A Novel Juxtamembrane Domain Isoform of HER4/ErbB4
ISOFORM-SPECIFIC TISSUE DISTRIBUTION AND DIFFERENTIAL PROCESSING IN RESPONSE TO PHORBOL ESTER*

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Human epidermal growth factor receptor 4 (HER4) is a member of the epidermal growth factor (EGF) receptor subfamily of receptor tyrosine kinases that is activated by neuregulins (NRG), betacellulin (BTC), and heparin-binding EGF-like growth factor. Sequencing of full-length human HER4 cDNAs revealed the existence of two HER4 isoforms that differed by insertion of either 23 or 13 alternative amino acids in the extracellular juxtamembrane (JM) region. The 23-amino acid form (HER4 JM-a) and the 13-amino acid form (HER4 JM-b) were expressed in a tissue-specific manner, as demonstrated by reverse transcriptase-polymerase chain reaction analysis of mouse and human tissues. Both isoforms were expressed in neural tissues such as cerebellum, whereas kidney expressed HER4 JM-a only and heart HER4 JM-b only. In situ hybridization using specific oligonucleotides demonstrated transcription of both JM-a and JM-b isoforms in the mouse cerebellum. Tyrosine phosphorylation analysis indicated that both receptor isoforms were activated to the same extent by NRG-1 and BTC, and to a lesser extent by NRG-1 and heparin-binding EGF-like growth factor. A functional difference was found, however, in response to phorbol ester treatment. Stimulation of cells with phorbol ester resulted in a loss of 125I-NRG-ester treatment. Stimulation of cells with phorbol ester difference was found, however, in response to phorbol ester heparin-binding EGF-like growth factor. A functional

Numerous growth factors exert their biological effects by interacting with receptor tyrosine kinases (RTK)1 at the sur-

face of the target cell. RTKs constitute a gene family of integral cell surface molecules that consist of functionally distinct domains (1). The N-terminal extracellular domain of a RTK interacts with specific ligands. A single transmembrane domain anchors the RTK to the cell membrane. An intracellular tyrosine kinase domain that is highly conserved among RTKs mediates ligand-dependent phosphorylation of tyrosine residues, creating binding sites for Src homology 2 domain-containing intracellular signaling molecules (2, 3). C-terminal to the tyrosine kinase domain is a C-terminal tail, the length of which varies between different RTK subfamilies but that usually contains tyrosine residues that can be phosphorylated by the tyrosine kinase.

Functional diversity is created by modifications of the RTK domain structure, for example by alternative splicing of the RNA precursor molecules encoding the protein or by proteolytic processing of the mature protein product. Well characterized examples of alternative splicing of exons encoding RTKs are the isoforms of fibroblast growth factor receptor-2 (FGFR-2) in which sequences encoding the second half of the third immunoglobulin-like domain located at the proximal extracellular domain are responsible for a specific affinity for either FGF-2 or FGF-7 (4). Alternative splicing also results in the production of soluble FGFR-1 or epidermal growth factor receptor (EGFR) extracellular domains (5, 6). There are numerous examples of release of RTK ectodomains by proteolytic processing, including the colony-stimulating factor-1 receptor, Fms (7); the hepatocyte growth factor receptor, Met (8); the Kit ligand receptor (9); the Gas6 receptor, Axl (10); and FGFR-1 (11).

Human epidermal growth factor receptor 4 (HER4) is the most recently described member of the EGFR-like subfamily of RTKs that consists of EGFR (HER1, ErbB1), HER2 (ErbB2, Neu), HER3 (ErbB3), and HER4 (ErbB4) (12–17). HER4 is a receptor for the neuregulins (NRG) (18), a group of alternatively spliced products of a single growth factor gene that includes acetylcholine receptor inducing activity, glial growth factor, heregulin, and Neu differentiation factor (19–22). Two EGF-like growth factors, betacellulin (BTC) and heparin-binding EGF-like growth factor (HB-EGF), which, unlike NRGs, are ligands for EGFR (23, 24), can also activate HER4 (25–28). Recently, a novel NRG-like gene, NRG-2, was identified and demonstrated to be a ligand for HER4 (29, 30). Activation of

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1 The abbreviations used are: RTK, receptor tyrosine kinase; GFG, fibroblast growth factor; FGFR, FGF receptor; EGF, epidermal growth factor; EGFR, EGF receptor; bp, base pair(s); kb, kilobase pair(s); DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; PMA, phorbol 12-myristate 13-acetate; HB-EGF, heparin-binding EGF-like growth factor; BTC, betacellulin; HER, human epidermal growth factor receptor; NRG, neuregulin.
HER4 in vitro leads to cellular proliferation, chemotaxis, or differentiation via activation of specific signal transduction cascades (27, 31–33). Expression of HER4 mRNA in several adult tissues, such as heart, kidney, brain, and skeletal muscle (17), suggests that HER4 is involved in signaling necessary for the maintenance of a variety of mature organs. High HER4 expression levels in human breast cancer cell lines have further implicated HER4 as having a role in tumorigenesis (17). The biological significance for HER4 has been demonstrated in targeted null mice lacking HER4 (34). These homozygous HER4 knockout mice die at embryonic day 10–11 and have cardiac and neural defects.

In the course of studying the interactions of HER4 with HB-EGF (27) and the role of HER4 signaling in the developing cerebellum (35), we sequenced two full-length coding sequences of HER4 cDNA fragments isolated from cDNA libraries made independently from either a human MDA-MB-453 breast cancer cell line (17) or from human fetal brain tissue (33), and we discovered structural differences in the two cDNAs. In this report, we demonstrate that these two cDNA clones represent alternatively spliced isoforms that differ by insertion of either 23 (JM-a) or 13 (JM-b) alternative amino acids in the proximal extracellular domain just N-terminal to the transmembrane domain (juxtamembrane domain). To date, no alternatively spliced isoforms of HER4 have been described. The two isoforms are differentially expressed in mouse tissues, in particular in neural tissues, heart, and kidney. When expressed in NIH 3T3 clone 7 cells, both isoforms could be activated by HB-EGF, NGF-x1, NGF-x1, and BTC. On the other hand, a functional difference was observed, in that pretreating the transfectants with a phorbol ester, phorbol 12-myristate 13-acetate (PMA), resulted in the loss of NGF-x1 binding and a reduction in total cell-associated HER4 protein levels in HER4 JM-a-transfected cells, but not in HER4 JM-b-transfected cells. These results suggest that the JM-a, but not the JM-b, isoform can be cleaved in the juxtamembrane domain. It was concluded that novel isoforms of HER4 exist and that they may have different biological functions.

**EXPERIMENTAL PROCEDURES**

**Sequencing of HER4 Expression Vectors**—Full-length HER4 coding region inserts were cloned into pcDM8 (Invitrogen) and pEV7 (33) mammalian expression vectors to generate cH4M2 and pEV7-HER4 plasmids, respectively. The origin of the HER4 insert in cH4M2 was a cDNA library produced from an MDA-MB-453 breast cancer cell line (17), and the origin of the HER4 insert in pEV7-HER4 was a cDNA library produced from human fetal brain tissue (33). The coding regions of both HER4 inserts were sequenced from one strand by chain termination sequencing using a Sequenase version 2.0 DNA sequencing kit (U. S. Biochemical Corp.). The primers used were designed according to the one published HER4 sequence (Ref. 17; GenBank accession number L07868). When compared with this sequence, there were two changes in the HER4 coding sequence of pEV7-HER4. These were: (i) a major alteration in the external juxtamembrane region (Figs. 1 and 3) in which 69 nucleotides (23 amino acids) are replaced by 39 different nucleotides (23 amino acids), and (ii) a minor 1-base replacement of G to A in pEV7-HER4, which would result in a Gly → Asp change in the middle of the C-terminal Cys-rich domain. This change may reflect polymorphism or might be a cloning artifact. There were also two differences in the cH4M2 HER4 insert compared with the published sequence, both in the very 5′ end. These were: (i) a change from ATG in the published sequence to CCA, resulting in a conserved Lys → Arg change, and (ii) a change from G to T in the published sequence to T, which would not result in an amino acid change. These two changes had been generated to optimize the Kozak consensus for an initiating Met and to optimize PCR-mediated amplification, respectively, and are not present in vivo.

**RT-PCR Amplification and Cloning of HER4 Isoform Sequences**—Total RNA was prepared from various tissues obtained from 19–21-g Swiss Webster mice (Charles River Laboratories) and from the myocardium of the left ventricle of a human heart (obtained from a valve operation) by using the RNAsol B reagent according to the manufacturer’s instructions (Tel-Test, Inc.). Total RNA (2.5 μg) was subsequently reverse transcribed to cDNA with Superscript II enzyme according to the manufacturer’s instructions (Life Technologies, Inc.) using random oligonucleotide primers (Life Technologies, Inc.). Specific fragments of this cDNA were amplified with 20 μl of 10× Taq DNA polymerase buffer (Boehringer Mannheim), 5 nmol of each dNTP (Boehringer Mannheim), and 50 μl of primers. The PCR products were visualized with UV light. A 1-μl DNA ladder (Life Technologies, Inc.) was used as a size marker.

For sequencing, juxtamembrane HER4 RT-PCR amplicons from mouse heart and kidney tissues were cloned into pBluescript vector (Stratagene) and sequenced in both orientations with T3 and T7 primers.

**Expression Vectors and Transfection**—To generate stable cell lines expressing HER4 JM-a or HER4 JM-b under direction of the same CMV promoter, mouse JM-a and JM-b HER4 sequences were ligated into cH4M2. To do this, a 2.3-kb BstEII-NeI fragment that included the juxtamembrane domain was digested and purified from the pEV7-HER4 (HER4 JM-b isoform) and ligated into cH4M2 (HER4 JM-a isoform) from which the corresponding BstEII-NsiI fragment had first been removed.

This procedure generated a cH4M2 JM-b expression plasmid that differed from the original cH4M2 JM-a expression plasmid only in the alternative juxtamembrane sequences within the 2.3-kb BstEII-NsiI fragment.
HER4 Juxtamembrane Isoforms

Both cH4M2 JM-a and a cH4M2 JM-b were separately co-transfected with an antibiotic resistance gene encoding plasmid (pMAMneo; CLONTECH) into NIH 3T3 clone 7 cells (33) using Lipofectin (Life Technologies, Inc.) according to the manufacturer’s recommendations. Clones transfected with pMAMneo plasmid alone were generated to be used as a transfection control. Cells were subsequently cultured in DMEM supplemented with 10% fetal bovine serum, 1% glutamine/penicillin/streptomycin supplement (GPS; Irvine Scientific), 4.5 g/liter glucose, and 500 μg/ml G418 (Geneticin; Life Technologies, Inc.). G418-resistant clones were screened for their HER4 expression levels by immunoprecipitation and Western blotting as described below.

Phosphorylated ERK Treatment of Cells—The media were aspirated and replaced with DMEM containing 0 or 100 ng/ml PMA (Sigma). One hundred ng/ml has been suggested to be the optimal concentration for stimulating HER4 processing in NIH 3T3 cells (37). PMA treatments were carried out at 37 °C for time periods ranging from 0 to 60 min for the 125I-NRG-1 binding assay and for 45 min for the immunoprecipitation and Western blot analysis of HER4 protein amounts and for the anti-HER4 immunocytochemistry.

Immunoprecipitation and Western Blot Analysis of HER4 Levels—To screen for HER4 expression levels, individual HER4-transfected clones were grown to confluence in six-well dishes, lysed, and immunoprecipitated with a 1:150 dilution of a rabbit polyclonal antibody recognizing the cytoplasmic domain of human HER4 (clone H4.77.16; Neomarkers) as described previously (27). The immunoprecipitates were separated on 6% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Millipore Inc.). The HER4 proteins on the membranes were subsequently detected by Western blotting using a HER4-specific antibody as described above.

HER4 Tyrosine Phosphorylation Analysis—Tyrosine phosphorylation of HER4 in response to growth factor stimulation was analyzed in cultures (six-well dishes) of NIH 3T3 clone 7 transfected clones similar to those used for screening of HER4 protein levels. Serum-starved cells were treated with or without 100 ng/ml HB-EGF, NRG-1, BTC in DMEM and the levels of HER4-specific tyrosine phosphorylation were measured as described (27). Recombinant human HB-EGF was kindly provided by Dr. J. Abraham (Scios Nova, Mountain View, and BTC in DMEM and the levels of HER4-specific tyrosine phosphorylation were measured as described (27). Recombinant human HB-EGF, NRG-1, BTC in DMEM and the levels of HER4-specific tyrosine phosphorylation were measured as described (27). Recombinant human HB-EGF was kindly provided by Dr. J. Abraham (Scios Nova, Mountain View, CA), recombinant human NRG-β1 (residues 177–241 corresponding to the EGF-like domain of heregulin-β1) was purchased from R&D Systems, and recombinant human BTC (KCN-TECH) was kindly provided by Dr. M. Sliwkowski (Genentech, Inc., South San Francisco, CA) and recombinant human BTC (GenBank accession no. L07868). The HER4 coding regions of the two plasmids were sequenced, and just one major difference was observed. The HER4 coding region of pEVE7-HER4 differed from the published MDA-MB-453-derived HER4 sequence (Ref. 17; GenBank accession no. L07868) by an in-frame alteration in which 69 nucleotides in the extracellular juxtamembrane domain of the published sequence were replaced with an unrelated sequence of 39 nucleotides. The deduced difference in amino acid sequence resulting from this replacement was a switch from 23 juxtamembrane amino acids in cH4M2 to 13 different amino acids in pEVE7-HER4 (Fig. 1). The alteration in nucleotide sequence is shown in Fig. 3. The 23-amino acid form was named HER4 JM-a, and the alternative 13-amino acid form was named HER4 JM-b, based on the location of the alternative sequences within the juxtamembrane (JM) domain and the chronology of their description. The virtual identity of the 3924-bp and 3894-bp full-length coding sequences of cH4M2 and pEVE7-HER4, respectively, with the exception of the juxtamembrane regions, suggested that the differential juxtamembrane sequences were generated as a result of alternative splicing of RNA derived from a single gene as opposed to originating from two genes.

RESULTS

Identification of HER4 Juxtamembrane Isoforms—Two plasmids containing full-length human HER4 cDNA inserts were obtained: cH4M2, which contained an insert originally cloned from a human MDA-MB-453 breast cancer cell line (17), and pEVE7-HER4, which contained an insert originally cloned from fetal human brain tissue (33). The HER4 coding region of pEVE7-HER4 differed from the published MDA-MB-453-derived HER4 sequence (Ref. 17; GenBank accession no. L07868) by an in-frame alteration in which 69 nucleotides in the extracellular juxtamembrane domain of the published sequence were replaced with an unrelated sequence of 39 nucleotides. The deduced difference in amino acid sequence resulting from this replacement was a switch from 23 juxtamembrane amino acids in cH4M2 to 13 different amino acids in pEVE7-HER4 (Fig. 1). The alteration in nucleotide sequence is shown in Fig. 3. The 23-amino acid form was named HER4 JM-a, and the alternative 13-amino acid form was named HER4 JM-b, based on the location of the alternative sequences within the juxtamembrane (JM) domain and the chronology of their description. The virtual identity of the 3924-bp and 3894-bp full-length coding sequences of cH4M2 and pEVE7-HER4, respectively, with the exception of the juxtamembrane regions, suggested that the differential juxtamembrane sequences were generated as a result of alternative splicing of RNA derived from a single gene as opposed to originating from two genes.

RT-PCR Analysis of HER4 JM Isoforms in Mouse and Human Tissues—To demonstrate that both the HER4 JM-a and JM-b isoforms existed in vivo, total RNA was isolated from several mouse tissues, subjected to reverse transcription, and analyzed by PCR using primers flanking the variable juxtamembrane region of mouse HER4. The expected size for a PCR product amplified from HER4 JM-a-derived cDNA was 273 bp and from HER4 JM-b-derived cDNA 243 bp, respectively. After separating the PCR products in a 2% agarose gel, bands corresponding to the expected sizes of either one or both isoforms were found but with subtle tissue distributions (Fig. 2, lanes 1–21, top panel). For example, cerebellum expressed both isoforms, kidney expressed solely the JM-a isoform, and heart expressed solely the JM-b isoform (Fig. 2, lanes 11, 5, and 1, respectively). As a control to ensure that all samples analyzed by PCR contained cDNA templates, the same reverse transcriptase products that were used to amplify HER4

incubated with a monoclonal anti-HER4 antibody (clone H4.77.16; Neo-markers) overnight at 4 °C. The cells were washed with PBS, and the primary antibody was detected using a Cy3-conjugated anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories). Photographs were obtained with an Olympus 1 × 70 inverted microscope.

FIG.1. Alternative HER4 juxtamembrane isoforms. A schematic diagram of HER4 with the deduced amino acid sequences of two alternative juxtamembrane isoforms (JM-a and JM-b) is shown. The alternative sequences are in boldface. The two contiguous amino acids that are at either end of the alternating juxtamembrane sequences are shown to help localize the juxtamembrane domains within the published sequence (17). Cys, cysteine-rich domains; TM, transmembrane domain; TK, tyrosine kinase domain.
JM fragments were used in PCR analysis with mouse β-actin-specific primers. A single β-actin PCR product of expected size (450 bp) was detected in all tissues (Fig. 2, lanes 1–21, bottom panel). When the PCR reaction was carried out in the absence of cDNA template, no PCR product was detected with either set of primers (Fig. 2, lane 22). RT-PCR was also carried out with selected human tissues. Human cerebellum was found to express both HER4 isoforms, but human heart only the JM-b isoform (data not shown), consistent with the mouse RT-PCR distribution results.

The identities of the amplified mouse RT-PCR products were confirmed by cloning the single HER4 juxtamembrane RT-PCR products derived from mouse kidney and heart into a pBlue-script vector and sequencing the inserts. Mouse and human HER4 juxtamembrane domains were found to be highly homologous. The RT-PCR product from mouse kidney (Fig. 2, lane 5, JM-a) contained a sequence differing in only 1 nucleotide out of 69 from the sequence of the expression plasmid cH4M2 encoding human HER4 JM-a (Fig. 3). The deduced JM-a amino acid sequence was identical between human and mouse (Fig. 3). In addition, the RT-PCR product from mouse heart (Fig. 2, lane 1, JM-b) contained a sequence differing in only 3 nucleotides out of 39 from the sequence of the expression plasmid pEV7-HER4 encoding human HER4 JM-b (Fig. 3). One of these nucleotide differences resulted in a difference (Met versus Thr) in the deduced JM-b amino acid sequence between human and mouse (Fig. 3). The presence of the novel JM-b sequence in human tissues was further confirmed by showing that a sequence identical to that obtained from the pEV7-HER4 expression vectors was sequenced from RT-PCR products obtained from kidney and heart tissues, and that the respective juxtamembrane domains are highly conserved between mouse and human.

**In Situ Hybridization Analysis of HER4 JM Isoforms**—The RT-PCR analysis of HER4 isoform expression in mouse tissues (Fig. 2) suggested that in neural-derived tissues both HER4 JM-a and HER4 JM-b mRNAs are produced simultaneously. To determine whether the two HER4 isoforms are expressed in the same or distinct anatomical regions, we studied the distribution of mRNAs encoding each isoform in the mouse cerebellum, a tissue that apparently expresses relatively high levels of each HER4 isoform. Using 35S-labeled antisense oligonucleotide probes corresponding to the different juxtamembrane sequences, and therefore specific for each isoform, both HER4 JM-a (Fig. 4A) and HER4 JM-b (Fig. 4B) were found to be expressed in the granule cell layer (GCL) and in the cerebellar white matter (WM) but not in the molecular layer (ML). The anatomical regions were visualized with hematoxylin staining in Fig. 4C. Qualitatively similar results were obtained using a riboprobe directed against the intracellular domain of HER4, which recognizes both isoforms (data not shown). The signals detected in the granule cell layer reflect the expression of both HER4 isoforms in the neuronal population within this layer. The signals in the white matter represent most probably the expression of both HER4 isoforms in oligodendrocytes, which are the main cell type in this region (38). In agreement with the results obtained with RT-PCR, a weak in situ hybridization signal was detected with the HER4 JM-a probe, but not with the HER4 JM-b probe, in mouse kidney sections (data not shown), demonstrating the specificity of the probes.

**Ligand Activation of the HER4 JM Isoforms**—Tissue-specific expression of HER4 JM-a or JM-b isoforms suggests that the isoforms may have different functions. To address this question, stable transfectants expressing either HER4 JM-a or HER4 JM-b were produced. To ensure that identical expression vectors were used, a 2.3-kb BstEII-NsiI fragment including the sequence coding for the juxtamembrane domain was isolated from HER4-pEV7 (HER4 JM-b) and ligated into cH4M2 (HER4 JM-a) to replace the corresponding BstEII-NsiI fragment. Both the original cH4M2 plasmid (cH4M2 JM-a) and the cH4M2 plasmid with a swapped juxtamembrane domain (cH4M2 JM-b) were subsequently transfected into NIH 3T3 clone 7 cells. Cell lines expressing similar levels of HER4 protein, as determined by immunoprecipitation and Western blotting, namely clones JM-a 2 and JM-b 42 (Fig. 5A, lanes 2 and 3), were incubated in the presence of HER4 ligands. The cells were lysed, and tyrosine phosphorylation of HER4 was assessed by Western blotting with an anti-phosphotyrosine antibody. Each of the ligands examined (HB-EGF, NRG-α1, NRG-β1, and BTC) stimulated tyrosine phosphorylation of both HER4 JM-a and HER4 JM-b well above control, and for a given ligand each.
of the receptor isoforms was activated to the same extent (Fig. 5B). The relative levels of HER4 activity induced by HB-EGF and NRG-α (Fig. 5B, lanes 2 and 3) were clearly lower than the levels achieved with NRG-β1 and BTC (Fig. 5B, lanes 4 and 5), consistent with previous reports (25, 27, 39). Control cells transfected with pMAMneo alone did not express detectable levels of endogenous HER4 protein (Fig. 5A, lane 1), nor were they activated by the HER4 ligands (Fig. 5B, control panel).

Phorbol Ester Treatment of Cells Expressing HER4 JM Isoforms—HER4 has been reported to be proteolytically cleaved in response to stimulation with a phorbol ester, PMA (37). Given that cleavage of many transmembrane proteins occurs in the external juxtamembrane region, we wanted to determine whether the two different HER4 juxtamembrane isoforms responded differently or similarly to PMA treatment. In a previous study (37), cleavage of the transmembrane form of HER4 was maximal after a 30–60-min treatment with a concentration of 100 ng/ml PMA, as ascertained by a reduction in the binding of radiiodinated NRG-β1 to the surface of the HER4-transfected cells. Therefore, we incubated cells expressing HER4 JM-a (clones JM-a 2 and JM-a 102) or HER4 JM-b (clones JM-b 15 and JM-b 42) with or without 100 ng/ml PMA for 45 min at 37 °C and then with 20 ng/ml [125I]-NRG-β1 for 1 h on ice, and measured the amount of radioactivity associated with the cells (Fig. 6A). PMA treatment resulted in a reduction in the amount of [125I]-NRG-β1 binding to two independent HER4 JM-a clones by about 80–85% but had no significant effect on [125I]-NRG-β1 binding to two independent HER4 JM-b clones (Fig. 6A). To determine whether the differences observed were a result of different kinetics of processing of the two receptor isoforms, the time course of PMA effects on the level of [125I]-NRG-β1 binding was measured (Fig. 6B). A reduction in the amount of [125I]-NRG-β1 binding to HER4 JM-a cells was detected by 20 min and was maximal by 40 min after PMA treatment, but no reduction of [125I]-NRG-β1 binding to HER4 JM-b cells was observed even at 60 min. When the total cellular HER4 protein levels in these cells were analyzed by a combination of immunoprecipitation and Western blotting, 100 ng/ml PMA treatment for 45 min resulted in a loss of cell-associated HER4 JM-a (Fig. 6C, clone JM-a 2, compare lane 4 with lane 3) but not of cell-associated HER4 JM-b (Fig. 6C, clone JM-b 15, compare lane 6 with lane 5). PMA treatment actually increased the [125I]-NRG-β1 binding to cells expressing HER4 JM-b (Fig. 6B) and the relative amount of cell surface HER4 JM-b (Fig. 6C), consistent with possible PMA stimulation of HER4 JM-b translocation to the cell surface and lack of HER4 JM-b cleavage.

To further confirm these differential effects of PMA, cells expressing HER4 JM-a or HER4 JM-b were stimulated for 45 min with 100 ng/ml PMA, fixed without permeabilization, and immunostained with an antibody recognizing the extracellular domain of HER4 (Fig. 7). PMA treatment of cells expressing HER4 JM-a led to a virtual total disappearance of HER4 immunoreactivity from the cell surface. In contrast, PMA did not reduce at all the HER4 immunoreactivity on the surface of cells expressing HER4 JM-b. Consistent with the results of [125I]-NRG-β1 binding (Fig. 6B) and HER4 immunoprecipitation and Western blotting (Fig. 6C), there was a slight increase in HER4 immunostaining after PMA treatment of cells expressing HER4 JM-b (Fig. 7).

Taken together, these results suggest that the two HER4 isoforms differ in their susceptibility to proteolytic cleavage in...
HER4-specific antibodies. The were determined by immunoprecipitation and Western blotting with without 100 ng/ml PMA for 45 min. Total cellular HER4 protein levels expressing HER4 JM-b (lanes 5
NRG-
then incubated with 20 ng/ml125I-NRG-
clones 15 and 42) were pretreated for 45 min with 100 ng/ml PMA and NIH 3T3 cells expressing HER4 JM-a (clones 2 and 102) or HER4 JM-b (clone 15; JM-b) were transfected with an antibiotic resistance gene encoding plasmid alone (Control) or together with plasmids encoding HER4 JM-a (clone 2; JM-a) or HER4 JM-b (clone 15; JM-b) were treated with 0 ng/ml (top panels) or 100 ng/ml (lower panels) PMA for 45 min. The cells were fixed, and the expression of cell surface HER4 was detected using a monoclonal antibody directed against the extracellular domain. Bar = 50 μm.

response to PMA. Although PMA treatment leads to a virtually total reduction in the amount of cell surface HER4 JM-a protein, it does not reduce the amount of cell surface HER4 JM-b protein.

DISCUSSION

We have demonstrated that HER4 exists in vivo in two alternatively spliced isoforms that differ in having either 23 or 13 alternative amino acids in the extracellular juxtamembrane domain immediately N-terminal to the transmembrane domain. The 23-amino acid isoform has been designated as HER4 JM-a and the 13-amino acid isoform as HER4 JM-b. The two isoforms appear to differ functionally in their response to phorbol ester, in that HER4 JM-a but not HER4 JM-b is processed. The juxtamembrane alterations are the only significant differences in the full-length coding sequences of cDNAs originating from two independent sources, human breast cancer cells and human fetal brain tissue, suggesting that they represent alternatively spliced forms derived from a single HER4 gene. This is consistent with the finding that a single HER4 gene is localized to q33.3–34 of human chromosome 2 (40). The two juxtamembrane sequences differ substantially, and the only conserved sequence in the two forms is Asp-Cys-Ile. Conservation of the only Cys residue in the juxtamembrane domain is consistent with the suggestion that the Cys residues in the proximal Cys-rich domain are critical for correct disulfide bonding (41).

The finding that there are alternative exons that encode alternative HER4 amino acid sequences is novel, inasmuch as these types of isoforms have not been described to date for members of the EGF receptor subfamily. So far, the only example of an alternatively spliced isoform of a receptor of the EGF receptor subfamily in normal mammalian tissues is the truncation of EGFR (Erbb1) that generates a soluble ectodomain (6). Alternative transcripts coding for truncated extracellular domains of EGFR, HER2, and HER3 are also generated as a result of a read-through of the splice donor site and the presence of a stop codon and a polyadenylation site within an intron (42–45). In addition, numerous aberrant forms of EGFR are expressed in tumor tissues in association with gene amplification and chromosomal rearrangements (12, 46–48). Alternative splicing of exons encoding the juxtamembrane domain may be a specific characteristic of HER4, inasmuch as our RT-PCR analysis of different mouse tissues did not reveal the existence of alternative EGFR or HER2 juxtamembrane isoforms.2 There may be other alternatively spliced HER4 isoforms as well, inasmuch as we have also detected alternative sequences in the HER4 cytoplasmic tail that either contain or do not contain the binding site for p85, the regulatory subunit of phosphatidylinositol 3-kinase.3 Taken together, HER4 resembles more the members of the FGF receptor subfamily, where the existence of alternatively spliced isoforms is a common phenomenon (49), rather than other members of the EGF receptor subfamily of RTKs.

What is the possible functional significance, if any, of having two HER4 juxtamembrane isoforms? One possibility is that alternative splicing of the juxtamembrane domain might lead to differential binding of HER4 ligands and activation of this receptor. The precedents are the FGF receptors, in which alternative splicing of the juxtamembrane region of FGFR-1, FGFR-2, and FGFR-3 de-

Fig. 6. Effect of PMA on 125I-NRG-β1 binding to cells transfected with HER4 JM isoforms. A, confluent six-well plate wells of NIH 3T3 cells expressing HER4 JM-a (clones 2 and 102) or HER4 JM-b (clones 15 and 42) were pretreated for 45 min with 100 ng/ml PMA and then incubated with 20 ng/ml 125I-NRG-β1. After washing, the amount of bound 125I-NRG-β1 was measured with a γ-counter. The amount of radioactivity bound to control transfected cells not expressing HER4 was subtracted. B, confluent 12-well plate wells of NIH 3T3 cells expressing HER4 JM-a (clone 102) or HER4 JM-b (clone 15) were pretreated for the time periods indicated with 100 ng/ml PMA and 125I-NRG-β1 binding was measured as in A. C, control transfectants (lanes 1 and 2), cells expressing HER4 JM-a (lanes 3 and 4; clone 2), and cells expressing HER4 JM-b (lanes 5 and 6; clone 15) were treated with or without 100 ng/ml PMA for 45 min. Total cellular HER4 protein levels were determined by immunoprecipitation and Western blotting with HER4-specific antibodies. The arrow points to a 180-kDa HER4 band.

Fig. 7. Effect of PMA on HER4 cell surface immunoreactivity. NIH 3T3 cells transfected with an antibiotic resistance gene encoding plasmid alone (Control) or together with plasmids encoding HER4 JM-a (clone 2; JM-a) or HER4 JM-b (clone 15; JM-b) were treated with 0 ng/ml (top panels) or 100 ng/ml (lower panels) PMA for 45 min. The cells were fixed, and the expression of cell surface HER4 was detected using a monoclonal antibody directed against the extracellular domain. Bar = 50 μm.

2 K. Elenius, G. Corfas, S. Paul, C. J. Choi, C. Rio, G. D. Plowman, and M. Klagsbrun, unpublished data.

3 K. Elenius and M. Klagsbrun, manuscript in preparation.
binds 125I-NRG, leading to internalization of HER4 JM-a, leading to diminished JM-a but not after treatment of cells expressing HER4 JM-b. blot analysis revealed a diminution of cell-associated 180-kDa expressing HER4 JM-b. 

(i) Immunoprecipitation and Western exposed to a phorbol ester, PMA, a differential response was demonstrated previously to induce proteolytic cleavage of HER4 in transfected NIH 3T3 cells (37). When NIH 3T3 clone 7 cells expressing either of the HER4 juxtamembrane isoforms were exposed to a phorbol ester, PMA, a differential response was noted, as follows. (i) PMA treatment prevented the binding of 125I-NRG-β1 to cells expressing HER4 JM-a but not to those expressing HER4 JM-b. (ii) Immunoprecipitation and Western blot analysis revealed a diminution of cell-associated 180-kDa HER4 protein after PMA treatment of cells expressing HER4 JM-a but not after treatment of cells expressing HER4 JM-b. (iii) Cell surface immunostaining with an antibody directed against the HER4 extracellular domain revealed loss of HER4 immunoactivity in response to PMA in cells expressing HER4 JM-a but not in cells expressing HER4 JM-b.

An alternative possibility is that phorbol ester treatment results in internalization of HER4 JM-a, leading to diminished binding of 125I-NRG-β1. However, our immunoprecipitation and Western blot experiments using whole cell lysates show a diminution of HER4 JM-a levels rather than the constant levels which might be expected if the HER4 was being translocated into the cell. Furthermore, unlike EGF-R, which is readily internalized after ligand binding, HER4 has been demonstrated not to be endocytosed effectively (55). Taken together, the most plausible explanation for the differential effects of phorbol ester on the JM-a and JM-b isoforms is that the HER4 JM-a isoform contains the cleavage site for protease-induced release of HER4 ectodomain release that is not present in the HER4 JM-b isoform. The ability of cells to express cleavable and/or non-cleavable forms of HER4 might result in another level of regulating the activities of the four known ligands for HER4.

The HER4 JM isoforms are expressed in vivo in a differential manner, suggesting that transcription of the HER4 gene and the splicing of its RNA precursor are regulated in a tissue-specific manner and that a level of specificity in isoform-specific function might exist. RT-PCR analysis of mouse tissues demonstrated that some tissues (lung, placenta, bladder, liver, and stomach) express little, if any, of the two isoforms, that some tissues (cerebellum, cerebral cortex, spinal cord, medulla oblongata, and eye) express both simultaneously, that some tissues (e.g. kidney) express solely JM-a whereas other tissues (e.g. heart) express solely JM-b. Besides RT-PCR, the expression of both JM-a and JM-b isoforms could be demonstrated in cerebellum by in situ hybridization using oligonucleotide probes that could hybridize with one but not the other isoform. This analysis suggested that both isoforms are transcribed by neurons in the granule cell layer and by oligodendrocytes in the white matter. Both of these cell types have been shown to express HER4 in vitro (56). The signal for HER4 JM-a was stronger in the granule cell layer, whereas the signal for HER4 JM-b was stronger in the white matter. Although these results are not quantitative, they suggest that the level of expression of the each isoform may be different in these two cell populations. Although the HER4 JM isoforms are expressed in a tissue-specific manner, preliminary results using RT-PCR indicate that there is no specific correlation in the expression pattern of either HER4 JM-a or HER4 JM-b with the expression pattern of either the NRG or HB-EGF ligands.

In summary, we have shown that two HER4 isoforms exist in vivo differing only in the juxtamembrane region and that they are most probably generated as a result of tissue-specific alternative splicing. These isoforms might differ functionally in their ability to be cleaved and release a soluble ectodomain. Taken together, these studies suggest that a novel mechanism exists to regulate HER4 function.

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