The ERBB4/HER4 Intracellular Domain 4ICD Is a BH3-Only Protein Promoting Apoptosis of Breast Cancer Cells

Anjali Naresh,[1] Weiwen Long,[2] Gregory A. Vidal,[2] William C. Wimley,[1] Luis Marrero,[3] Carolyn I. Sartor,[4] Sian Tovey,[5] Timothy G. Cooke,[5] John M.S. Bartlett,[5] and Frank E. Jones[1]

Departments of [1]Biochemistry and [2]Structural and Cellular Biology, Tulane University Health Sciences Center, Tulane Cancer Center; [3]Gene Therapy Program, The Morphology and Imaging Core Laboratory, Louisiana State University Health Sciences Center, New Orleans, Louisiana; [4]Department of Radiation Oncology, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, North Carolina; and [5]Endocrine Cancer Group, Section of Surgical and Translational Research, Glasgow University, Glasgow, Scotland, United Kingdom

Abstract

ERBB4/HER4 (referred to here as ERBB4) is a unique member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases. In contrast to the other three members of the EGFR family (i.e., EGFR, ERBB2/HER2/NEU, and ERBB3), which are associated with aggressive forms of human cancers, ERBB4 expression seems to be selectively lost in tumors with aggressive phenotypes. Consistent with this observation, we show that ERBB4 induces apoptosis when reintroduced into breast cancer cell lines or when endogenous ERBB4 is activated by a ligand. We further show that ligand activation and subsequent proteolytic processing of endogenous ERBB4 results in mitochondrial accumulation of the ERBB4 intracellular domain (4ICD) and cytochrome *c* efflux, the essential and committed step of mitochondrial regulated apoptosis. Our results indicate that 4ICD is functionally similar to BH3-only proteins, proapoptotic members of the BCL-2 family required for initiation of mitochondrial dysfunction through activation of the proapoptotic multi-BH domain proteins BAX/BAK. Similar to other BH3-only proteins, 4ICD cell-killing activity requires an intact BH3 domain and 4ICD interaction with the antiapoptotic protein BCL-2, suppressed 4ICD-induced apoptosis. Unique among BH3-only proteins, however, is the essential requirement of BAK but not BAX to transmit the 4ICD apoptotic signal. Clinically, cytosolic but not membrane ERBB4/4ICD expression in primary human breast tumors was associated with tumor apoptosis, providing a mechanistic explanation for the loss of ERBB4 expression during tumor progression. Thus, we propose that ligand-induced mitochondrial accumulation of 4ICD represents a unique mechanism of action for transmembrane receptors, directly coupling a cell surface signal to the tumor cell mitochondrial apoptotic pathway. (Cancer Res 2006; 66(12): 6412-20)

Introduction

Regulation of cell death pathways represents an important mechanism, whereby tumor cells gain a selective growth advantage and evade therapeutic eradication. Death decisions within mammalian cells are primarily regulated by the interplay between proapoptotic and antiapoptotic members of the BCL-2 family (1). In general, malignant cell survival is favored with enhanced protein expression of antiapoptotic and oncogenic BCL-2 family members, including BCL-2, BCL-XL, and MCL-1. These antiapoptotic proteins have in common four conserved BCL-2 homology domains (BH1-BH4). In contrast, the proapoptotic BCL-2 family members belong to two distinct functional classes: the BH3-only and multi-BH domain proteins. The BH3-only proteins, including BID, BIM, BIK, HRK, NOXA, and PUMA, share a single BH3 domain that is essential for cell-killing activity. They are activated in response to diverse developmental and cell stress stimuli and converge their apoptotic signal at the mitochondria or endoplasmic reticulum through activation of the multi-BH domain proteins BAX and BAK. Likewise, BAX and BAK require a BH3-only protein signal to initiate mitochondrial membrane permeabilization and subsequent cytochrome *c* efflux, the essential and committed step of the intrinsic apoptotic pathway. Cytosolic cytochrome *c* activates a cascade of potent cysteine proteases, referred to as caspases, which degrade numerous substrates typical of apoptotic cells. Although the BCL-2 family clearly influences tumorigenesis, the exact molecular mechanisms that underlie the complex interplay between BCL-2 family members and ultimately regulate mitochondrial dysfunction in tumor cells remain elusive.

Three members of the epidermal growth factor receptor (EGFR) family, EGFR, ERBB2/HER-2/*neu* (referred to here as ERBB2), and ERBB3, are associated with mitogenic pathways directly contributing to aggressive tumor phenotypes (2); however, the final member of this family, ERBB4, suppresses tumor cell proliferation (3, 4), possibly through modulation of cell death pathways (4). The influence of the EGFR family on human cancer is most apparent in breast cancer where ERBB2 is a key player during the progression of primary tumors to widespread and often lethal metastatic disease (2). In contrast, the levels of ERBB4 protein expression in breast cancer inversely correlate with tumor grade (5, 6), disease recurrence (7), and overall worsening patient prognosis (8, 9). Paradoxically, ERBB4 expression is essential for normal breast function (10, 11), prompting us to investigate the molecular mechanisms underlying the loss of ERBB4 expression during breast tumor progression. Consistent with clinical observations, initial studies of ligand-activated ERBB4 in breast cancer cell lines identified an antiproliferative function for

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

A. Naresh, W. Long, and G.A. Vidal contributed equally to this work.

This work is dedicated to Dr. Roy S. Weiner for his stalwart commitment to cancer patients and to the physicians and researchers of the Tulane Cancer Center.

Requests for reprints: Frank E. Jones, Department of Biochemistry, SL43, Tulane University Health Sciences Center, Tulane Cancer Center, 1430 Tulane Avenue, New Orleans, LA 70112-2699. Phone: 504-988-6585; Fax: 504-584-2739; E-mail: fjones@tulane.edu or John M.S. Bartlett, E-mail: j.m.bartlett@clinmed.gla.ac.uk.

doi:10.1158/0008-5472.CAN-05-2368

ERBB4 (3), and our most recent findings suggest that ERBB4 induces apoptosis when reintroduced into transformed cells (4).

In addition to its unique ability to suppress malignant cell growth, ERBB4 is also the only EGFR family member to undergo ligand induced proteolytic processing. Ligand activation of ERBB4 results in tumor necrosis factor-α converting enzyme (TACE) cleavage and shedding of the 120-kDa receptor ectodomain, whereas the 80-kDa intracellular domain remains tethered to the cell membrane (12). Subsequent intramembrane cleavage by presenilin-dependent γ-secretase activity results in release of the ERBB4 intracellular domain (4ICD; refs. 4, 13, 14). Interestingly, 4ICD accumulates within several subcellular compartments, including endosomes (15), the nucleus (16), and mitochondria (4). A physiologic role for 4ICD has been identified in the nucleus where 4ICD directly regulates gene expression (4, 16, 17) by functioning as a nuclear chaperone for the transcription factor signal transducers and activators of transcription 5A (STAT5A) followed by 4ICD binding to DNA at STAT5A target promoters (16). Although both ERBB4 transcriptional coregulation and cell-killing activity require proteolytic processing at the cell surface to release 4ICD (4), the molecular mechanisms underlying ERBB4 apoptotic activity remain to be established.

Here, we show that ligand activation and proteolytic processing of ERBB4 in multiple breast cancer cell lines results in mitochondrial accumulation of 4ICD, cytochrome *c* release from permeabilized mitochondria, and apoptotic cell death. We further show that 4ICD harbors a BH3 domain, which is essential for 4ICD proapoptotic activity. Thus, 4ICD represents a novel BH3-only protein member of the BCL-2 family, capable of directly coupling a cell surface signal to mitochondrial dysfunction.

Materials and Methods

Plasmid constructs. Generation of the plasmids pERBB4-EGFP and pERBB4KD-EGFP has been described elsewhere (16). The plasmid p4ICD-EGFP expressing 4ICD (residues 673-1309) fused at the COOH terminus to enhanced green fluorescent protein (EGFP) was created by replacing the 2.4-kb *Sal*I-*Bcl*I fragment from pBlERBB4 (16), with the *Sal*I-*Bcl*I digested PCR product generated with the oligonucleotide primers 5′-CCACGCGTC-GACCCTGACATTTGCTGTT (nucleotides 2038-2052; NM005235) and 5′-GGATGGTTGGGCTCAGAC (nucleotides 2410-2392; pBl4ICD-Flag). The 2.9-kb *Sal*I-*Acl*I fragment of pERBB4-EGFP was replaced with the 0.8-kb *Sal*I-*Acl*I fragment from pBl4ICD-Flag, and the resultant *Sal*I-*Sac*I fragment was replaced with the Kozak translational start sequence. The construct p4ICDmuBH3-EGFP containing base substitutions L986A and D991A was created by inserting the oligonucleotide linker 5′-TCGAGACCCTCAAAGA-TACCTAGTTATTCAGGGTGATGATCGTATGA (nucleotides 2967-3013) into the corresponding *Xho*I-*Hin*dIII sites of p4ICD-EGFP. The construct p4ICDKD-EGFP was created by replacing the 0.6-kb *Kpn*I-*Acl*I fragment of p4ICD-EGFP with the same fragment from pERBB4KD-EGFP. The construct pBl4ICDdelBH3-Flag with an in-frame deletion (residues 740-1020) containing the putative BH3 domain was generated by collapsing the *Kpn*I/*Sca*I sites of pBl4ICDFlag.

Cell lines. The cell line hTERT-HME was purchased from Clontech (Palo Alto, CA), and the human breast cancer cell lines MDA-MB-231, MDA-MB-361, T47D, MCF-7, and SKBr3 were purchased from the American Type Cell Culture (Manassas, VA). All cell lines were maintained according to the manufacturer's recommendations. The MCF-7/BCL-2 cell line stably overexpressing human BCL-2 has been described elsewhere (18). Generation and growth conditions of the mouse embryonic fibroblast (MEF) cell lines with deletions of BAX ($BAX^{-/-}$), BAK ($BAK^{-/-}$), or both ($BAX/BAK^{-/-}$) have been described elsewhere (19).

Apoptosis assays. Apoptosis was determined visually by examining cells at 40 hours after transfection using an inverted Leica DMIRB fluorescent microscope and calculating the percentage of EGFP-positive cells displaying morphologic signs of apoptosis following 4′,6-diamidino-2-phenylindole staining. All samples were prepared in duplicate, and each experiment was repeated at least thrice. Significant differences between data sets was determined using the paired Student's *t* test.

Apoptosis was also determined by Annexin V-APC/7-AAD (BD PharMingen, San Diego, CA) staining of cells cotransfected with pEGFPN3 and pLXSN, pLXSN-ERBB4, or pLXSN-ERBB4KD exactly as described by the manufacturer. The level of apoptosis was determined by flow cytometry analysis of EGFP-positive cells using a Becton Dickinson FACSCalibur. Data was analyzed using CellQuest (Becton Dickinson, Mountain View, CA) software according to the manufacturer's instructions. All samples were prepared in duplicate and each experiment was repeated at least thrice. Significant differences between data sets was determined using the paired Student's *t* test.

Apoptosis induced DNA fragmentation of heregulin β1 (R&D Systems, Minneapolis, MN)–stimulated T47D cells was determined by terminal deoxynucleotidyl transferase–mediated nick-end labeling (TUNEL) assay. In these experiments, T47D cells were stimulated with 50 ng/mL of heregulin β1 for 24 or 48 hours, and DNA fragmentation was detected using ApopTag Fluorescein *In situ* Apoptosis Detection kit (Chemicon) exactly as described by the manufacturer. Where indicated, T47D cells were preincubated for 1 hour before heregulin β1 stimulation with 20 μmol/L of the broad spectrum caspase inhibitor Z-VAD-FMK (zVAD; BD PharMingen).

Suppression of ERBB4 expression. To suppress expression of endogenous ERBB4, T47D cells were transfected with erbB-4/HER-4 siRNA SMARTpool or Nonspecific siRNA Negative Control Pool using siIMPORTER transfection reagent (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer's instructions. Suppression of ERBB4 was confirmed by Western blot analysis of total cell lysates as described previously (20) using antibodies directed against ERBB4 (Santa Cruz Biotechnology, Santa Cruz, CA) and α-tubulin (Upstate Biotechnology) as a loading control.

***In vitro* transcription/translation and pull-down assay.** *In vitro* transcription/translation was done with linearized pBluescript II SK, pBl4ICD-Flag, pBl4ICDdelBH3-Flag, or pcDNA-BCL-2 (generously supplied by Stanley Korsmeyer, Harvard Medical School, Boston, MA) using the TnT Quick Coupled Transcription/Translation System (Promega, Madison, WI) supplemented with 20 μCi of Redivue *L*-[^{35}S]methionine (Amersham, Arlington Heights, IL) exactly as described by the manufacturer. The pull-down assay was done using standard procedures with ANTI-FLAG M2-Agarose (Sigma, St. Louis, MO) as the affinity reagent.

Mitochondrial staining *in situ*. Mitochondrial localization of ERBB4-EGFP in transfected SKBr3 cells was visualized at 24 hours after transfection by incubating transfected cells in growth media containing 250 nmol/L Mito Tracker Red (Molecular Probes, Eugene, OR) for 30 minutes at 37°C. Cells were fixed in 4% paraformaldehyde, counterstained with Hoechst, and coverslipped with Prolong Antifade Media (Molecular Probes). The slides were analyzed by deconvolution microscopy on a Leica DMRXA automated upright epifluorescent microscope (Leica Microsystems, Bannockburn, IL).

Subcellular fractionation. Subcellular fractions were prepared from ca. 3×10^7 cells using modifications of procedures described elsewhere (21, 22). Cells were harvested by scraping and resuspended into 600 μL of hypotonic RSB buffer [10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, with Complete Protease Inhibitors (Roche Diagnostics, Indianapolis, IN)] and incubated on ice for 20 minutes. Cells were disrupted with 25 strokes of a Dounce homogenizer fitted with a B pestle followed by 20 passages through a 27-gauge needle. A 2.5× MS buffer [12.5 mmol/L Tris-HCl (pH 7.5), 525 mmol/L mannitol, 175 mmol/L sucrose, 2.5 mmol/L EDTA (pH 7.5)] was added to the iso-osmotic concentration of 1× MS (400 μL). Cell lysates were centrifuged at 1,300 × *g* for 6 minutes at 4°C. In some experiments, this cleared lysate was processed, as described below, to assay for BAK oligomerization. A crude mitochondria pellet was obtained by centrifugation of the cleared lysate at 16,100 × *g* for 20 minutes at 4°C. The supernatant was centrifuged at 100,000 × *g* for 1 hour at 4°C. The supernatant from this spin was saved as the cytosolic fraction (Cyto), and the pellet was dissolved in 200 μL of radioimmunoprecipitation assay buffer

[10 mmol/L Tris (pH 8), 140 mmol/L NaCl, 1% sodium deoxycholate, 0.1% SDS, 1% NP40] and saved as the endoplasmic reticulum/microsomal fraction. The crude mitochondria pellet from above was resuspended in 1 mL of ice-cold 1× MS buffer, and mitochondria were purified at the interface of a 1.0:1.5 mol/L sucrose step gradient by centrifugation in a swinging bucket rotor at 60,000 × *g* for 1 hour at 4°C. The isolated mitochondria were diluted to 0.25 mol/L sucrose by adding 4 volumes of dilution buffer [5 mmol/L Tris-HCl (pH 7.5), 1 mmol/L EDTA (pH 7.5)] and then pelleted by centrifugation at 16,100 × *g* for 20 minutes at 4°C. The mitochondria pellet (Mito) was dissolved in 200 μL of RIPA buffer. Fifty micrograms of total protein were separated by PAGE and analyzed by Western blot using antibodies directed against ERBB4 (Santa Cruz Biotechnology), BAX (Upstate Biotechnology), BAK (Upstate Biotechnology), calnexin (Stressgen Bioreagents), cytochrome *c* (BD Biosciences, San Jose, CA), and TOM40 (Santa Cruz Biotechnology).

Assay for BAK oligomerization. The cleared cell lysate from above was cross-linked with 1 mmol/L bismaleimidohexane (Pierce Chemical Co., Rockford, IL) for 30 minutes. The reaction was quenched by incubating with 1 mmol/L β-mercaptoethanol for 15 minutes, and cross-linked mitochondria were pelleted by centrifugation at 16,100 × *g* for 20 minutes. BAK oligomerization was analyzed by Western blot.

Western blot analysis of poly(ADP-ribose) polymerase cleavage. Cleavage of the caspase-3 substrate poly(ADP-ribose) polymerase (PARP) was examined in heregulin β1–stimulated T47D cells by Western blot analysis. Briefly, T47D cells were mock stimulated or stimulated with 50 ng/mL heregulin β1 for 12, 24, or 48 hours, and cell lysates were prepared in a high salt extraction buffer [20 mmol/L HEPES (pH 7.9), 350 mmol/L NaCl, 1 mmol/L MgCl2, 0.1 mmol/L EDTA, 0.5 mmol/L DTT, 1% NP40, 20% glycerol, and supplemented with 1 mmol/L phenylmethylsulfonyl fluoride, and Complete Protease Inhibitor (Roche Diagnostics)]. Fifty micrograms of each sample were separated by PAGE and analyzed by Western blot as described elsewhere (20) using rabbit anti-PARP at 1:1,000 (Roche Diagnostics).

Tumor microarray construction. The tissue microarrays were generated from retrospectively identified, archival formalin-fixed, paraffin-embedded primary breast cancer cases diagnosed at the Glasgow Royal Infirmary between 1984 and 1993. None of the patients in this sample set received neoadjuvant therapy. Three 0.6-mm^2 cores of primary human breast cancer tissue were removed from representative tumor areas on each paraffin block identified by a pathologist. These cores were used to construct tissue microarray blocks in triplicate (80-120 cores per block). Cores of normal skin, smooth muscle, testes, lymph node, placenta, and tonsil were also included in the tissue microarray as controls.

Immunohistologic and statistical analysis of primary human breast tumors. Immunohistochemical staining for ERBB4/HER4 was done on tissue microarray samples using an antibody directed against the ERBB4 COOH terminus (Neomarkers, Fremont, CA) exactly as described elsewhere (9). The *in situ* TUNEL assay was done using the ApopTag Plus Peroxidase *In situ* Apoptosis Detection kit (Chemicon International, Temecula, CA) exactly as described by the manufacturer. Membrane or cytosolic ERBB4 staining and TUNEL staining was scored by first determining the intensity of tumor cell staining on a scale of 1 to 4, with 4 being the most intense staining. This number was multiplied by the percentage of tumor cells in each tissue microarray exhibiting positive staining and then divided by 100. Using this criteria, staining was recorded on a scale of 1 to 4 with 4 being the most intense widespread staining (examples of TUNEL staining are presented in Supplementary Fig. S1). Samples with staining of 3 to 4 were considered to be unequivocal positives and used for statistical analysis. In addition, all samples were examined by a total of four observers with a consensus obtained on all staining of 3 to 4. Examples of apoptosis staining from 1 to 4 are included as Supplementary Data.

Results

ERBB4 induces apoptosis of breast cancer cell lines. ERBB4 expression seems to be selectively lost as primary breast tumors progress to a more aggressive phenotype (5, 6). In addition, established human breast cancer cell lines either express low levels of ERBB4 protein or fail to express ERBB4 altogether (23). We have previously shown that reintroduction of ERBB4 into a breast cancer cell line resulted in an antiproliferative response (3). To determine the molecular mechanism underlying this apparent ERBB4-mediated growth suppression, we determined the effect of ectopic ERBB4 expression on the viability of multiple breast cancer cell lines, each differing in their expression of the estrogen receptor, the ERBB2 breast oncogene, or endogenous ERBB4. Reintroduction of ERBB4 into each breast tumor cell line tested resulted in apoptotic cell death of >60% of ERBB4-EGFP expressing cells (Fig. 1*A*). Estrogen receptor, ERBB2, or endogenous ERBB4 expression failed

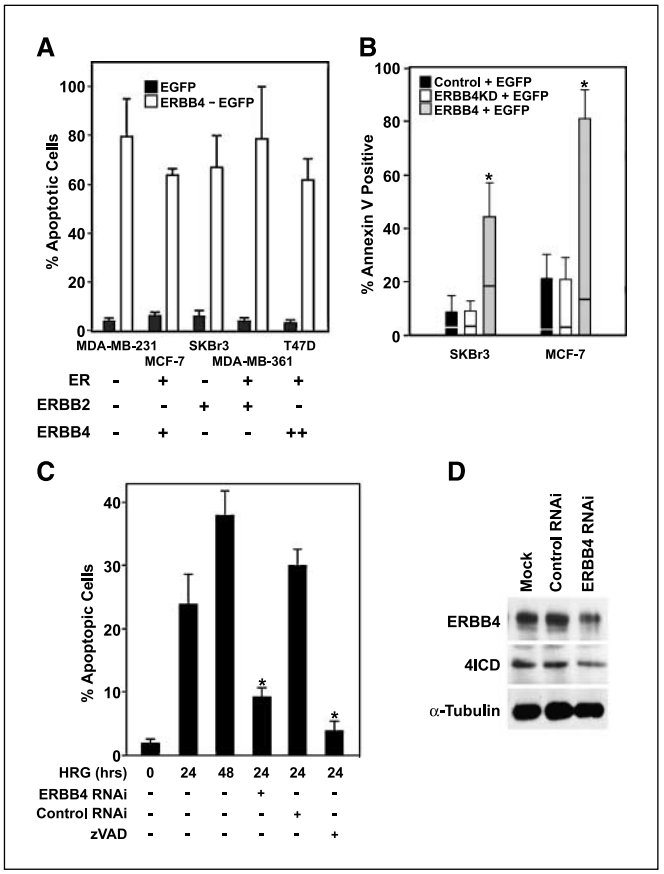

Figure 1. ERBB4 induces apoptosis of breast cancer cell lines. *A,* the indicated cell lines were transfected with ERBB4-EGFP or pEGFPN3 vector control. The expression of estrogen receptor (*ER*), ERBB2, and ERBB4 in each cell line is indicated. The percentage of apoptotic EGFP positive cells was determined by morphology and chromosomal condensation following 4′,6-diamidino-2-phenylindole staining at 40 hours after transfection. *Columns,* mean of three experiments; *bars,* SE. *B,* Annexin V assay for apoptosis. The indicated breast cancer cell lines were cotransfected with pEGFPN3 and control, ERBB4, or ERBB4KD expression vectors. The percentage of apoptotic EGFP-positive cells was determined by flow cytometry analysis following Annexin V/7-AAD staining at 40 hours after transfection. *Columns,* mean of three experiments; *bars,* SE. The lower region of each column indicates percentage of 7-AAD-positive cells. *, significant differences in each data set as determined by Student's *t* test. *C,* ligand activation of endogenous ERBB4 results in apoptosis. T47D breast cancer cells were stimulated with 50 ng/mL heregulin β1 for 24 or 48 hours, and apoptosis was assayed by TUNEL. In some experiments, cells were pretreated with zVAD at 20 μmol/L for 1 hour before addition of heregulin β1. To suppress ERBB4 expression, cells were pretreated for 72 hours with erbB-4/HER-4 siRNA SMARTpool (ERBB4 RNAi) or Nonspecific siRNA Negative Control Pool (Control RNAi), and TUNEL assay was done after a 24-hour heregulin β1 stimulation. *D,* Western blot analysis showing RNAi-mediated down-regulation of ERBB4. T47D cells were mock treated or treated with control or ERBB4 RNAi for 80 hours, and 50 μg of total cell lysate was analyzed by ERBB4 or α-tubulin Western blot.

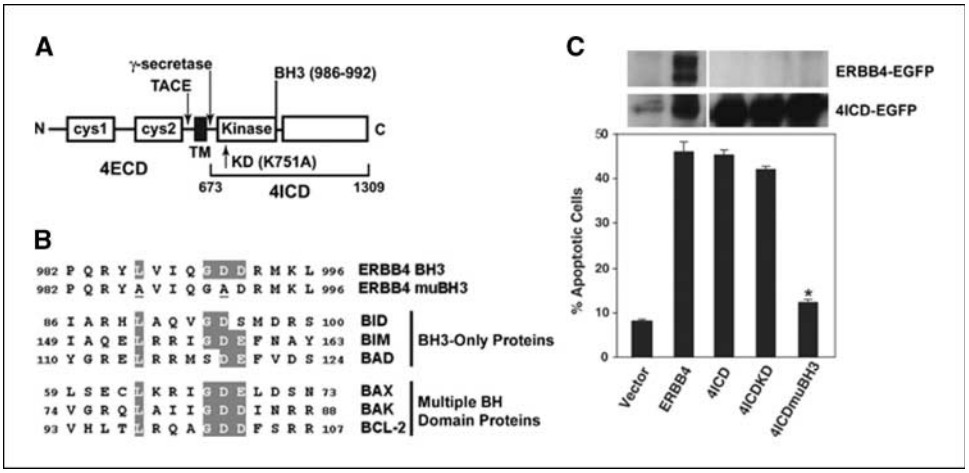

Figure 2. An intact BH3-domain is required for ERBB4 cell-killing activity. *A,* schematic of ERBB4 functional domains. The ERBB4 extracellular domain (4ECD) consists of an NH_2-terminal ligand-binding region composed of two cysteine-rich regions (cys1 and cys2). Proteolytic processing by tumor necrosis factor-α converting enzyme (*TACE*) and γ-secretase results in membrane release of the ERBB4 intracellular domain (4ICD; residues 673-1309), which harbors a potential BH3 domain (residues 986-992). *B,* alignment of the BH3 domains between ERBB4 and regulatory BCL-2 family members. BH3 domain consensus residues are stippled, and residues altered in 4ICDmuBH3 are underlined. *C,* cell-killing activity of 4ICD requires an intact BH3 domain. SKBr3 cells were transfected with pEGFPN3 vector control, ERBB4-EGFP, 4ICD (residues 673-1309) fused to EGFP (4ICD-EGFP), the same construct harboring mutations within the kinase domain (4ICDKD-EGFP) or the BH3 domain (4ICDmuBH3-EGFP), and the percentage of apoptotic EGFP positive cells was determined at 40 hours after transfection by morphology and chromosomal condensation following 4′,6-diamidino-2-phenylindole staining. *Columns,* mean of three experiments; *bars,* SE. *, sample significantly different from 4ICD and 4ICDKD but not EGFP control as determined by paired Student's *t* test. Equivalent levels of ectopic ERBB4 and 4ICD expression was confirmed by Western blot analysis (*top*) of 50 μg of total cell lysate.

to affect ERBB4 cell-killing activity. ERBB4 apoptotic activity was substantiated by examining Annexin V binding to ERBB4-transfected MCF-7 and SKBr3 breast cancer cell lines. Annexin V binds to cell surface phosphatidyl serine, a specific marker for early apoptosis. Ectopic expression of ERBB4, but not the ERBB4 kinase mutant ERBB4KD, induced Annexin V binding to ~40% and 80% of transfected SKBr3 and MCF-7 cells, respectively (Fig. 1*B*).

We next determined if ligand activation of endogenous ERBB4 induced breast tumor cell killing. Stimulation of the ERBB4 expressing T47D breast cancer cell line, a rare cell line that overexpresses ERBB4, with the ERBB4 ligand heregulin β1 resulted in >20% of stimulated cells undergoing apoptosis after 24 hours with nearly 40% of the cells apoptotic following a 48-hour heregulin β1 stimulation (Fig. 1*C*). Heregulin β1 also induced apoptosis of the MCF-7 breast cancer cell line (Fig. 3C), another cell line that expresses ERBB4. Suppression of ERBB4 expression in the T47D cell line by RNA interference (RNAi; Fig. 1*D*) inhibited heregulin β1–induced apoptosis confirming that ERBB4 mediates the apoptotic response of T47D cells to heregulin β1 (Fig. 1*C*). Control RNAi did not affect apoptosis induced by heregulin β1 (Fig. 1*C*). Heregulin β1–induced apoptosis was dramatically suppressed by preincubating T47D cells with the broad spectrum caspase inhibitor zVAD (Fig. 1*C*). These latter results imply that heregulin β1–induced apoptosis involves activation of cellular caspases.

Cell-killing activity of the ERBB4 intracellular domain (4ICD) requires an intact BH3 domain; however, intrinsic kinase activity is dispensable. Having identified a dominant apoptotic function for ERBB4, we next designed experiments to identify the cellular apoptotic pathway recruited by ERBB4. We identified a potential BH3 domain within 4ICD (Fig. 2*A* and *B*), raising the possibility that 4ICD functions as a proapoptotic BH3-only protein member of the BCL-2 family. Significantly, ectopic expression of 4ICD in SKBr3 cells exhibited apoptotic activity at levels equivalent to the ERBB4 holoreceptor (Fig. 2*C*). In contrast to the ERBB4 holoreceptor, which required an intact kinase domain for cell-killing activity, 4ICD induced significant levels of apoptosis in the absence of kinase activity (Fig. 2*C*). Mutations of 4ICD predicted to inactivate the BH3 domain (Fig. 2*B*), however, abolished 4ICD-mediated apoptosis (Fig. 2*C*). These results provide evidence that 4ICD has independent apoptotic activity and raises the possibility that 4ICD functions as a proapoptotic BH3-only protein member of the BCL-2 family. Similar BH3 domain base substitutions in full-length ERBB4 inactivated the intrinsic kinase essential for ERBB4 cell-killing activity; thus, the contribution of the BH3 domain to apoptosis induced by the ERBB4 holoreceptor was uninterruptible (data not shown).

4ICD forms a functional interaction with BCL-2. Similar to other BH3-only proteins (24, 25), the 4ICD BH3 domain sequence (residues 975-1002) can be modeled to form an amphipathic α-helix, which threads into the hydrophobic binding pocket of BCL-XL (Fig. 3*A*). This interaction between BH3-only proteins and antiapoptotic BCL-2 family members suppresses BH3-only protein cell-killing activity (1). We, therefore, determined if the 4ICD BH3 domain mediated a functional interaction with BCL-2. Consistent with the idea that the 4ICD BH3 domain is a protein interaction motif, we coimmunoprecipitated *in vitro* translated BCL-2 with 4ICD but not with a 4ICD deletion mutant lacking the BH3 domain (4ICDdelBH3; Fig. 3*B*). Overexpression of the antiapoptotic BCL-2 oncogene disrupts apoptosis, in part, by binding to and sequestering BH3-only proteins (26). Likewise, heregulin β1–induced apoptosis was suppressed by stable overexpression of BCL-2 in the MCF-7 cell line (Fig. 3*C*). Furthermore, BCL-2 overexpression also repressed apoptosis induced by ectopic expression of ERBB4 (Fig. 3*D*). ERBB4-induced cell killing was, however, restored in the presence of BCL-2 by coexpression with BAD (Fig. 3*D*). BAD also harbors a BH3 domain and regulates apoptosis by binding to BCL-2, thereby releasing BH3-only proteins with intrinsic cell-killing activity (27). Taken together, these results strongly implicate 4ICD as a proapoptotic BH3-only protein and

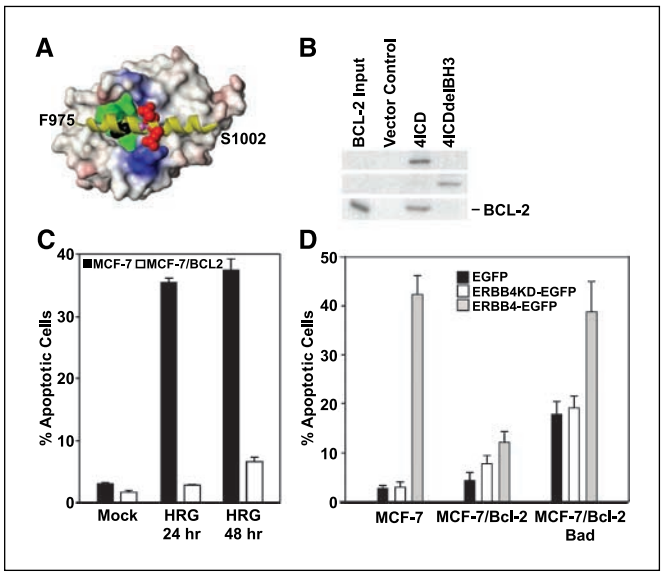

Figure 3. 4ICD forms a functional interaction with BCL-2. *A,* molecular model of the potential BH3 domain of ERBB4 interacting with BCL-XL (25). The helical backbone of the ERBB4 BH3 motif (residues 975-1002) is shown in yellow ribbon, and the conserved residues of the motif are in CPK (spacefilling) mode. Leucine (L986) is black; glycine (G990) is magenta; and the essential aspartic acid residues (D991 and D992) are red. The hydrophobic pocket of the binding site is green. The remainder of the surface of BCL-XL is colored by electrostatic potential showing the basic residues (*blue*) of BCL-XL that interact with the conserved acidic residues of the BH3 motif. *B,* 4ICD interacts with BCL-2. Equivalent amounts of *in vitro* translated BCL-2 were incubated with *in vitro* translated 4ICD-Flag, 4ICDdelBH3-Flag, which lacks the BH3 domain, or the empty vector control. The [^{35}S]methionine-labeled translation products were coimmunoprecipitated with a Flag-specific antibody coupled to agarose. The BCL-2 input represents one tenth of the amount added to each coimmunoprecipitate. *C,* BCL2 suppresses heregulin β1 (*HRG*)–induced apoptosis of MCF-7 cells. MCF-7 and MCF-7 cells stably overexpressing BCL-2 (MCF-7/BCL-2) were treated with 50 ng/mL heregulin β1 for 24 or 48 hours, and the percentage of morphologically apoptotic cells was determined by 4′,6-diamidino-2-phenylindole staining. *Columns,* mean of three experiments; *bars,* SE. *D,* BCL-2 suppresses ERBB4-induced apoptosis. MCF-7 and MCF-7/BCL-2 cells were transfected with ERBB4-EGFP, ERBB4KD-EGFP, or pEGFPN3, alone or in combination with a vector expressing BAD. The percentage of morphologically apoptotic EGFP-positive cells was determined by 4′,6-diamidino-2-phenylindole staining at 40 hours after transfection. *Columns,* mean of three experiments; *bars,* SE.

show that 4ICD cell-killing activity can be regulated by members of the BCL-2 family.

Heregulin β1–induced mitochondrial accumulation of 4ICD results in mitochondrial permeabilization. We next determined the molecular mechanism(s) underlying ERBB4 cell-killing activity. An important functional characteristic of BH3-only proteins is their ability to regulate apoptosis by localizing to and integrating signals at the mitochondria (1). We, therefore, determined if ectopically expressed ERBB4 localized to the mitochondria of transfected SKBr3 cells. Mitochondria were stained with MitoTracker Red (CMXRos), and CMXRos colocalization with ectopic ERBB4-EGFP was determined by deconvolution microscopy. The results indicate that a substantial perinuclear population of ERBB4 localizes to the mitochondria of SKBr3 cells undergoing apoptosis (Fig. 4*A*). We have previously shown that a point mutation introduced into the juxtamembrane region of ERBB4 (V673I) abolished γ-secretase processing of ERBB4 and subsequent membrane release of 4ICD. Significantly, and in contrast to wild-type ERBB4, this ERBB4 processing mutant (referred to as ERBB4V673I) was not detected in isolated mitochondria and lacks cell-killing activity (4). ERBB4V673I was, therefore, used as a negative control in our mitochondrial localization experiments. Similar to ERBB4, ERBB4V673I also accumulates in the perinuclear region of transfected SKBr3 cells, but ERBB4V673I fails to colocalize with CMXRos (Fig. 4*B*), providing additional evidence that ERBB4V673I is excluded from mitochondria. We confirmed these observations by Western blot analysis of mitochondria isolated from SKBr3 cells ectopically expressing ERBB4 or ERBB4V673I each lacking an EGFP fusion. Consistent with our previous results (4), 4ICD was the predominant form of ERBB4 localizing to mitochondria of ERBB4-transfected cells, whereas both the ERBB4 holoreceptor and 4ICD were excluded from the mitochondrial fraction of ERBB4V673I-transfected cells (Fig. 4*C*). We next determined if heregulin β1 stimulation of endogenous ERBB4 promoted mitochondrial accumulation of 4ICD. Mitochondria were isolated from T47D cells following a 1-hour heregulin β1 stimulation, and ERBB4 was detected by Western blot analysis. Consistent with ectopic expression of ERBB4 in SKBr3 cells, heregulin β1 stimulation of T47D breast cancer cells resulted in ERBB4 proteolytic processing, 4ICD membrane release, and mitochondrial accumulation of endogenous 4ICD (Fig. 4*D*). Low but detectable levels of mitochondrial ERBB4 holoreceptor was also observed (Fig. 4*D*). These results show that the 4ICD BH3-only protein is the predominant form of ERBB4 localizing to mitochondria in response to heregulin β1.

We next determined the effect of heregulin β1 stimulation and mitochondrial accumulation of 4ICD on mitochondrial membrane integrity. During activation of the intrinsic apoptotic pathway, the outer mitochondrial membrane is permeabilized, resulting in the efflux of cytochrome *c* from the intermembrane space. Subsequent cytosolic accumulation of cytochrome *c* is a hallmark of mitochondrial dysfunction essential for activation of cellular caspases and the intrinsic cell death cascade (1). Significantly, heregulin β1 stimulation of T47D cells resulted in a dramatic increase in cytosolic cytochrome *c* (Fig. 4*D*) and cleavage of the caspase-3 substrate PARP (Fig. 4*E*). These results indicate that heregulin β1–induced mitochondrial accumulation of 4ICD promotes mitochondrial permeabilization, cytochrome *c* efflux, and caspase activation, culminating in cell death. Detection of the integral mitochondrial membrane protein TOM40 was used to confirm purity of subcellular fractions (Fig. 4*C* and *D*).

BAK is the essential mediator of 4ICD-induced apoptosis. Cell death signals that activate the intrinsic apoptotic pathway must converge at the mitochondria through the multiple BH domain proteins BAX and BAK (28). Activation and subsequent oligomerization of mitochondrial BAX or BAK results in mitochondrial permeabilization and is the essential and committed step of the intrinsic apoptotic pathway. We, therefore, determined the effect of heregulin β1 stimulation of ERBB4 in T47D cells on endogenous BAX and BAK activation. When activated, BAX monomers translocate from the cytosol and oligomerize within the endoplasmic reticulum (22) and mitochondrial membranes (29) to initiate mitochondrial permeabilization, whereas activated BAK monomers residing within the mitochondrial membrane oligomerize to induce mitochondrial dysfunction (30). Heregulin β1 stimulation of T47D cells failed to stimulate endoplasmic reticulum or mitochondrial accumulation of BAX (Fig. 5*A*), suggesting that BAX is not involved in the heregulin β1/4ICD apoptotic pathway. Calnexin and TOM40 were included as controls for endoplasmic reticulum and mitochondrial fractions, respectively. In contrast, heregulin β1 stimulation of T47D cells promoted a depletion of BAK monomers with a corresponding increase in BAK dimer formation (Fig. 5*B*, compare *lanes 2* and *4*).

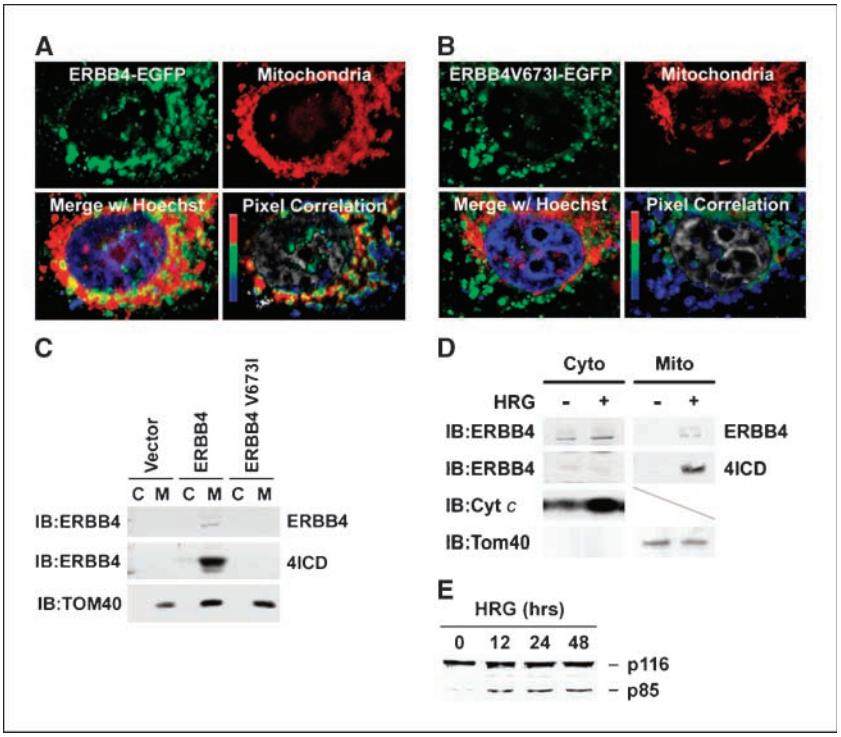

Figure 4. Heregulin β1 (*HRG*) induces mitochondrial accumulation of 4ICD and subsequent permeabilization of mitochondria. *A* and *B,* mitochondrial localization of ERBB4. SKBr3 cells were transfected with (*A*) ERBB4-EGFP or (*B*) the γ-secretase processing mutant, ERBB4V673I-EGFP (4); stained with MitoTracker Red (CMXRos) at 24 hours after transfection; and observed by deconvolution microscopy. Colocalization was measured using a pixel correlation algorithm with red, indicating highly significant levels of colocalization. *C,* accumulation of 4ICD in purified mitochondria. SKBr3 cells were transfected with vector control, ERBB4, or ERBB4V673I. Cells were harvested at 40 hours after transfection, and cell lysates were fractionated into cytosol-rich S100 (*C*) and mitochondria (*M*). Forty micrograms of each fraction were analyzed by Western blot using antibodies directed against ERBB4 and the mitochondrial membrane protein TOM40. *D,* ligand stimulation of endogenous ERBB4 results in mitochondrial accumulation of 4ICD and cytochrome *c* release. T47D breast cancer cells were mock stimulated or stimulated with 50 ng/mL heregulin β1 for 60 minutes at room temperature. Cell lysates were prepared and fractionated into cytosol-rich S100 (*Cyto*) and mitochondria (*Mito*). Forty micrograms of each fraction were analyzed by Western blot using antibodies directed against ERBB4, cytochrome *c*, and the mitochondrial membrane protein TOM40. *E,* heregulin β1 stimulation of T47D cells results in PARP cleavage. T47D cells were mock stimulated (0 time point) or stimulated with 50 ng/mL heregulin β1 for 12, 24, or 48 hours, and cell lysates were analyzed by Western blot for p116 PARP and the caspase-3 cleavage product p85 PARP.

ERBB4-specific RNAi, but not the nonspecific control RNAi pool, abolished heregulin β1–induced loss of BAK monomers and accumulation of BAK dimers (Fig. 5*B*, compare *lanes 6* and *8*). Taken together, these results strongly suggest that heregulin β1 activation of ERBB4 results in activation of the BAK pore-inducing complex, leading to mitochondrial permeabilization and cytochrome *c* release. To further substantiate a role for BAK as the exclusive mediator of ERBB4 cell killing, we transfected MEF cells or MEF cells lacking BAX, or BAK, or both with ERBB4-EGFP and determined the levels of apoptosis in each cell line. In direct concordance with our observations of heregulin β1–stimulated T47D cells, ERBB4 induced apoptosis in >90% of transfected MEF and BAX-null MEF cells but only 10% of transfected BAK-null or BAX/BAK-null MEF cells (Fig. 5*C*). Collectively, our results indicate that heregulin β1 activation of ERBB4 and subsequent mitochondrial accumulation of 4ICD promote mitochondrial

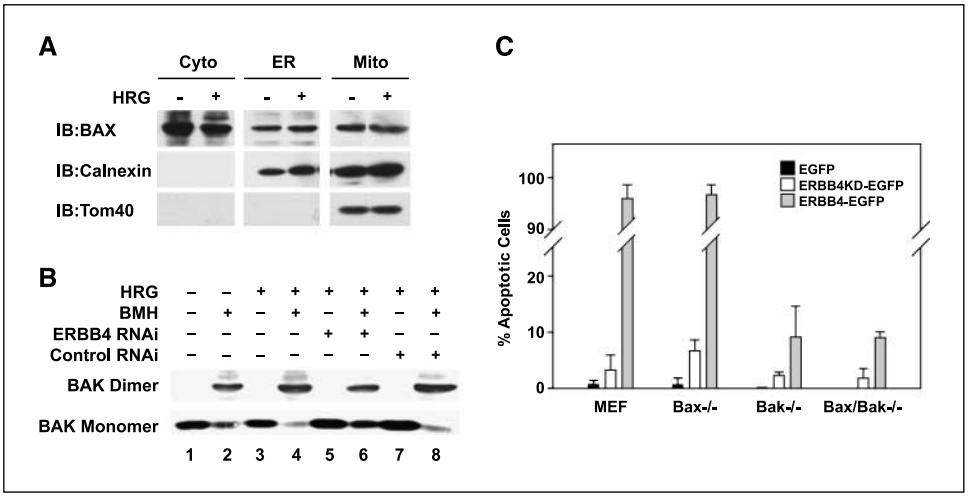

Figure 5. BAK is an essential mediator of 4ICD-induced apoptosis. *A,* ligand stimulation of ERBB4 fails to mobilize BAX. T47D breast cancer cells were stimulated with 50 ng/mL heregulin β1 (*HRG*) for 1 hour. Cell lysates were fractionated into a cytosol-rich S100 (*Cyto*), a light microsomal fraction composed of endoplasmic reticulum and endosomes (*ER*), and mitochondria (*Mito*). Forty micrograms of each fraction were analyzed by Western blot using antibodies directed against BAX, the endoplasmic reticulum marker calnexin, and the mitochondrial membrane protein TOM40. *B,* ligand stimulation of ERBB4 results in BAK oligomerization. Crude mitochondrial extracts were prepared from mock-stimulated or heregulin β1–stimulated T47D breast cancer cells and treated with 1 mmol/L bismaleimidohexane cross-linker for 30 minutes at room temperature. ERBB4 expression was suppressed by pretreating cells with ERBB4 RNAi or Control RNAi. BAK monomers and cross-linked BAK dimers were detected by Western blot analysis. *C,* ERBB4 cell killing of MEF cells requires BAK. MEF, $BAX^{-/-}$, $BAK^{-/-}$, or $BAX/BAK^{-/-}$ MEF cells were transfected with ERBB4-EGFP, ERBB4KD-EGFP, or pEGFPN3, and the percentage of morphologically apoptotic EGFP-positive cells was determined by 4′,6-diamidino-2-phenylindole staining at 40 hours after transfection. *Columns,* mean of three experiments; bars, SE.

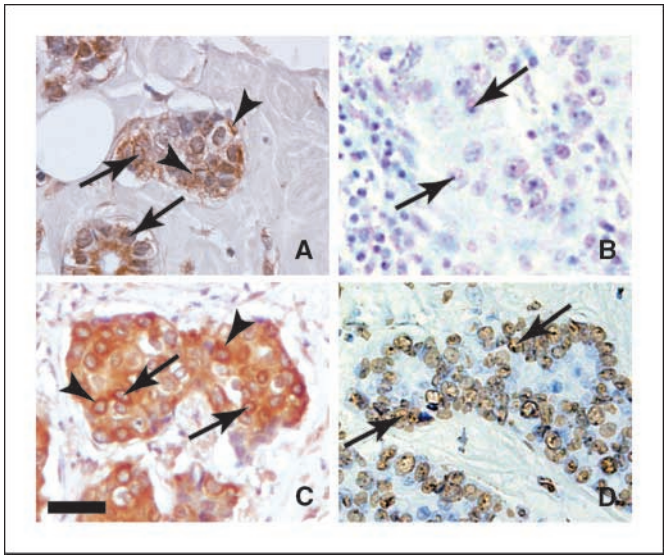

Figure 6. Cytosolic accumulation of 4ICD is associated with increased apoptosis in primary human breast tumors. A cohort of 136 therapy naive archived primary human breast tumors prepared as tissue microarrays were stained by immunohistochemistry for (*A* and *C*) ERBB4 using an antibody directed against the ERBB4 COOH terminus or (*B* and *D*) apoptosis indicated by DNA fragmentation (TUNEL assay) using an ApopTag *In situ* Apoptosis Detection kit. *A* and *B,* the majority of tumors with membrane associated ERBB4 (*A, arrowheads*) were TUNEL negative (*B*, nuclei indicated by *arrows*). *C* and *D,* cytosolic 4ICD expression (*C, arrowheads*) was significantly associated with increased tumor cell apoptosis (χ^2 analysis, $P < 0.0001$). *D,* TUNEL-positive nuclei (*arrows*). Arrowheads in (*A*) indicate membrane staining of ERBB4. Arrowheads in (*C*) indicate perinuclear accumulation of 4ICD. Arrows in all panels indicate nuclei. Bar, 50 μm (*C*).

dysfunction and cell killing through activation of the apoptosis "gateway" protein BAK.

Cytosolic 4ICD is associated with breast tumor apoptosis. Our current results imply that cytosolic accumulation of 4ICD may suppress tumor cell proliferation by activating cellular apoptotic pathways; however, can these findings be translated to a clinically relevant setting? To address this question, we analyzed a cohort of 136 therapy naive archived primary breast tumors for ERBB4 expression by immunohistochemistry and apoptosis using an *in situ* DNA fragmentation assay (Supplementary Fig. S1). Of the primary tumors analyzed, 17 of 136 (12.5%) displayed strong staining (3+) for membrane ERBB4; 15 of 136 (11%) exhibited strong cytoplasmic staining of 4ICD; and 15 of 136 (11%) of the primary tumors exhibited extensive (3+) apoptosis. Eight tumor samples were positive for cytosolic 4ICD expression and TUNEL (Fig. 6*C* and *D*), indicating a highly significant association (χ^2 analysis, $P < 0.0001$). Cytosolic 4ICD staining was most prominent in perinuclear regions (Fig. 6*C, arrowheads*), where the majority of mitochondria reside. An additional tumor sample positive for cytosolic 4ICD and TUNEL is illustrated in Supplementary Fig. S1. Interestingly, nearly all tumors with ERBB4 localized to cell membranes were also TUNEL negative (Fig. 6*A* and *B*). Indeed, only 1 of the 17 tumors with membrane ERBB4 was TUNEL positive (χ^2 analysis, $P > 0.6$).

Discussion

The aberrant genetic milieu that contributes to tumor development and progression is highly variable and complex. One generalization that has gained recent experimental and clinical support, however, is that successful tumor development requires evasion of cellular apoptotic pathways. In breast cancer, several mechanisms may contribute to apoptotic resistance, including overexpression of the antiapoptotic proteins BCL-2 and BCL-XL (31, 32). Here, we present evidence that the EGFR family member ERBB4 functions as a unique proapoptotic protein. We propose that loss of ERBB4 expression during breast carcinogenesis disrupts an important apoptotic pathway and contributes to the ability of breast tumors to avoid apoptotic cell death.

Our findings provide evidence that ERBB4 suppresses breast cancer cell growth through activation of the intrinsic apoptotic pathway, by functioning as a proapoptotic BH3-only protein. Although members of the BH3-only protein class of the BCL-2 family are functionally diverse, these proteins share several critical mechanistic properties. For example, an intact BH3 domain is required to initiate apoptotic signals at the mitochondrial membrane through activation of BAX or BAK. The BH3-only protein apoptotic signal may however be disengaged at the mitochondria through interaction with antiapoptotic proteins including BCL-2, BCL-XL, or MCL-1. Likewise, ERBB4 cell-killing activity requires an intact BH3 domain and is inhibited by an interaction with BCL-2. Furthermore, ligand stimulation of endogenous ERBB4 results in mitochondrial accumulation of the BH3 domain–bearing 4ICD, oligomerization of BAK, and subsequent cytochrome *c* efflux from permeabilized mitochondria (Fig. 7).

As a transmembrane receptor, however, ERBB4 would represent a unique member of the BH3-only protein class. Indeed, our results describe a novel mechanism of action for a transmembrane receptor, with ERBB4 directly coupling an external stimulus to mitochondrial dysfunction and cell death through membrane release of 4ICD. Another mechanistic difference between ERBB4 and other BH3-only proteins is the essential contribution of BAK to ERBB4 cell killing. Other BH3-only proteins examined to date efficiently transmit their apoptotic signals through either BAX or BAK (19, 26, 33). Although the functional significance of a coupled ERBB4/BAK apoptotic pathway remains to be determined, clinical studies imply that the two proteins are coregulated in breast

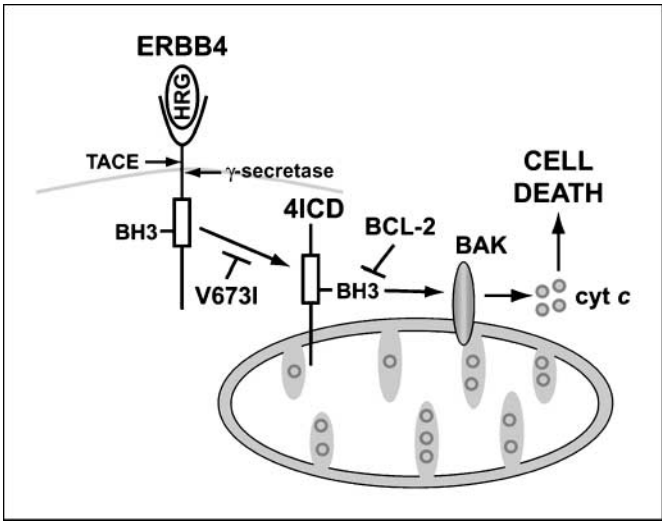

Figure 7. Model of ERBB4 cell-killing activity. Ligand-activated ERBB4 is proteolytically processed at the cell surface to release the 4ICD BH3-only protein. We propose that mitochondrial accumulation of 4ICD results in BAK activation, cytochrome *c* release from permeabilized mitochondria, and cell death. See text for additional details.

cancer. Indeed, both ERBB4 and BAK are associated with estrogen receptor–positive (7, 31, 34) and low-grade breast tumors (6, 32). In contrast, BAX expression is associated with estrogen receptor–negative (31) and high-grade tumors (32). Another clinical study suggests that BAK is the multi-BH domain protein critical for regulating apoptosis in breast cancer (35). Thus, both experimental and clinical evidence support the notion that suppression of early-stage breast carcinogenesis is influenced by a 4ICD/BAK-coupled apoptotic program.

Critical for the appropriate function of BH3-only proteins is a mechanism to regulate apoptotic activity, thereby preventing deleterious apoptosis. The activities of several BH3-only proteins, including BIM, HRK, BBC3, NOXA, and PUMA, are transcriptionally regulated, whereas the apoptotic activities of BID, BAD, and BIK are regulated by different posttranslational mechanisms (36, 37). Posttranslational regulation of ERBB4 apoptotic activity seems to involve both activation of its intrinsic tyrosine kinase and subsequent proteolytic processing at the cell surface to release 4ICD. Interestingly, an intact kinase domain, essential for apoptosis induced by the ERBB4 holoreceptor, was dispensable for 4ICD-mediated cell killing, suggesting that signaling pathways coupled to the ERBB4 holoreceptor are not required for cell killing. In support of this contention, we have previously shown that an ERBB4 mutant that lacks γ-secretase processing, therefore failing to release 4ICD while retaining canonical signal transduction pathways, lacked cell-killing activity (4). Furthermore, here, we show that cytosolic 4ICD but not membrane-tethered ERBB4 was associated with tumor apoptosis, providing additional evidence that ERBB4 signaling from the cell surface has little, if any, effect on tumor apoptosis. Thus, we propose that the ERBB4 tyrosine kinase provides an essential contribution to apoptosis by supplying a mechanism for ERBB4 proteolytic processing, thereby releasing the apoptotic activity of 4ICD.

Although we show a significant association between cytosolic 4ICD and tumor apoptosis, the lack of apoptosis in some primary breast tumors expressing cytosolic 4ICD indicates that cytosolic accumulation of 4ICD is insufficient for tumor cell apoptosis. There exist several possible explanations for the lack of apoptosis in tumors with cytosolic 4ICD. Cytosolic staining of 4ICD indicates successful cleavage of ERBB4 by TACE; however, γ-secretase-mediated cleavage is required to release 4ICD from cellular membranes. Thus, cytosolic staining of 4ICD may represent endosome-tethered 4ICD in tumors lacking γ-secretase activity. Alternatively, these tumors may have altered expression of regulatory BCL-2 family members. For example, our results predict that tumor overexpression of BCL-2 or the absence of BAK would disengage the 4ICD apoptotic signal. Nevertheless, our results suggest that proteolytic processing of membrane-bound ERBB4 and subsequent mitochondrial accumulation of the 4ICD BH3-only protein represents a clinically important event contributing to tumor cell death.

In addition to 4ICD mitochondrial localization and apoptotic function, we have recently shown that 4ICD regulates gene expression as a nuclear protein (4, 16, 17). These divergent 4ICD functional activities can be reconciled in part because in the normal breast 4ICD seems to be retained in the nucleus (10) through interaction with the STAT5A transcription factor at target promoters (16). We propose that cellular transformation disrupts nuclear retention of 4ICD, leading to 4ICD mitochondrial accumulation and eventually apoptosis. This hypothesis is supported by recent experiments showing enhanced cell-killing activity of ERBB4 containing a mutated nuclear localization signal (4). In addition, disruption of 4ICD nuclear retention results in a 4ICD mechanistic switch from a transcriptional coregulator to a mitochondrial proapoptotic protein.[6] We are currently investigating the exact molecular mechanisms regulating 4ICD subcellular localization and functional activities influencing normal breast function and transformation.

In conclusion, we have shown that ligand stimulation and subsequent proteolytic processing of ERBB4 results in membrane release and mitochondrial accumulation of 4ICD, a proapoptotic BH3-only protein. Furthermore, 4ICD directly initiates mitochondrial permeabilization through activation of the apoptotic "gateway" protein BAK, distinguishing ERBB4 from transmembrane "death receptors" that must recruit a BH3-only protein to initiate mitochondrial dysfunction (1). This unique apoptotic function for a cell surface receptor provides the first mechanistic description of a cell death pathway directly integrating an activated transmembrane receptor with the tumor cell mitochondrial apoptotic machinery. Moreover, the potent cell-killing activity of 4ICD provides a mechanistic explanation for the selective loss of ERBB4 expression during the metastatic progression of breast cancer and supports a novel tumor suppressor function for ERBB4.

Acknowledgments

Received 7/6/2005; revised 3/24/2006; accepted 3/30/2006.

Grant support: National Cancer Institute/NIH grants RO1CA95783 and RO1CA96717 (F.E. Jones) and U.S. Army Medical Research and Materiel Command grants W81XWH-06-1-0531 (A. Naresh), DAMD17-03-1-0418 (W. Long), and DAMD17-03-1-0395 (G.A. Vidal).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Diane Clark, Laura McDonald, and Lacey Sullivan for excellent laboratory management; other members of the Jones lab for providing intellectual and technical support; Nicolette Solano for outstanding administrative assistance; the late Stanley Korsmeyer and Anthony Letai for helpful and stimulating discussions during the evolution of this project; Stanley Korsmeyer for providing the MEF cell lines and BCL-2 cDNA; Charles Hemenway for helpful suggestions; and Amy Johnson for editing this article.

[6] A. Naresh and F.E. Jones, unpublished observations.

References

1. Adams JM. Ways of dying: multiple pathways to apoptosis. Genes Dev 2003;17:2481–95.
2. Yarden Y, Sliwkowski MX. Untangling the ErbB signaling network. Nat Rev Mol Cell Biol 2001;2:127–37.
3. Sartor CI, Zhou H, Kozlowska E, et al. HER4 mediates ligand-dependent antiproliferative and differentiation responses in human breast cancer cells. Mol Cell Biol 2001;21:4265–75.
4. Vidal GA, Naresh A, Marrero L, Jones FE. Presenilin-dependent γ-secretase processing regulates multiple ERBB4/HER4 activities. J Biol Chem 2005;280:19777–83.
5. Srinivasan R, Gillett CE, Barnes DM, Gullick WJ. Nuclear expression of the c-erbB-4/HER4 growth factor receptor in invasive breast cancers. Cancer Res 2000;60:1483–7.
6. Kew TY, Bell JA, Pinder SE, et al. c-*erb*B-4 protein expression in human breast cancer. Br J Cancer 2000;82:1163–70.
7. Barnes NL, Khavari S, Boland GP, Cramer A, Knox WF, Bundred NJ. Absence of HER4 expression predicts recurrence of ductal carcinoma *in situ* of the breast. Clin Cancer Res 2005;11:2163–8.
8. Suo Z, Risberg B, Kalsson MG, et al. EGFR family expression in breast carcinomas. c-erbB-2 and c-erbB-4

receptors have different effects on survival. J Pathol 2002;196:17–25.
9. Witton CJ, Reeves JR, Going JJ, Cooke TG, Bartlett JM. Expression of the HER1–4 family of receptor tyrosine kinases in breast cancer. J Pathol 2003;200:290–7.
10. Long W, Wagner K-U, Lloyd KCK, et al. Impaired differentiation and lactational failure in ErbB4-deficient mammary glands identify ERBB4 as an obligate mediator of Stat5. Development 2003;130:5257–68.
11. Tidcombe H, Jackson-Fisher A, Mathers K, Stern DF, Gassmann M, Golding JP. Neural and mammary gland defects in ErbB4 knockout mice genetically rescued from embryonic lethality. Proc Natl Acad Sci U S A 2003; 100:8281–6.
12. Rio C, Buxbaum JD, Peschon JJ, Corfas G. Tumor necrosis factor-alpha-converting enzyme is required for cleavage of erbB4/HER4. J Biol Chem 2000;275:10379–87.
13. Lee HJ, Jung KM, Huang YZ, et al. Presenilin-dependent γ-secretase-like intramembrane cleavage of ErbB4. J Biol Chem 2002;277:6318–23.
14. Ni C-Y, Murphy MP, Golde TE, Carpenter G. γ-secretase cleavage and nuclear localization of ErbB-4 receptor tyrosine kinase. Science 2001;294:2179–81.
15. Zhou W, Carpenter G. Heregulin-dependent trafficking and cleavage of ErbB-4. J Biol Chem 2000;275: 34737–43.
16. Williams CC, Allison JG, Vidal GA, et al. The ERBB4/HER4 receptor tyrosine kinase regulates gene expression by functioning as a STAT5A nuclear chaperone. J Cell Biol 2004;167:469–78.
17. Clark DE, Williams CC, Duplessis TT, et al. ERBB4/HER4 potentiates STAT5A transcriptional activity by regulating novel STAT5A serine phosphorylation events. J Biol Chem 2005;280:24175–80.
18. Burow ME, Weldon CB, Tang Y, McLachlan JA, Beckman BS. Oestrogen-mediated suppression of tumour necrosis factor alpha-induced apoptosis in MCF-7 cells: subversion of Bcl-2 by anti-oestrogens. J Steroid Biochem Mol Biol 2001;78:409–18.
19. Wei MC, Zong WX, Cheng EH, et al. Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. Science 2001;292:727–30.
20. Jones FE, Welte T, Fu X-Y, Stern DF. ErbB4 signaling in the mammary gland is required for lobuloalveolar development and Stat5 activation during lactation. J Cell Biol 1999;147:77–87.
21. Clayton DA. Isolation of mitochondria from cells and tissues. In: Spector DL, Goldman RD, Leinwand LA, editors. Cells: a laboratory manual. Plainview: Cold Spring Harbor Laboratory Press; 1997. p. 41.1–.7.
22. Zong WX, Li C, Hatzivassiliou G, et al. Bax and Bak can localize to the endoplasmic reticulum to initiate apoptosis. J Cell Biol 2003;162:59–69.
23. Sawyer C, Hiles I, Page M, Compton M, Dean C. Two erbB-4 transcripts are expressed in normal breast and in most breast cancers. Oncogene 1998;17:919–24.
24. Sattler M, Liang H, Nettesheim D, et al. Structure of Bcl-xL-Bak peptide complex: recognition between regulators of apoptosis. Science 1997;275:983–6.
25. Liu X, Dai S, Zhu Y, Marrack P, Kappler JW. The structure of a Bcl-xL/Bim fragment complex: implications for Bim function. Immunity 2003;19:341–52.
26. Cheng EH, Wei MC, Weiler S, et al. BCL-2, BCL-X(L) sequester BH3 domain-only molecules preventing BAX- and BAK-mediated mitochondrial apoptosis. Mol Cell 2001;8:705–11.
27. Letai A, Bassik MC, Walensky LD, Sorcinelli MD, Weiler S, Korsmeyer SJ. Distinct BH3 domains either sensitize or activate mitochondrial apoptosis, serving as prototype cancer therapeutics. Cancer Cell 2002;2:183–92.
28. Bouillet P, Strasser A. BH3-only proteins: evolutionarily conserved pro-apoptotic Bcl-2 family members essential for initiating cell death. J Cell Sci 2002;115: 1567–74.
29. Wolter KG, Hsu YT, Smith CL, Nechushtan A, Xi XG, Youle RJ. Movement of Bax from the cytosol to mitochondria during apoptosis. J Cell Biol 1997;139: 1281–92.
30. Griffiths GJ, Dubrez L, Morgan CP, et al. Cell damage-induced conformational changes of the proapoptotic protein Bak *in vivo* precede the onset of apoptosis. J Cell Biol 1999;144:903–14.
31. Eguchi H, Suga K, Saji H, Toi M, Nakachi K, Hayashi SI. Different expression patterns of Bcl-2 family genes in breast cancer by estrogen receptor status with special reference to pro-apoptotic Bak gene. Cell Death Differ 2000;7:439–46.
32. Sierra A, Castellsague X, Coll T, et al. Expression of death-related genes and their relationship to loss of apoptosis in T_1 ductal breast carcinomas. Int J Cancer 1998;79:103–10.
33. Zong W-X, Lindsten T, Ross AJ, MacGregor GR, Thompson CB. BH3-only proteins that bind pro-survival Bcl-2 family members fail to induce apoptosis in the absence of Bax and Bak. Genes Dev 2001;15:1481–6.
34. Knowlden JM, Gee JMW, Seery LT, et al. c-*erb*B3 and c-*erb*B4 expression is a feature of the endocrine responsive phenotype in clinical breast cancer. Oncogene 1998;17:1949–57.
35. Rochaix P, Krajewski S, Reed JC, Bonnet F, Voigt JJ, Brousset P. *In vivo* patterns of Bcl-2 family protein expression in breast carcinomas in relation to apoptosis. J Pathol 1999;187:410–5.
36. Han J, Flemington C, Houghton AB, et al. Expression of bbc3, a pro-apoptotic BH3-only gene, is regulated by diverse cell death and survival signals. Proc Natl Acad Sci U S A 2001;98:11318–23.
37. Puthalakath H, Strasser A. Keeping killers on a tight leash: transcriptional and post-translational control of the pro-apoptotic activity of BH3-only proteins. Cell Death Differ 2002;9:505–12.