

## Role of the ErbB-4 Carboxyl Terminus in $\gamma$ -Secretase Cleavage\*

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**The ErbB-4 receptor tyrosine kinase has a PDZ domain recognition motif at its carboxyl terminus. The first step in ErbB-4 proteolytic processing is a metalloprotease-dependent cleavage of the receptor ectodomain, which is not influenced by deletion of this motif. Metalloprotease cleavage of ErbB-4 produces a membrane-associated 80-kDa fragment that is a substrate for subsequent  $\gamma$ -secretase cleavage, which releases the cytoplasmic domain from the membrane and allows nuclear translocation of this fragment. Deletion of the PDZ domain recognition motif does abrogate the  $\gamma$ -secretase cleavage of ErbB-4. The wild-type 80-kDa ErbB-4 fragment forms an association complex with presenilin, thought to be the catalytic moiety of  $\gamma$ -secretase activity. However, this association is significantly impaired by loss of the PDZ domain recognition motif from ErbB-4. Deletion of this ErbB-4 motif prevents the nuclear localization of the ErbB-4 cytoplasmic domain. Data also show that the basal cleavage of wild-type ErbB-4 by this proteolytic system can produce a sufficient level of ErbB-4 processing to negatively influence cell growth and that loss of the PDZ domain recognition motif abrogates this response.**

ErbB-4 is a type I receptor tyrosine kinase that binds heregulin (HRG)<sup>1</sup> (1). HRG also binds to ErbB-3. In addition some ErbB-1 ligands (heparin-binding epidermal growth factor, betacellulin, and epiregulin) are reported to activate ErbB-4.

Recently, ErbB-4 was shown to be processed by a novel proteolytic pathway (2), analogous to that which generates cell fate determination responses after activation of the Notch non-tyrosine kinase receptor (3). The amyloid precursor protein (APP) is also processed in a similar manner (4). The first step in this pathway is cleavage within the ectodomain close to the plasma membrane. In the case of ErbB-4, this cleavage is stimutable by TPA (5) and, in some cell lines, by HRG (6). This proteolytic event results in the release of a 120-kDa ErbB-4 fragment into the extracellular milieu and the cellular retention of an 80-kDa membrane-associated fragment. Ectodomain cleavage requires the transmembrane metalloprotease tumor necrosis factor- $\alpha$ -converting enzyme (7).

After ectodomain cleavage of ErbB-4, the cell-associated 80-kDa fragment containing the transmembrane and cytoplasmic domains is cleaved by a  $\gamma$ -secretase activity releasing the cytoplasmic domain into the cytosol (2, 8). Subsequently, the cytoplasmic domain fragment of ErbB-4 translocates into the nucleus. The function of the 80-kDa fragment in the nucleus is not known, although the carboxyl-terminal region of this fragment does display weak activity in the GAL4 transcription activation assay (2). Importantly,  $\gamma$ -secretase inhibitors block the induction of cell death by HRG when T47D carcinoma cells are placed in serum-free media (2). The nuclear localization of other receptor tyrosine kinases, including ErbB-1 (9), ErbB-3 (10), and fibroblast growth factor receptor 1 (11), have been described, but these do not appear to involve proteolytic processing of the receptors.

At this time relatively little is known about how transmembrane metalloprotease tumor necrosis factor- $\alpha$ -converting enzyme and  $\gamma$ -secretase recognize their substrates. In this study, evidence is provided that ErbB-4 PDZ domain recognition motif is necessary for  $\gamma$ -secretase cleavage.

### EXPERIMENTAL PROCEDURES

**Materials**—HRG  $\beta$ 1 was purchased from R&D Systems. The cDNA for human ErbB-4 and rabbit antiserum to a carboxyl-terminal sequence (residues 1108–1264) of human ErbB-4 was generously provided by Dr. Matthias Kraus (University of Alabama, Birmingham, AL) and used for Western blotting. Polyclonal antibodies C-18 to an epitope in the carboxyl terminus of ErbB-4 and H-70 to an epitope in the amino terminus of presenilin 1 were obtained from Santa Cruz Biotechnology. Monoclonal anti-phosphotyrosine antibody (13-5900) and horseradish peroxidase-conjugated Protein A were purchased from Zymed Laboratories Inc. Protein A-Sepharose CL-4B, enhanced chemiluminescence (ECL) reagents, and TPA were obtained from Sigma. The  $\gamma$ -secretase inhibitor Compound E (12) was provided by Dr. Todd Golde (Mayo Clinic, Jacksonville, FL). Nuclear export signal inhibitor leptomycin B was provided by Dr. Minoru Yoshida (University of Tokyo, Japan). G418 sulfate was obtained from Mediatech Inc. All other chemicals were reagent grade from Sigma.

**Cell Culture**—COS7 and NIH3T3 cell lines were obtained from American Tissue Culture Collection. These cells and all NIH3T3-derived cells, described below, were routinely grown in 5% CO<sub>2</sub> at 37 °C in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin (Mediatech Inc.). Experimental cultures were generally grown in 100-mm culture dishes until 90% confluence unless otherwise noted in the text.

**Gene Transfection and Establishment of ErbB-4 Wild-type (WT) and  $\Delta$ TVV Cell Lines**—LTR-W.T.ErbB4 is an ErbB-4 expression vector under the control of a LTR promoter and is described elsewhere (13). It contains a neomycin selection marker. To produce a cDNA construct to express ErbB-4 with the carboxyl-terminal three residues TVV truncated, an 18-mer upstream primer, 5'-GACTACCTGCAGGAGTAC-3', was synthesized to match a sequence within ErbB-4 cDNA containing a *Bam*HI site. Also, a 31-mer downstream primer 5'-ACGCGTCGACTTAATTCCGGGTGTCTGTAAGG-3', was made containing a *Sal*I site next to stop code TAA, which terminates translation 9 bp before the wild-type ErbB-4 stop sequence. PCR amplification of LTR-W.T.ErbB4 cDNA with the above primers produced a 160-bp fragment that was then cut with *Bam*HI and *Sal*I to generate cohesive ends. The LTR-W.T.ErbB4 construct was processed to remove the small fragment

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<sup>1</sup> The abbreviations used are: HRG, heregulin; PDZ, PSD-95/discs large/ZO-1; APP, amyloid precursor protein; WT, wild-type; FBS, fetal bovine serum; LTR, long terminal repeat; DMEM, Dulbecco's modified Eagle's medium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PBS, phosphate-buffered saline.

between *Bam*HI and *Sal*I (next to the stop codon), and the PCR product was inserted into the exact site. The resulting construct, called LTR- $\Delta$ TVV, contains ErbB-4 cDNA with 9 bp corresponding to the carboxyl-terminal three residues, *i.e.* TVV, deleted. The mutant construct was verified by enzymatic digestion and DNA sequencing.

For gene transfection, COS7 and NIH3T3 cells were transfected with LTR-WT.ErbB4 or LTR- $\Delta$ TVV using Effectene reagent (Qiagen) according to the manufacturer's protocol. Typically, 2.0–2.5  $\mu$ g of DNA was used to transfect cells in a 100-mm dish. COS7 cells were assayed for ErbB-4 receptor function 36–48 h after transfection. Stably transfected NIH3T3 cell lines were obtained after selection in G418 sulfate (400  $\mu$ g/ml) for 2 months. Each stably transfected cell line, either WT ErbB-4 or  $\Delta$ TVV mutant, represents a pool of several individual colonies. For each receptor species, two stable cell lines from independent transfections were established and assayed.

**Cell Treatment and Lysis and Fractionation**—Cell lysates were obtained essentially as described previously (5) with some modifications. Briefly, 90% confluent cell monolayers in 100 mm dishes were incubated overnight in DMEM containing 0.2% FBS (or 1% FBS, as indicated in the text). Cells were then incubated at 37 °C with the indicated additions for the indicated times. Next, the cells were washed three times with  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free phosphate-buffered saline ( $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS) and lysed in 1 ml of cold TGH lysis buffer (1% Triton X-100, 10% glycerol, 20 mM Hepes (pH 7.2), 100 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 1 mM  $\text{Na}_3\text{VO}_4$ , and 50 mM NaF) or mild TGH lysis buffer, having reduced levels of Triton X-100 (0.2%) and NaCl (20 mM). The lysed cells were scraped from the dish and placed in a 1.5-ml microcentrifuge tube. The lysates were incubated on ice for 30 min and then centrifuged (14,000  $\times g$ , 10 min) at 4 °C, and the supernatants were saved for Western blotting or immunoprecipitation. Preparation of membrane and cytosol fractions was described previously (5).

**Immunoprecipitation and Western Blotting**—To analyze ErbB-4 in total cell lysates, ~1–2 mg of cell lysate were immunoprecipitated and underwent Western blotting or ~50  $\mu$ g underwent blotting without immunoprecipitation. To assess ErbB-4 in cytosol and membrane fractions, equal volumes of each fraction representing equal numbers of cells were immunoprecipitated and then blotted.

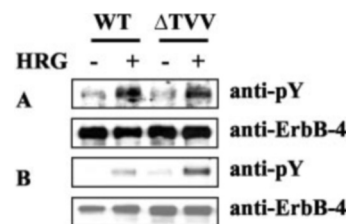
To precipitate proteins, ~4  $\mu$ g of ErbB-4 or presenilin 1 antibody was incubated (2 h at 4 °C) with lysates followed by a 1-h incubation with Protein A-Sepharose CL-4B. Immune complexes were washed three times with the same lysis buffer (TGH or mild TGH), resuspended in 1 $\times$  Laemmli buffer, and boiled for 5 min. Subsequently, samples were electrophoresed in a 7.5% SDS-polyacrylamide gel and transferred to nitrocellulose membranes for Western blotting. Membranes were blocked by a 1-h incubation at room temperature in TBST buffer (0.05% Tween 20, 150 mM NaCl, 50 mM Tris, pH 7.4) containing 3% bovine serum albumin (for tyrosine phosphorylation detection) or 5% nonfat dry milk (for all others). Subsequently, ErbB-4 antiserum or phosphotyrosine antibody was added for 2 h at room temperature with shaking. Membranes were then washed thrice with TBST buffer, incubated with horseradish peroxidase-conjugated Protein A for 1 h, and after being washed with TBST buffer, visualized by ECL.

**Immunofluorescence Staining in Fixed Cells**—Cells grown on coverslips were fixed with methanol at –20 °C for 5 min. The cells were then rehydrated with PBS for 10 min and, after a 45-min blocking period using 10% goat serum (Sigma) in PBS, were incubated with C-18 ErbB-4 antibody for 45 min. After thorough washing (three times in PBS), secondary antibody Alexa Fluor<sup>TM</sup> 546 goat anti-rabbit IgG (H+L) conjugate (Molecular Probes) was added for 30 min. The cells were then mounted in Aqua Poly/Mount (Polysciences, Inc.). A Zeiss LSM 410 confocal microscope equipped with  $\times 40$  (1.3 numerical aperture) oil immersion objective lens, and the single fluorochrome filter set for Alexa 546 (Molecular Probes) was used for visualization and recording images.

## RESULTS

**Ligand Responsiveness of the ErbB-4  $\Delta$ TVV Mutation**—The carboxyl terminus of ErbB-4 encodes a PDZ domain recognition motif that has been demonstrated to facilitate interaction with PSD95, a multi-PDZ domain-containing protein that is highly expressed in neuronal cells (14, 15). However, the biological significance of ErbB-4 interaction with PDZ domains is not known.

To test the possible functional role of the PDZ domain binding motif in ErbB-4, the three carboxyl-terminal residues that



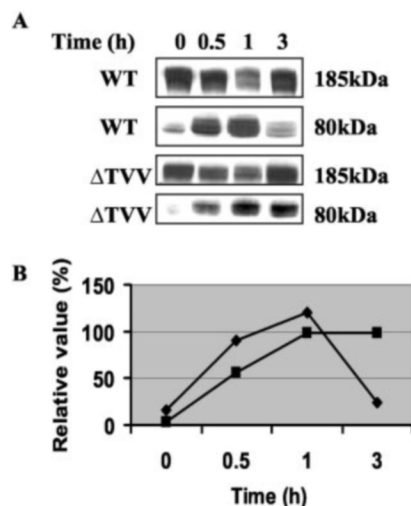
**FIG. 1. Ligand activation of WT ErbB-4 and  $\Delta$ TVV mutant.** A, COS7 cells transiently expressing WT ErbB-4 or  $\Delta$ TVV mutant ErbB-4 were treated with HRG (20 ng/ml) for 10 min. The cells were then immediately washed three times with cold  $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS and then lysed in TGH buffer. After clarification, the lysate (0.8 mg) was immunoprecipitated with C-18 ErbB-4 antibody. Western blots were carried out with anti-phosphotyrosine followed by membrane stripping and reblotting with ErbB-4 antibody. B, NIH3T3 cells stably expressing WT ErbB-4 or the  $\Delta$ TVV mutant ErbB-4 were incubated overnight in DMEM medium with 1% FBS, treated with HRG (20 ng/ml, 10 min), and lysed for immunoprecipitation and Western blotting as described in the legend for A.

define this motif (16), were deleted to create the ErbB-4 mutant designated  $\Delta$ TVV. We first tested by transient expression in COS7 cells the capacity of HRG to stimulate tyrosine phosphorylation of both WT ErbB-4 and the  $\Delta$ TVV mutant. The results (Fig. 1A) demonstrate that both forms of ErbB-4 are activated by exogenous HRG. Subsequently, permanent cell lines expressing WT ErbB-4 or the  $\Delta$ TVV mutant were obtained by transfection of NIH3T3 cells, which do not endogenously express any ErbB family members. In these cells, HRG also activated both receptor isoforms to approximately equivalent extents (Fig. 1B). Consistent with growth factor-dependent activation of both ErbB-4 species, HRG also activated Erk1 and Erk2 protein kinases when added to cells expressing WT ErbB-4 or the  $\Delta$ TVV mutant (data not shown).

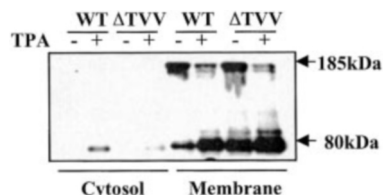
The above results show that loss of the PDZ domain recognition motif does not abrogate ligand-dependent receptor activation and by inference cell surface expression. This result is slightly in contrast to a previous report which concluded that the ErbB-4 PDZ domain recognition motif was required for maximal growth factor activation of this receptor (15).

**Influence of the  $\Delta$ TVV Mutation on ErbB-4 Proteolytic Processing**—The  $\Delta$ TVV mutation of ErbB-4 was evaluated to assess the potential role of the PDZ domain recognition motif in the proteolytic processing pathway, which requires the participation of at least two integral membrane proteases. The initial step in this pathway is the metalloprotease cleavage of ErbB-4, which produces an 80-kDa membrane-associated fragment (5). When cells expressing WT ErbB-4 or the  $\Delta$ TVV mutant were treated with TPA, the 80-kDa fragment was produced at approximately the same rate from both receptor species for the first 60 min, as shown in Fig. 2. However, at a later time (3 h) the 80-kDa fragment generated in cells expressing the ErbB-4  $\Delta$ TVV mutant remained at a significant level, whereas the level of the fragment generated in cells expressing the wild-type receptor had significantly declined. These results indicate that whereas the  $\Delta$ TVV mutation did not influence the metalloprotease cleavage of ErbB-4, the cell-associated fragment generated from the mutant receptor was significantly more metabolically stable.

One means by which the membrane-associated 80-kDa ErbB-4 fragment is metabolized is by  $\gamma$ -secretase cleavage. This cleavage converts the membrane-associated fragment, designated m80, to a cytosolic fragment, designated s80 (2). Therefore, the TPA-dependent formation of the m80 and s80 ErbB-4 fragments from the WT and  $\Delta$ TVV ErbB-4 receptors were examined 30 min after the addition of the agonist. Detection of anti-ErbB-4 reactive bands in the membrane fraction (Fig. 3) shows that the full-length (180-kDa) WT ErbB-4 and



**FIG. 2. Influence of  $\Delta$ TVV mutation on ErbB-4 ectodomain cleavage.** A, NIH3T3 cells expressing WT ErbB-4 or  $\Delta$ TVV were cultured until 90% confluent, starved overnight in DMEM medium with 1% FBS, and then treated with TPA (100 ng/ml) for the times indicated. TGH lysates were prepared for Western blotting with ErbB-4 antibody to detect the full-length (180-kDa) ErbB-4 molecule and the 80-kDa cell-associated fragment. B, the bands were quantitated by scanning densitometry. To compare the value of the 80-kDa band in the two cell lines, the amount of 80-kDa fragment was normalized to the amount of 180-kDa ErbB-4 present at time 0 h.

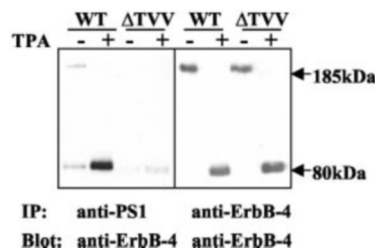


**FIG. 3. Influence of  $\Delta$ TVV mutant on  $\gamma$ -secretase cleavage of ErbB-4.** NIH3T3 cells expressing WT ErbB-4 or  $\Delta$ TVV were cultured until 90% confluent. The cells were then starved overnight in DMEM with 0.2% FBS and treated for 30 min with TPA (100 ng/ml) or vehicle  $\text{Me}_2\text{SO}$ . The cells were then disrupted by homogenization, and the membrane and cytosolic fractions were prepared as described under "Experimental Procedures." Aliquots of membrane and cytosolic fractions were then immunoprecipitated with ErbB-4 antibody (C-18) and blotted with another ErbB-4 antibody.

$\Delta$ TVV mutant proteins are degraded in the presence of TPA and that the m80 fragment is generated from each. However, the s80 ErbB-4 fragment is readily detected only in cells that express the WT receptor. Only a very low level of s80 is produced in cells expressing the  $\Delta$ TVV mutant. After quantitating the s80 bands, it is estimated that production of this fragment is reduced by 90–95% in the  $\Delta$ TVV mutant.

The above results indicate that introduction of the  $\Delta$ TVV mutation selectively abrogates conversion by  $\gamma$ -secretase of the mutant m80 ErbB-4 fragment to the s80 fragment.  $\gamma$ -Secretase catalytic activity is generally considered to be a property of presenilin proteins; however, this point remains unclear (17). Other substrates of  $\gamma$ -secretase, such as APP and Notch, have been found to co-precipitate with presenilins (PS1, PS2) (18–22).

A co-precipitation strategy was employed to ascertain whether presenilin associates with ErbB-4 and whether this is influenced by the receptor's PDZ domain recognition motif. The results, as shown in Fig. 4 (left panel), demonstrate that when PS1 is immunoprecipitated from untreated cells expressing the WT ErbB-4 receptor, anti-ErbB-4 reactive proteins are detected representing the full-length ErbB-4 (180-kDa) and the



**FIG. 4. Association of presenilin with ErbB-4 and the 80-kDa ErbB-4 fragment.** NIH3T3 cells expressing WT ErbB-4 or  $\Delta$ TVV were cultured until 90% confluent and then incubated overnight in DMEM with 0.2% FBS. Subsequently, the cells were treated with TPA (100 ng/ml) or vehicle  $\text{Me}_2\text{SO}$  as a control for 30 min. The cells were lysed in mild TGH lysis buffer containing reduced levels of Triton X-100 (0.2%) and NaCl (20 mM). Aliquots of the lysates were then immunoprecipitated as indicated with anti-PS1 or anti-ErbB-4, and the precipitates (IP) were blotted with anti-ErbB-4.

80-kDa ErbB-4 fragment. When TPA is added to these cells, however, the level of 80-kDa ErbB-4 fragment detected in the PS1 immunoprecipitate is greatly increased, as is the amount of 80-kDa fragment in these cells. Comparison of the amount of full-length ErbB-4 in untreated cells and 80-kDa fragment in TPA-treated cells suggests that PS1 associates preferentially with the 80-kDa fragment, which mostly represents the m80  $\gamma$ -secretase substrate. In contrast, an almost undetectable level of ErbB-4 reactivity is found in PS1 immunoprecipitates from cells expressing the  $\Delta$ TVV mutant. The data in the right panel show that in both cell types equal levels of full-length ErbB-4 are present and that in the presence of TPA the 80-kDa fragment is also detected at equivalent levels in both cell types. The results suggest that s80 is not generated from the m80  $\Delta$ TVV ErbB-4 mutant, because the latter fragment is unable to associate with PS1. The presence of PS2 in these cells was not detectable.

If the absence of a PDZ domain recognition motif prevents efficient  $\gamma$ -secretase cleavage of ErbB-4, then it would be expected that the  $\Delta$ TVV mutant receptor would fail to be translocated to the nucleus after the addition of TPA. The data in Fig. 5 show that whereas TPA addition to cells expressing the WT ErbB-4 receptor does provoke nuclear localization of ErbB-4 immunoreactivity, similar treatment of cells expressing the  $\Delta$ TVV mutant reveals no nuclear ErbB-4 signal even if leptomycin B (23) is added to prevent nuclear export. The data additionally demonstrate that if leptomycin B is added but the cells are not stimulated with TPA, ErbB-4 reactive material is present in the nucleus of cells expressing the WT receptor but not in cells that express the  $\Delta$ TVV mutant. This basal level of nuclear localized ErbB-4 is prevented in the cells expressing WT receptor by addition of the  $\gamma$ -secretase inhibitor Compound E (12).

**Influence of  $\Delta$ TVV Mutation on Cell Growth**—When T47D mammary carcinoma cells are placed in serum-free medium, HRG-induced ErbB-4 cleavage leads to cell death in a manner that requires  $\gamma$ -secretase activity (2). Also, overexpression of the ErbB-4 cytoplasmic domain in COS cells results in cell death (8). Although a basal level of ErbB-4 cleavage exists in all cell systems, this was not sufficient to influence survival of the T47D cells. To investigate further the potential biological significance of basal ErbB-4 cleavage, the survival of the transfected NIH3T3 cells used in the experiments described herein was evaluated.

The data in Fig. 6 show that when cells expressing WT ErbB-4 or the  $\Delta$ TVV mutant are placed in serum-free medium, the WT ErbB-4 cells initially survived and slightly increased in cell number, but by day 3 nearly all had died. In contrast, cells that express the  $\Delta$ TVV mutant ErbB-4 survive under the same



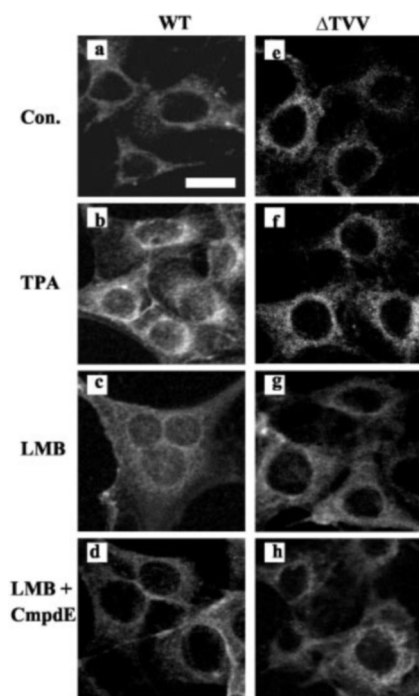


FIG. 5. **PDZ domain recognition motif-dependent ErbB-4 nuclear localization.** NIH3T3 cells expressing WT ErbB-4 or  $\Delta$ TVV were cultured on coverslips until 80% confluent and then incubated overnight in DMEM with 0.2% FBS (a, b, e, f), or with leptomycin B (10 ng/ml) (c, g), or with leptomycin B (LMB, 10 ng/ml) and compound E (Cmpd E, 10 nM) (d, h) as indicated. TPA (100 ng/ml) was then added (b, f) for 90 min. All cells were then fixed and immunostained with C-18 ErbB-4 antibody for confocal microscopy as described under "Experimental Procedures." Bar, 25  $\mu$ m, applies to each image.

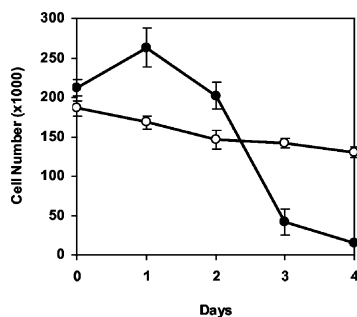


FIG. 6. **Influence of  $\Delta$ TVV mutation on cell death.** NIH 3T3 cells expressing WT ErbB-4 (●) or the  $\Delta$ TVV mutant (○) were plated ( $\sim 2 \times 10^5$  cells/well in 12-well dishes. On the next day (day 0), the cells were washed with DMEM twice and cultured in serum-free DMEM. Cell numbers were determined at 24-h intervals thereafter, using a Coulter counter.

culture conditions for at least 4 days. This result indicates that cells expressing the  $\gamma$ -secretase cleavable WT ErbB-4 are sensitive to cell death under these conditions, but the  $\Delta$ TVV mutant does not produce a signal that provokes cell death. This also suggests that in these NIH3T3 cells, basal ErbB-4 cleavage is sufficient to provoke a cell response.

To confirm that  $\gamma$ -secretase activity is necessary for this growth response, the experiment was repeated in the presence of Compound E, a potent  $\gamma$ -secretase inhibitor (12). The cells were plated as described in Fig. 6, and cell numbers were determined 3 days later (Table I). The results demonstrate that only 14% of the cells expressing the WT ErbB-4 survived under these conditions, whereas 88% of the cells expressing the  $\Delta$ TVV mutant survived. The addition of Compound E almost completely prevented cell death in cells expressing the WT ErbB-4

TABLE I

*Influence of  $\gamma$ -secretase inhibitor on cell growth*

Approximately  $2 \times 10^5$  cells expressing WT ErbB-4 or the  $\Delta$ TVV mutant were plated in complete medium. On the following day the medium was removed and replaced with serum-free medium. Compound E (10 nM) was added as indicated. Three days later cell numbers were determined. Each cell count represents triplicate determination.

Additions	Final cell number ( $\times 1000$ )	
	WT	$\Delta$ TVV
None	27.3 $\pm$ 11.4	176.7 $\pm$ 12.3
Compound E	283.7 $\pm$ 32.9	153.7 $\pm$ 11.5

receptor but did not significantly alter the response of cells that express the  $\Delta$ TVV mutant.

## DISCUSSION

The major conclusion of the experimental results presented in the manuscript is that PDZ domains have a central role in the mechanism by which ErbB-4 is recognized and processed by  $\gamma$ -secretase activity. This proteolytic step seems to involve intramembraneous cleavage of ErbB-4 and results in the release of the receptor's cytoplasmic domain (s80) into the cytosol and nucleus (2).

The immediate substrate for  $\gamma$ -secretase activity is not the full-length ErbB-4 but rather a membrane-associated fragment (m80) that is derived from the native receptor by metalloprotease-mediated cleavage of the receptor ectodomain (2). The steps in this proteolytic pathway for ErbB-4 are analogous to those described for the proteolytic processing of Notch, a non-tyrosine kinase receptor, and APP, a nonreceptor transmembrane molecule. The intramembraneous cleavage of all three substrates requires  $\gamma$ -secretase activity. However, the cleavage sites in Notch and APP are dissimilar in sequence and relative location within the transmembrane domain (17). Hence,  $\gamma$ -secretase recognition is not thought to depend on substrate primary sequence information, as is the case with many other proteases. The transmembrane sequence of ErbB-4 is unlike that of Notch or APP.

The data presented herein show that the carboxyl-terminal PDZ domain recognition motif of ErbB-4 is required for  $\gamma$ -secretase cleavage of the ErbB-4 m80 fragment. This motif is also necessary for association of PS1 with the m80 ErbB-4 fragment. These results imply that the target of this ErbB-4 motif plays an important role in mediating intramembraneous cleavage of ErbB-4. Others have identified PSD95 as one target of the ErbB-4 carboxyl-terminal sequence (14, 15). PSD95, which is highly expressed in neuronal cells, contains three PDZ domains, two of which recognize ErbB-4. In neuronal tissue PSD95 functions to organize specialized complexes of transmembrane proteins (16). In the non-neuronal cells used in the experiments described herein, we have been able to detect PSD95 family members in ErbB-4, but not PS1, immunoprecipitates (data not shown).

Interestingly, the carboxyl terminus of the CooH-terminal fragment of PS1 also contains a PDZ domain recognition sequence, and PDZ domain containing-proteins that recognize PS1 through this motif have been reported (22, 24). Hence, it seems plausible to suggest that ErbB-4 association with  $\gamma$ -secretase involves PDZ domain-containing proteins acting as a scaffold to bridge the two molecules. Both APP and Notch have also been shown to associate with PS1 using co-precipitation methodology. However, the associations are not known to be direct and may involve other molecules. In the APP/PS1 association, evidence exists that a PDZ domain-containing protein interacts by a PDZ domain with PS1 and by another domain with APP (22). Because PDZ domain-containing proteins often multimerize, it is possible that multiple proteins

having PDZ domains could facilitate ErbB-4 association with  $\gamma$ -secretase activity. Alternatively, it is possible that mutation of the ErbB-4 PDZ domain recognition motif alters the cell surface trafficking of ErbB-4 and prevents  $\gamma$ -secretase cleavage.

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