γ-Secretase Cleavage and Nuclear Localization of ErbB-4 Receptor Tyrosine Kinase

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ErbB-4 is a transmembrane receptor tyrosine kinase that regulates cell proliferation and differentiation. After binding of its ligand heregulin (HRG) or activation of protein kinase C (PKC) by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), the ErbB-4 ectodomain is cleaved by a metalloprotease. We now report a subsequent cleavage by γ-secretase that releases the ErbB-4 intracellular domain from the membrane and facilitates its translocation to the nucleus. γ-Secretase cleavage was prevented by chemical inhibitors or a dominant negative presenilin. Inhibition of γ-secretase also prevented growth inhibition by HRG. γ-Secretase cleavage of ErbB-4 may represent another mechanism for receptor tyrosine kinase–mediated signaling.

ErbB receptors, including the epidermal growth factor (EGF) receptor ErbB-1, ErbB-2, ErbB-3, and ErbB-4, are growth factor–dependent transmembrane tyrosine kinases that control cell proliferation and differentiation (*1*). Ectodomain cleavage of ErbB-4, but not other ErbB receptors, can be stimulated by the PKC activator TPA (*2*) or by HRG (*3*) (also known as neu differentiation factor), the growth factor that binds to ErbB-4. This cleavage requires the metalloprotease TACE (*4*), a transmembrane molecule with an ectodomain protease. Products of this cleavage event are an 80-kD fragment that contains the cytoplasmic and transmembrane domains and a few ectodomain residues, plus a 120-kD ectodomain fragment that is released into the extracellular medium (*2*). We have now detected a soluble (s80) ErbB-4 fragment in addition to the membrane-associated (m80) fragment in the cell lysates of TPA-treated T47-14 cells, which are NIH 3T3 cells that ectopically express ErbB-4 (Fig. 1A). The s80 ErbB-4 fragment was detected after the cells were treated with TPA for 15 min. The ratio of s80 to m80 was about 1:8. The low level of detectable s80 may explain why this fragment was not reported previously.

TPA or HRG induces ectodomain cleavage of ErbB-4 in certain cancer cell lines that express endogenous ErbB-4 (*3*), such as T47D mammary carcinoma cells, and both agents also induced formation of the s80 and m80 ErbB-4 fragments (Fig. 1B). Although contamination of small amounts of membrane could account for the appearance of an 80-kD fragment in the soluble fraction, no native ErbB-4 or ErbB-2 (*5*) was detected in the soluble fraction.

Generation of a cytosolic fragment from a transmembrane protein involves cleavage within the transmembrane domain by γ-secretase or by a Site-2 protease (*6*), or cleavage within the cytoplasmic domain by proteases such as the proteasome (*7*) or caspases (*8*). Because neither the pan-caspase inhibitor zVAD-FMK nor the specific proteasome inhibitor lactacystin inhibited formation of the ErbB-4 s80 or m80 fragments in TPA-treated cells (*5*), their involvement in s80 generation was excluded. However, treatment of T47-14 cells with various γ-secretase inhibitors [zYIL-cho (*9*), H5106, also called L-685,458 (*10*), and compound E (*11*)] inhibited formation of the s80 ErbB-4 fragment, but not the m80 fragment, in response to subsequent TPA addition (Fig. 2A). This selective effect of γ-secretase inhibitors on formation of the two fragments further indicates that membrane contamination of the soluble fraction did not account for the s80 fragment.

The polytopic membrane proteins presenilin-1 (PS1) or presenilin-2 (PS2) may be the catalytic component of an active γ-secretase complex (*12*). Generation of the ErbB-4 s80 fragment in 293 cells treated with TPA increased when the cells coexpressed exogenous wild-type PS1 (Fig. 2B). In contrast, coexpression of an exogenous dominant negative (DN) PS1 mutant, which lacks the first two transmembrane domains of PS1 (*13*), prevented formation of the s80 ErbB-4 fragment by endogenous enzyme activity but did not alter TPA-induced production of the m80 fragment. Similar results were obtained with H4 glioma cell lines that overexpressed either wild-type PS1 or another DN PS1 mutant (D385E) (*5*). Hence, both chemical and biological data demonstrate γ-secretase–mediated formation of the s80 ErbB-4 fragment.

Other γ-secretase substrates include the transmembrane proteins Notch and β-amyloid precursor protein (APP) (*12*). Processing of Notch and APP by γ-secretase requires the prior metalloprotease-dependent cleavage of their ectodomains. To determine whether formation of the s80 ErbB-4 fragment also requires metalloprotease-mediated cleavage of the ErbB-4 ectodomain, we incubated T47-14 cells with the metalloprotease inhibitor BB-94 before treatment with TPA (Fig. 2C). BB-94 blocked formation of both membrane and soluble 80-kD ErbB-4 fragments and also prevented a decrease in the level of native ErbB-4. This finding indicates that γ-secretase processing of ErbB-4 required prior ectodomain cleavage and that the direct γ-secretase substrate is not the intact ErbB-4 molecule, but rather the m80 fragment. Although the site of γ-secretase cleavage that

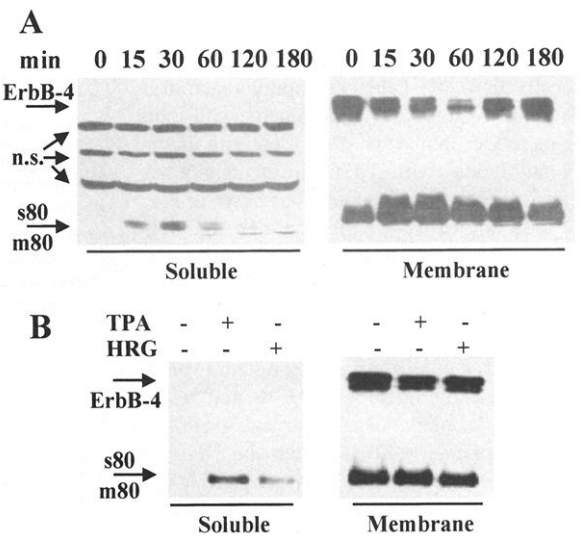

Fig. 1. Detection of ErbB-4 fragments in soluble and membrane fractions. (**A**) Confluent monolayers of T47-14 cells were grown and then treated with TPA (100 ng/ml) for the indicated times, and cell lysates were separated into soluble and membrane fractions for Western blot analysis with an antibody to the ErbB-4 cytoplasmic domain as described (*2*). Nonspecific (n.s.) bands are indicated. (**B**) Confluent monolayers of T47D cells were incubated for 30 min with TPA (100 ng/ml) or HRG (20 ng/ml) as indicated. Soluble and membrane fractions were prepared and immunoprecipitated with C-18 antibody to ErbB-4 COOH-terminal domain as described (*2*). The precipitates were analyzed by Western blot with the antibody to the ErbB-4 cytoplasmic domain indicated in (A). Native ErbB-4 (185 kD) and the membrane-bound 80-kD (m80) and soluble 80-kD (s80) fragments are indicated.

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Table 1. Requirement of γ-secretase for HRG-dependent growth inhibition. T47D cells were plated in Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum. After overnight incubation, the cell number was determined (748,000 cells per well) and the medium was changed to serum-free DMEM. Additions of HRG (20 ng/ml), EGF (25 ng/ml), or compound E (10 nM) were made as indicated. After 2 days, the medium with fresh additions was changed; final cell numbers were determined after 4 days. Changes relative to no additions (100%) are also shown.

Additions	Final cell number ($\times 10^3$)	Relative change (%)
None	1007 ± 65	100
HRG	373 ± 39	37
HRG + compound E	978 ± 94	97
EGF	1418 ± 188	141
EGF + compound E	1664 ± 192	165
Compound E	863 ± 134	86

releases the s80 fragment from m80 has not been identified, it is likely to be within the ErbB-4 transmembrane domain, analogous to the γ-secretase cleavage of Notch and APP. Cleavage of ErbB-4 within the transmembrane domain could sufficiently destabilize the membrane association energetics of the s80 fragment such that it is released into the cytosol.

ErbB-4 has been detected in the nuclei of tumor cells and, unlike ErbB-1, -2, or -3, harbors potential nuclear localization and export signals within its cytoplasmic domain (*14, 15*). Therefore, we used the γ-secretase inhibitor H5106 to determine whether nuclear localization of ErbB-4 is dependent on γ-secretase activity. Although a small amount of ErbB-4 was detected by immunochemistry in the nuclei of untreated T47-14 cells, nuclear staining markedly increased after treatment with TPA (Fig. 3). However, when cells were treated with TPA in the presence of H5106, nuclear localization was abolished, indicating that γ-secretase processing of ErbB-4 was necessary for nuclear localization of ErbB-4 immunoreactivity. Similar results were obtained with the γ-secretase inhibitors zYIL-cho and compound E and with T47D breast carcinoma cells (*5*). The immunodetection shown in Fig. 3 used an ErbB-4 antibody to a COOH-terminal epitope. When an antibody to the ErbB-4 ectodomain (C-18, Santa Cruz) was used, there was no evidence of nuclear immunoreactivity (*5*). This is consistent with the presence of a cytoplasmic domain ErbB-4 fragment in the nuclei of TPA-treated cells.

When fusion proteins comprising green fluorescent protein (GFP) at either the NH_2- or COOH-terminus of the ErbB-4 cytoplasmic domain were transiently expressed in COS-7 cells, they were detected in both the nucleus and cytoplasm (Fig. 4). The NH_2-terminal GFP fusion protein was more extensively localized in the nucleus. When the nuclear export inhibitor leptomycin B (LMB) was added to cells, both GFP–ErbB-4 fusion proteins predominantly localized to nuclei. In contrast, in either the absence or presence of LMB, a GFP fusion protein containing the cytoplasmic domain of ErbB-2 localized to the cytoplasm only. These findings suggest that after γ-secretase cleavage of the m80 ErbB-4 fragment, the s80 fragment is released into the cytoplasm and shuttles between the nucleus and the cytoplasm.

Our results indicate that the fate of ErbB-4 is analogous to that of the Notch and APP cytoplasmic domains, which translocate to

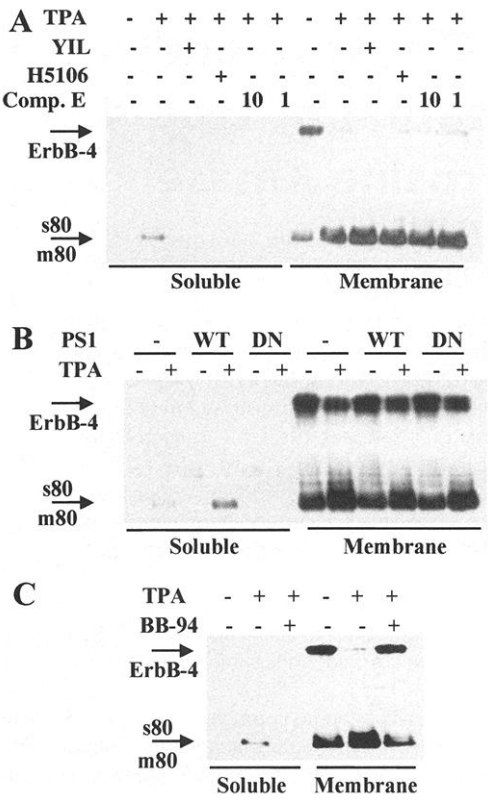

Fig. 2. γ-Secretase activity requirement for formation of soluble ErbB-4 fragment. (**A**) Confluent T47-14 cells grown as described (*2*) were treated for 30 min with the γ-secretase inhibitors YIL (60 μM), H5106 (10 μM), or compound E (1 or 10 nM). TPA (100 ng/ml) was then added for 30 min and soluble and membrane fractions were prepared. Native ErbB-4 and ErbB-4 fragments were detected as in Fig. 1B. (**B**) 293 cells, untransfected or stably expressing wild-type (WT) PS1 or a dominant negative (DN) PS1 mutant (PS1TM1-2) as described (*13*), were treated with TPA (100 ng/ml) for 30 min as indicated. Soluble and membrane fractions were assayed as in Fig. 1B for ErbB-4 and the s80 and m80 ErbB-4 fragments. (**C**) Confluent T47-14 cells were grown as in (A) and preincubated with the metalloprotease inhibitor BB-94 (10 μM) as described (*2*) for 30 min before addition of TPA (100 ng/ml) for an additional 30 min. Soluble and membrane fractions were prepared and assayed as in Fig. 1B for ErbB-4 and the s80 and m80 fragments.

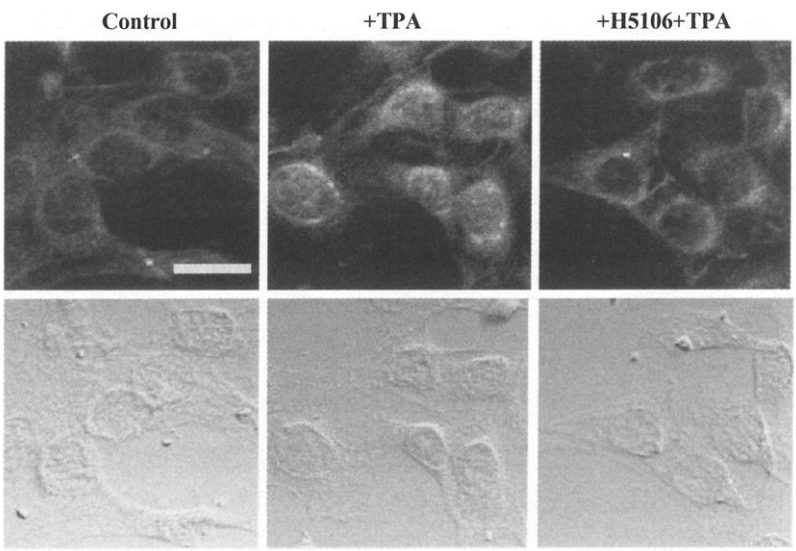

Fig. 3. Immunochemical localization of ErbB-4. Subconfluent T47-14 cells, grown on cover slips as described (*2*), were treated with dimethyl sulfoxide (control cells) or TPA (100 ng/ml) for 90 min, or were preincubated with H5106 (10 μM) for 30 min before addition of TPA for an additional 90 min. After fixation with –20°C methanol, the cells were rehydrated, blocked with goat serum, and immunostained with C-18 antibody to the ErbB-4 COOH-terminal domain as described (*14*). The primary antibody was detected with goat antibody to rabbit immunoglobulin G conjugated with Alexa Fluor 546 and examined by confocal microscopy. Differential interference contrast microscopy images are also presented (bottom panels). Scale bar, 25 μm.

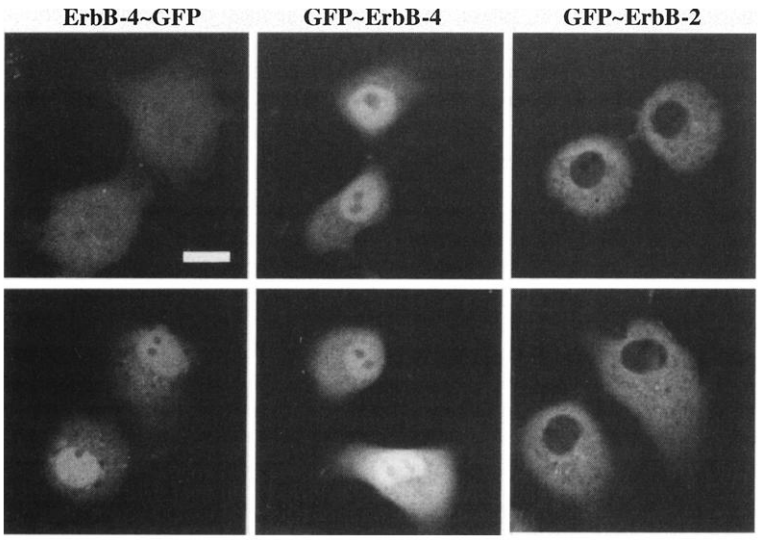

Fig. 4. Localization of GFP-ErbB fusion proteins. The columns labeled ErbB-4–GFP and GFP–ErbB-4 show GFP fusions at the COOH- or NH_2-terminus, respectively, of the ErbB-4 cytoplasmic domain (residues 676 to 1308); the right column shows GFP fusion at the NH_2-terminus of ErbB-2 (residues 676 to 1255). Fusions were constructed and expressed in COS-7 cells as described (*17*). After 48 hours, the cells were examined by confocal microscopy. The cells in the bottom panels were incubated for 1 hour with LMB (100 ng/ml) before microscopy. Scale bar, 25 μm.

the nucleus and promote transcription after γ-secretase cleavage (*12, 16*). We have used the GAL4 transactivation system in COS-7 cells to assess the possible transactivation potential of the ErbB-4 cytoplasmic domain. The results indicate that the COOH-terminal domain of ErbB-4 has weak transcriptional activity (about three times that of the control GAL4 DNA binding domain), whereas the ErbB-4 kinase domain and the complete cytoplasmic domain have no increased activity relative to the control (*17*). When expressed as GFP fusion proteins, the ErbB-4 COOH-terminal domain localized to the nucleus, but the kinase domain was present mainly in the cytosol (*17*). Recently it was reported that the EGF receptor (ErbB-1) is localized to the nucleus and that its COOH-terminal domain functions as a transcription factor in several assays, including the GAL4 system (*18*).

HRG association with ErbB-4 inhibits growth and induces the differentiation of mammary carcinoma cell lines (*19, 20*). When serum-deprived T47D mammary carcinoma cells were treated with HRG, the total cell number decreased (Table 1). In contrast, treatment of the cells with EGF increased the cell number. Addition of the γ-secretase inhibitor compound E completely abrogated the HRG-induced cell loss but had no effect on the growth-stimulatory effect of EGF. This result indicates that γ-secretase activity is necessary for this biological response to HRG. HRG responses in T47D cells could involve ErbB-3 or ErbB-2 in addition to ErbB-4, but we have not detected proteolytic cleavage of ErbB-3 or ErbB-2 in these cells (*5*).

ErbB-4 is a receptor tyrosine kinase that is proteolytically processed by consecutive ectodomain and intramembrane cleavages, resulting in the nuclear translocation of its cytoplasmic domain. Although ectodomain cleavage of several other receptor tyrosine kinases has been reported, it is not known whether any are also processed by γ-secretase activity. Because the s80 ErbB-4 fragment contains a tyrosine kinase domain, nuclear substrates may become phosphorylated by the s80 fragment, further extending the receptor's mechanism of action.

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15. Given that the nuclear export signal consensus sequence is $XR_{2-4}XR_2XR_1X$ (where X represents Leu, Ile, or Val, and R represents any amino acid), there are three putative nuclear export signal sequences in the ErbB-4 cytoplasmic domain sequence: Leu^{713}-Lys-Glu-Thr-Glu-Leu-Lys-Arg-Val-Lys-Val-Leu, Leu^{780}-Val-Arg-Leu-Leu-Gly-Val-Cys-Leu, and Leu^{939}-Pro-Gln-Pro-Pro-Ile-Cys-Thr-Ile-Asp-Val.
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17. For supplementary figures showing that the ErbB-4 COOH-terminal domain has activity in the GAL4 transactivation assay and is localized in the nucleus as a GFP fusion protein, see *Science* Online (www.sciencemag.org/cgi/content/full/1065412/DC1).
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Chromosome Dynamics in the Yeast Interphase Nucleus

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Little is known about the dynamics of chromosomes in interphase nuclei. By tagging four chromosomal regions with a green fluorescent protein fusion to *lac* repressor, we monitored the movement and subnuclear position of specific sites in the yeast genome, sampling at short time intervals. We found that early and late origins of replication are highly mobile in G_1 phase, frequently moving at or faster than 0.5 micrometers/10 seconds, in an energy-dependent fashion. The rapid diffusive movement of chromatin detected in G_1 becomes constrained in S phase through a mechanism dependent on active DNA replication. In contrast, telomeres and centromeres provide replication-independent constraint on chromatin movement in both G_1 and S phases.

Most information available on the nuclear organization of chromosomes is based on the analysis of fluorescent probes in formaldehyde-fixed cells. It is generally assumed that chromosomes, which occupy distinct "territories" in mammalian nuclei (*1*), are relatively static when compared to metaphase chromosomes. This notion of nuclear order is