAPT did not change cell-cell aggregation in CHO parental cell lines (Fig. 4B). We then tested whether inhibition of γ-secretase modulates cell migration, a complex cellular event that involves cytoskeletal reorganization, detachment from the extracellular matrix, and generation of new cell-cell junctions. Using a wound healing assay system (33), we found that 21 hours of DAPT treatment inhibited cell migration of CHO cells stably expressing β2 by ~40% as compared to control DMSO-treated cells (Fig. 4C, D). These data demonstrate that γ-secretase activity is required for cell-cell adhesion and cell migration in CHO cells stably expressing β2.

**β2-CTF and NE-CTF independently inhibit cell migration.**

In cells transfected with β2, γ-secretase inhibition elevates β2-CTF levels. To directly assess whether accumulation of β2-CTFs inhibits cell migration, we generated N-terminally truncated deletion mutant constructs of β2-V5/His, and as a control, of nectin-V5/His (Fig. 5A). Following a murine Igκ-chain signal peptide (pSecTag, Invitrogen) and a short linker, the β2 and nectin-1 sequences of these CTFs started two amino acids from the putative transmembrane domains of the proteins. When transiently overexpressed in CHO cells, already stably expressing dominant negative PS1 (D385A), both CTFs were predominantly localized to cell surface membranes (Fig. 5B). Stable expression of β2-CTF and nectin-CTF in CHO cells did not cause detectable levels of apoptotic cell death, as assessed by two markers of apoptosis, poly-D-(ribose) polymerase (PARP) cleavage (Supplemental Fig. S1, S2) and generation of active caspase 3 (data not shown). Given that β2-expressing cells treated with γ-secretase inhibitors contain both full-length β2 and β2-CTFs (see Fig. 4C), we decided to replicate similar conditions by stably co-expressing both forms of the protein in CHO cells (Fig. 5C). As a control, CHO cell lines stably co-expressing full-length nectin-1 and NE-CTF were also generated for these experiments (Fig. 5C). Stable expression of full-length β2 and β2–CTF decreased cell migration in wound healing assays by 81-93%, while full-length nectin-1 and NE-CTF reduced cell migration by 84% (Fig. 5D, E). These inhibitory effects on cell migration were not due to decreased rates of cell division, because we could not detect any growth rate difference among the different cell lines (data not shown). To test whether reduced ICD signaling could account for the observed decrease in cell migration, we generated cells co-expressing β2–ICD or NE–ICD together with full-length β2 (Supplemental Fig. S3). Interestingly, overexpression of β2 or nectin-ICD does not induce statistically meaningful changes in cell migration (Fig. 5F). These data indicate that the inhibitory effects of CTFs on cell migration derive from increased CTF itself. Taken together, our data suggest that CTFs generated by ectodomain shedding of β2 and nectin are processed by γ-secretase activity to prevent adverse effects of the CTFs on cell migration.

**DAPT treatment inhibits PDGF-induced cell migration of SH-SY5Y neuroblastoma cells.**

To show that γ-secretase cleavage of endogenous substrates, including β2, is required for cell migration of neuronal cells, we used SH-SY5Y neuroblastoma cells as a model system. SH-SY5Y cells have been reported to express β2 mRNA in a previous study (52). In preliminary studies, we reconfirmed that this cell line expresses β2 mRNA by using RT-PCR (data not shown). To show that endogenous β2 and nectin-1 in SY5Y cells undergo α and γ-secretase-mediated cleavages, we performed Western blot analysis using antibodies against the C-termini of endogenous β2 and nectin-1. As expected, endogenous β2- and nectin-CTFs specifically increased when cells were co-treated with DAPT and TPA (Fig. 6A). To test whether γ-secretase cleavage modulates migration SH-SY5Y cells, we employed a modified Boyden chamber assay using PDGF as a chemoattractant (34) (Fig. 6B). We found that PDGF induced cell migration by 4.6 folds as compared to the BSA control in the lower chambers. However, DAPT decreased PDGF-
induced cell migration by 43% (Fig. 6C). These data support the notion that γ-secretase cleavage of endogenous β2 and/or nectin-1 promotes cell migration of neuronal cells.

**DISCUSSION**

In this study we show that β2, an auxiliary subunit of the voltage-gated sodium channel and a cell adhesion protein, is a substrate for both α- and PS/γ-secretase-mediated cleavages. We also found that increased levels of β2-CTFs, either by inhibiting γ-secretase activity or by expressing recombinant β2-CTF, block cell aggregation and migration in CHO cells. Interestingly, similar results were found when the CTF, but not ICD, of another γ-secretase substrate and cell adhesion protein, nectin, was overexpressed. These data indicate that PS/γ-secretase-mediated processing of γ-secretase substrates, such as β2-CTF and NE-CTF, is required for cell-cell adhesion and migration, supporting a role for PS/γ-secretase in cellular functions requiring these processes such as neurite outgrowth and axon guidance.

β2-CTF and NE-CTF are likely to inhibit cell migration via a complex interplay amongst different cellular events, including direct interference with cell adhesion or altered intracellular signaling. Considering that only two extracellular amino acids are preserved in our CTF constructs, CTF effects on cell migration are probably mediated by the cytoplasmic domain. However, direct overexpression of ICDs did not inhibit cell migration. The cytoplasmic domain of the β2 subunit recruits ankyrin in response to cell adhesion, thereby anchoring the extracellular space to the spectrin cytoskeletal system(27,53). Likewise, nectin-1 which is also a PS/γ-secretase substrate, is anchored to the cytoskeleton via several adaptor proteins including afadin, and α-, and β-catenin(8,54). E-cadherin, another binding partner of α-, and β-catenin, undergoes γ-secretase-mediated cleavage which releases a cytoplasmic E-cadherin fragment together with β-catenin and α-catenin(17). Fe65, an adaptor protein of APP, is also released and translocated to the nucleus in a γ-secretase-dependent manner(37,55,56). These studies support a function for PS/γ-secretase in modulating the release of adaptor signaling proteins, which are attached to the cytoplasmic domains of γ-secretase substrate proteins. Thus, signaling through interactions among CTFs, adaptor molecules and the cytoskeletal system might be relevant to the CTF-mediated inhibition of cell migration.

β2 is involved in both cell adhesion and sodium channel function, thus its misprocessing may interfere with neuronal function at multiple levels. Additionally, a recent study suggests that β subunits are involved in neurite outgrowth(57). Moreover, another recent study demonstrated that the accumulation of membrane-tethered DCC-CTF (one of the reported γ-secretase substrate proteins) increased neurite outgrowth in *in vitro* and *in vivo* model systems(58). Here we show that β2-CTF, which is similar to membrane-tethered DCC-CTF, directly inhibits cell aggregation and migration in cells that do not express the α subunit of the VGSC. It will be interesting to see whether elevated levels of β2-CTFs also affect neurite outgrowth, which is mechanistically close to cell migration. Considering the function of β2 in controlling cell surface sodium channel density, elevated β2-CTFs may also alter sodium channel function in neuronal systems. Several studies have previously shown that co-expression of α and β subunits results in stabilization of cell surface sodium channel levels(59,60). Conversely, absence of the β2 subunit in β2 knock-out mice leads to a general decrease in active voltage gated sodium channels on the cell surface(61). Therefore, it will be interesting to study whether PS/γ-secretase-mediated proteolytic cleavage of β2 modulates sodium channel activity.

The identification of additional PS/γ-secretase substrates may raise questions about the predicted safety of γ-secretase inhibitors in anti-amyloid therapy. Complete blocking of γ-secretase activity should elevate CTF levels of currently known γ-secretase substrate proteins, such as β2 and nectin-1. Studies show strong developmental defects including abnormal neuronal migration in PS1-/-/ mice and neurodegeneration in 6 months old conditional PS1/2 knock-out mice(21,62). However, partial inhibition of γ-secretase activity may reduce Aβ generation without significantly affect cleavage of other substrates. Even better, development of γ-secretase inhibitors designed to specifically block Aβ generation such as NSAID would be desirable for treatment of AD patients(63-65).
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FOOTNOTES

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1 Abbreviations used are: AD, Alzheimer’s disease; Aβ, amyloid β-peptide; APP, β-amyloid precursor protein; PS, presenilin PS1, presenilin 1; PS2, presenilin 2; TPA, 12-O-tetradecanoylphorbol-13-acetate; cell adhesion molecules (CAMs); AICD, APP intracellular domain; NICD, Notch intracellular domain; β2, Voltage-gated sodium channel β2 subunit; β2-CTF, Voltage-gated sodium channel β2 subunit C-terminal fragment; β2 –ICD, Voltage-gated sodium channel β2 subunit intracellular domain; NE-CTF, nectin-1 C-terminal fragment; NE-ICD, nectin-1 intracellular domain.

FIGURE LEGENDS

Fig. 1. The ectodomain of β2 is first cleaved by an α-secretase activity. A. Sequence comparison of the APP-ε/Notch-S3 domains in nine human PS1/γ-secretase substrates and in β2. B. Expected proteolytic processing of β2, based on previously identified PS/γ-secretase substrates. C. Western blot analysis of CHO cells expressing β2-V5/His. β2-CTF levels are increased by TPA treatment, further enhanced by treatment with a γ-secretase inhibitor, DAPT, which is expected to block further processing of β2-CTFs by γ-secretase. D. DAPT treatment elevates β2-CTF levels in CHO cells while co-treatment with the α-secretase inhibitor TAPI-1 (10 μM) blocks this increase. E. Co-expression of β2 with the α-secretase ADAM10 increases levels of the 15 kDa β2-CTF. F.L., full-length; NTF, N-terminal fragment.

Fig. 2. Following ectodomain shedding, β2-CTF is further processed by PS/γ-secretase. A. Western blot analysis of CHO cells expressing β2-V5/His. β2-CTF levels are increased by three different γ-secretase inhibitors, DAPT, L-685,458, and WPE31C; this increase is further potentiated by TPA. B. DAPT increases β2-CTF levels in a dose-dependent manner. C. Expression of the dominant negative form of PS1, PS1 (D385A), also results in the accumulation of the 15 kDa β2-CTF (arrowhead), only slightly intensified by TPA treatment. D. β2-CTF levels are elevated by the absence of functional presenilins in PS1/PS2 double knock out ES cells, slightly increased by TPA. E. DAPT treatment also increases β2-CTF levels in a rat neuroblastoma cell line (B104) stably expressing human β2-V5/His. F. Mouse cortical neurons (DIV16) produce detectable levels of endogenous β2-CTF following DAPT treatment.

Fig. 3. Cell-free generation of β2-ICD is PS/γ-secretase activity-dependent. A. A 12-kDa β2-ICD is only detectable when membrane fractions from β2-transfected CHO cells are incubated at 37 °C, and in the absence of the γ-secretase inhibitor DAPT. B. Western blot analysis of membrane and soluble fractions from a cell-free γ-secretase assay. In addition to DAPT, the γ-secretase inhibitor L-685,458 also blocks β2-ICD generation. The β2-ICD does not appear in the soluble fraction, because it remains loosely associated with membranes. A sodium hydroxide (NaOH) wash, which also removes actin associated with the membranes but not the control transferrin receptor, is needed for dissociation of β2-ICD from membranes.

Fig. 4. DAPT inhibits β2-mediated cell-cell adhesion and cell migration in a wound-healing assay. A. Representative photomicrographs showing cell-cell aggregation after 60 minutes of incubation. Cell clumps were generated specifically in CHO cells stably expressing β2 (CHO-β2, see arrow head in bottom left panel), blocked by 500 nM DAPT (bottom right panel). CHO(-): wild-type CHO cells without the expression of β2. B. Quantitation of the extent of cell-cell aggregation shows that DAPT inhibits cell-
cell aggregation of cells stably expressing β2. N(t): total particle number at time t of incubation; N(0): initial particle number. C. Representative photomicrographs showing that 500 nM DAPT treatment reduces wound healing in CHO-β2 cell lines. Dotted lines delimit the initially wounded regions, as determined by superimposing the picture taken immediately after the wound was made. D. The extent of wound healing was quantified by counting the number of migrated cells after 21 hours. Statistical analysis was performed using ANOVA followed by a post hoc Tukey’s analysis. *p<0.05 compared to DMSO treated cells (n=3 per each condition).

Fig. 5. Overexpressed recombinant β2- or NE-CTFs inhibit cell migration in a wound-healing assay. A. Schematic representation of recombinant β2-CTF, NE-CTF, β2-ICD, and NE-ICD proteins used in the assay (cloned into pSecTag/FRT/V5-His TOPO). B. Subcellular localization of β2- and NE-CTF expressed in CHO cells stably expressing dominant negative PS1 (D385A). C. Western blot analysis by using an anti-V5 antibody of two different stable clones co-expressing full-length β2 and β2-CTF (CHO-β2 F.L.+β2-CTF), and one clone with nectin and NE-CTF (CHO-nectin F.L.+NE-CTF). CTF levels in these cells lines increased by 2-3 fold compared to parental cell lines (CHO-β2 F.L. and CHO-nectin F.L.) expressing only full-length proteins, respectively. D. Representative photomicrographs showing that wound healing activity of CHO cell lines co-expressing full length and CTF of β2 (CHO-β2 F.L.+β2-CTF) or nectin-1 (CHO-nectin F.L.+NE-CTF) was decreased as compared to CHO cells expressing only full-length β2 (CHO-β2 F.L.) or nectin-1 (CHO-nectin F.L.), respectively. Dotted lines delimit the initially wounded regions, as determined by superimposing the picture taken immediately after the wound was made. E. Quantitative analysis of the experiment shown in D. Statistical analyses used were a Student’s t test (left panel) and ANOVA followed by a post hoc Tukey’s analysis (right panel). The control for the right panel is nectin F.L. while β2 F.L. for the left panel (***p<0.001 compared with control; n=3 per each condition). F. Cell cultures co-expressing β2 F.L. (β2 F.L) and β2-ICD (β2 F.L.+β2-ICD) or NE-ICD (β2 F.L.+NE-ICD) were generated and their wound-healing activity was measured. Co-expression of β2-ICD or NE-ICD in CHO-β2 F.L cells doesn’t show any statistically meaningful effect on cell migration (n=6 per each condition).

Supplemental Figures. S1. Western blot analysis of CTFs stably expressed in CHO cells. Multiple bands, probably aggregates, were observed only when β2-CTF, not NE-CTF, was expressed in CHO cells. CHO-NE-CTF, a CHO cell line stably expressing NE-CTF; CHO-β2-CTF, a CHO cell line stably expressing β2-CTF. S2. Western blot analysis of PARP cleavage in cell lines overexpressing β2-CTF and NE-CTF, respectively. The cleaved form of PARP, a marker for apoptosis, was not detected in any of the cell lines while it was seen in staurosporine-treated cells. S3. The mixed cell cultures co-expressing full-length β2 and β2-ICD (CHO-β2 F.L.+β2-ICD) or full-length β2 and NE-ICD (CHO-β2 F.L.+NE-ICD) were generated and confirmed by Western blot analysis by using an anti-V5 antibody which recognizes both full-length proteins and ICDs.
A

Temp. (°C):

|          | 0  | 37 |
|----------|----|----|
| DAPT (nM): | 0  | 0  | 1  |
|          | 45 | β2 F.L. |
|          | 14 | β2-CTFs |
|          |    | β2-ICD |

B

Temp. (°C):

|          | 0  | 37 |
|----------|----|----|
| m100 DAPT (μM): | 0  | 0  | 0.5 | 0  |
| s100 L-685, 386 (μM): | 0  | 0  | 0   | 1  |
|          | 66 | β2 F.L. |
|          | 45 | β2-CTF |
|          | 30 | β2-ICD |
|          | 14 | β2-ICD |

Transferrin receptor

Actin

0.1 M NaOH wash
A

B

C

Relative amount of cells migrated

BSA  PDGF + DMSO  PDGF + DAPT

0 5000 10000 15000
Presenilin/γ-secretase-mediated cleavage of the voltage-gated sodium channel β2 subunit regulates cell adhesion and migration
Doo Yeon Kim, Laura A. MacKenzie Ingano, Bryce W. Carey, Warren P. Pettingell and Dora M. Kovacs

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Additions and Corrections

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Dominant-negative inhibition of pheromone receptor signaling by a single point mutation in the G protein α subunit.

Yuh-Lin Wu, Shelley B. Hooks, T. Kendall Harden, and Henrik G. Dohlman

Pages 35291 and 35292, Figs. 5A and 6A: We failed to note that Dr. David Stone and co-workers had published experiments similar to those in Figs. 5A and 6A (See Refs. 28, 30, and 45). On the basis of those experiments, Dr. Stone and his colleagues had proposed that the mutated G protein α subunit must interact with the receptor in order to inhibit the pheromone signal. We apologize to Dr. Stone for this oversight.

Vol. 280 (2005) 9409–9415

Curcumin regulates expression and activity of matrix metalloproteinases 9 and 2 during prevention and healing of indomethacin-induced gastric ulcer.

Snehasikta Swarnakar, Krishnendu Ganguly, Parag Kundu, Aditi Banerjee, Pallab Maity, and Anamika V. Sharma

Page 9412, Table I: In the original submission, the data presented in Table I had been prepared by Dr. Ranajit K. Banerjee and Dr. Ishita Chattopadhyay and was based on previous work performed in Dr. Banerjee’s laboratory.

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Presenilin-γ-secretase-mediated cleavage of the voltage-gated sodium channel β2-subunit regulates cell adhesion and migration.

Doo Yeon Kim, Laura A. MacKenzie Ingano, Bryce W. Carey, Warren H. Pettingell, and Dora M. Kovacs

Page 23251: The grant footnote should read “This work was supported by grants from the NIA/National Institutes of Health and the John Douglas French Alzheimer’s Foundation.”

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Differential responses of the Nrf2-Keap1 system to laminar and oscillatory shear stresses in endothelial cells.

Tomonori Hosoya, Atsushi Maruyama, Moon-Il Kang, Yukie Kawai, Takahiro Shibata, Koji Uchida, Eiji Warabi, Noriko Noguchi, Ken Itoh, and Masayuki Yamamoto

Dr. Eiji Warabi and Dr. Noriko Noguchi were inadvertently omitted from the author list. Their affiliation is: Laboratory for Systems Biology and Medicine, Research Center for Advanced Science and Technology, University of Tokyo, 4-6-1 Komaba, Meguro, Tokyo 153-8904, Japan.

Page 27250, “Acknowledgments”: As a result of adding Dr. Warabi to the author list, the “Acknowledgment” should now read: “We thank Dr. T. O’Connor for help in the preparation of the manuscript.”