Secretase-dependent Tyrosine Phosphorylation of Mdm2 by the ErbB-4 Intracellular Domain Fragment*

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ErbB-4 is a receptor tyrosine kinase that is activated by the binding of its cognate ligands, such as heregulin (neuregulin) (1). As with other ErbB receptors, ligand binding provokes homo- and heterodimerization, particularly with ErbB-2, leading to tyrosine kinase activation and the initiation of signal transduction pathways. Although ErbB-4 activation can provoke a mitogenic response, frequently the cellular response is the promotion of cell differentiation. Genetic studies in mice reveal an ErbB-4 requirement for differentiation of the mammary gland in the adult (2–4) and for neural and cardiac development during embryogenesis (5). In cell culture ErbB-4 activation is required for the differentiation of mammary cells (6) and PC12 cells (7). The molecular basis for the capacity of ErbB-4 to influence cell differentiation pathways is not known.

ErbB-4 is also novel within this receptor family in regard to its sequential proteolytic processing by α- and γ-secretases. Treatment of cells with heregulin (8) or 12-O-tetradecanoylphorbol-13-acetate (9) initiates a metalloprotease-dependent ectodomain cleavage of ErbB-4 between His-651 and Ser-652 (10), placing this initial cleavage site eight residues prior to the transmembrane domain, which is typical of an α-secretase activity. Ectodomain cleavage or ErbB-4 is abrogated in TACE null cells (11), and recombinant TACE is able to cleave a peptide representing ErbB-4 residues 646–657 between His-651 and Ser-652 (10). Therefore, it seems likely that the α-secretase cleavage site is eight residues prior to the transmembrane domain and that the transmembrane domain is cleaved in vivo.

The α-secretase cleavage event is followed by a second, γ-secretase event (1). As with other ErbB receptors, ligand binding provokes homo- and heterodimerization, particularly with ErbB-2, leading to tyrosine kinase activation and the initiation of signal transduction pathways. Although ErbB-4 activation can provoke a mitogenic response, frequently the cellular response is the promotion of cell differentiation. Genetic studies in mice reveal an ErbB-4 requirement for differentiation of the mammary gland in the adult (2–4) and for neural and cardiac development during embryogenesis (5). In cell culture ErbB-4 activation is required for the differentiation of mammary cells (6) and PC12 cells (7). The molecular basis for the capacity of ErbB-4 to influence cell differentiation pathways is not known.

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There are two products of ectodomain cleavage: a 120-kDa ectodomain fragment, which can be recovered in the medium, and a membrane-associated 80-kDa (m80) fragment that begins with Ser-652 and includes the transmembrane and cytoplasmic domains of ErbB-4 (9, 10).

The m80 fragment is utilized as a substrate by γ-secretase activity that cleaves within the transmembrane domain at a site known as the α-secretase cleavage site (11). The α-secretase cleavage event is followed by a second, γ-secretase event (1). As with other ErbB receptors, ligand binding provokes homo- and heterodimerization, particularly with ErbB-2, leading to tyrosine kinase activation and the initiation of signal transduction pathways. Although ErbB-4 activation can provoke a mitogenic response, frequently the cellular response is the promotion of cell differentiation. Genetic studies in mice reveal an ErbB-4 requirement for differentiation of the mammary gland in the adult (2–4) and for neural and cardiac development during embryogenesis (5). In cell culture ErbB-4 activation is required for the differentiation of mammary cells (6) and PC12 cells (7). The molecular basis for the capacity of ErbB-4 to influence cell differentiation pathways is not known.

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EXPERIMENTAL PROCEDURES

Materials—Heregulin β1 was purchased from R & D Systems. The γ-secretase inhibitor Compound E was a generous gift from Todd Golde (Mayo Clinic, Jacksonville, FL). Anti-ErbB-4, anti-Mdm2, anti-p21, and anti-Shc were purchased from Santa Cruz. Anti-Shc and anti-phosphotyrosine were from BD Laboratories, and FLAG and tubulin antibodies were obtained from Sigma. Anti-p53 was from BD Biosciences. HIp53 cells were a gift from Jennifer Pietenpol (Vanderbilt University). 32D B-4 cells expressing ErbB-4 (Jma CYT-2 isoform) were obtained from James Staros (Vanderbilt University). HIp53 cells were a gift from Jennifer Pietenpol (Vanderbilt University). 32D B-4 cells expressing ErbB-4 (Jma CYT-2 isoform) were obtained from James Staros (Vanderbilt University). The p211990 (H9253) promoter luciferase construct (32) was provided by Moshe Oren (Weizmann Institute). Renilla luciferase reporter construct (pRLCMV) was obtained from Promega.


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from Promega. The FLAG-ubiquitin construct was generated by insertion of a DNA coding for ubiquitin into the HindIII and XbaI sites of the FLAG CMV2 vector (Kodak).

**Cells and Transfections—**Cos7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. AR75–1 and H1299 cell lines were maintained in RPMI with 10% fetal bovine serum. 32D cells were cultured in RPMI with 15% fetal bovine serum with 5% WEHI condition medium as a source of interleukin-3. HP53 cells were grown in F12 with 10% fetal bovine serum. Transfection was performed using Lipofectamine 2000 (Invitrogen) reagent according to the manufacturer’s instructions.

**Immunoprecipitation and Western Blotting—**ZR-75–1, 32D, H1299, and HP53 cells were harvested with RIPA buffer (1% Nonidet P-40, 1% DOC, 0.1% SDS, 10 mM phosphate buffer, 120 mM NaCl, 2.7 mM KCl) supplemented with complete mini-protease inhibitor mixture and phosphatase inhibitor mix II. Lysates were precipitated using appropriate antibodies. The protein concentration of lysates was determined and equal amounts of protein were mixed with SDS sample buffer and separated on SDS-PAGE prior to Western analysis. Cos7 cells were lysed with TGH buffer (1% Triton X-100, 10% glycerol, 20 mM HEPES (pH 7.2), 100 mM NaCl) and subjected to immunoprecipitation.

**In Vivo Ubiquitination Assay—**The ubiquitination of Hdm2 in vivo was detected by co-transfection of H1299 cells with 4 μg of green fluorescent protein-tagged s80 along with 10 μg of Hdm2 and FLAG-tagged ubiquitin in various combinations. Twenty-four hours post-transfection the cells were washed with ice-cold phosphate-buffered saline. Cells were collected with RIPA buffer (1% Nonidet P-40, 1% DOC, 0.1% SDS, 10 mM phosphate buffer, 120 mM NaCl, 2.7 mM KCl) and lysed at 4°C for 30 min. Soluble protein fraction was separated by centrifugation at 13,000 rpm for 30 min. Each lysate was boiled at 95°C for 6 min and incubated with Hdm2 antibodies for 2 h before adding recombinant protein G-Sepharose 4B beads. After overnight incubation, beads were washed three times with RIPA buffer, and immunoprecipitates were eluted with SDS sample buffer and separated on 7.5% SDS-PAGE. The gel was subjected to Western analysis using FLAG antibodies (M2 monoclonal antibody; Sigma) to detect the ubiquitinated Hdm2.

**Tyrosine Phosphorylation of Mdm2—Secretase Liberation of the ErbB-4 ICD Fragment and its Translocation to the Cytoplasm and Nucleus.** In an attempt to identify such a novel substrate(s), substrates of the c-Abl tyrosine kinase, which is localized in the cytoplasm and nucleus (33), were tested. The results for one such c-Abl substrate, Mdm2 (34), are shown in Fig. 1. Mdm2 is a well characterized ubiquitin ligase that negatively regulates p53 levels in cells (35) and that is predominantly localized in the cytoplasm and nucleus (33).

The data in Fig. 1 show that when heregulin is added to ZR-75–1 cells, Hdm2 (human Mdm2) is tyrosine phosphorylated, and this event is prevented by preincubation with the pan ErbB kinase inhibitor PD 153035 (Fig. 1A) or the γ-secretase inhibitor Compound E (1B). To test whether the secretase inhibitor might interfere non-selectively in the phosphorylation of substrates by heregulin receptors, the phosphorylation of Shc was examined under the same conditions. The data in Fig. 1C show that Compound E does not interfere with the heregulin-dependent tyrosine phosphorylation of Shc nor a co-precipitation of a protein of 170 kDa that likely represents the activated heregulin receptor. The secretase processing of ErbB-4 involves as a prerequisite initial step α-secretase cleavage of the ectodomain (12). Therefore, heregulin-dependent generation of the ErbB-4 ICD fragment was tested in the presence of the metalloprotease inhibitor BB2983 (Fig. 1D). The results of this experiment demonstrate that heregulin-induced phosphorylation of Hdm2 requires α- and γ-secretase activities.

Because secretase processing of ErbB-4 requires ~10–15 min following the addition of heregulin (14, 17), the time course for heregulin-induced phosphorylation of Hdm2 was compared with that for the phosphorylation of Shc, which does not require proteolytic processing of ErbB-4. The data presented in Fig. 2 show that the following the addition of heregulin Shc is maximally phosphorylated within 5 min, whereas the phosphorylation of Hdm2 proceeds with a delay of 5–10 min and is gradually phosphorylated thereafter. The relative delay in Hdm2 phosphorylation is likely due to the time required for growth factor-induced secretase processing of ErbB-4 and translocation of the s80 ICD to subcellular compartments.

The preceding experiments utilize ZR-75–1 cells that express ErbB-3 and ErbB-4, each of which binds heregulin, as well as their dimerization partner ErbB-2. Hence it is possible that the observed phosphorylation of Hdm2 is mediated by an ErbB-3/ErbB-2 dimer. This seems unlikely, however, as neither ErbB-3 nor ErbB-2 is known to be a secretase substrate.

To test that heregulin-dependent phosphorylation of Hdm2 requires only ErbB-4, ErbB-4 (JmaCyt 2 isoform) was expressed in 32D cells, a mouse monocyte precursor cell line that does not otherwise express any ErbB family members (37). When heregulin is added to 32D cells expressing ErbB-4, Mdm2 is tyrosine phosphorylated in a γ-secretase-dependent manner, whereas no phosphorylation is detectable in the parental 32D cells treated with heregulin (Fig. 3). This demonstrates that ErbB-4 is sufficient for heregulin-dependent Mdm2 phosphorylation and that neither ErbB-3 nor ErbB-2 is necessary.

These results demonstrate that Mdm2 is a novel substrate...
for receptor tyrosine kinases in that secretase liberation of the ErbB-4 intracellular domain leads to the phosphorylation of a protein, which is predominantly localized in the nucleus and which is not otherwise phosphorylated by the receptor. This is the first report of such a mechanism to expand the repertoire of substrates for receptor tyrosine kinases.

Interactions of ErbB-4 ICD Fragment and Mdm2—The results cited above predict that the ICD fragment of ErbB-4 may be sufficient to tyrosine phosphorylate Mdm2. This is tested by the experiment shown in Fig. 4A. Co-expression of the s80 ICD fragment and Hdm2 in Cos7 cells leads to constitutive tyrosine phosphorylation of Hdm2. In this experiment there is a detectable level of Hdm2 phosphorylation in the absence of exogenous ErbB-4 ICD, but this is significantly increased by the presence of the ICD fragment. The data also show that expression of a kinase-negative (K751R) s80 ICD mutant does not provoke Hdm2 phosphorylation.

The data in Fig. 4A also show that co-expression of the s80 ICD and Hdm2 results in a lower level of Hdm2 compared with cells that express Hdm2, but not s80. Therefore, densitometry was used to evaluate the level of tyrosine-phosphorylated Hdm2 relative to the amount of Hdm2 for the conditions in Fig. 4A. The results indicate that the s80 ICD enhances Hdm2 phosphorylation by a factor of ~10-fold.

This result indicates that the s80 ICD fragment of ErbB-4 is sufficient to provoke Mdm2 phosphorylation and implies that the sole role of heregulin in this event is to initiate the secretase processing of ErbB-4. Given the capacity of the ICD fragment to phosphorylate Hdm2, the association of the two mole-
Cytosolic p53-dependent transcription, by expression of the s80 ICD fragment and p53 significantly inhibit p21, the transcriptional activity of p53 and that the co-expression of Hdm2 and p53 reduces, by using a p21 luciferase reporter. The data shown in Fig. 7 demonstrates that following the addition of Ponasterone A, p21 levels are increased more rapidly (at 0–6 h) in cells that express the s80 ICD fragment than in cells that do not express the s80 ICD fragment.

In this experiment, changes in the level of the cell cycle inhibitor p21 were followed (Fig. 6, middle panel). The level of p21 was reduced when the s80 ICD fragment is co-expressed. Because Hdm2 is an E3 ubiquitin ligase that can stimulate its own degradation by autoubiquitination (35), we tested the capacity of the s80 ICD fragment to influence the ubiquitination of Hdm2. The results, presented in Fig. 5, clearly demonstrate that expression of the s80 ICD fragment enhances Hdm2 ubiquitination.

ErbB-4 ICD Fragment Influence on p53—Because Mdm2 is the prime negative regulator of p53 levels in cells, we tested whether the ErbB-4 ICD fragment affects on p53 levels. The data would suggest that the presence of the s80 ICD fragment should, by decreasing Mdm2 levels, increase p53 levels. To test this we utilized a p53 null cell line that has been engineered to inducibly express p53 under the influence of Ponasterone A (38). This system allows assessment of the influence of the ICD fragment on relatively low levels of p53 found shortly after the addition of Ponasterone A. HIp53 cells were transiently transfected with the s80 ICD fragment, and after 24 h Ponasterone A was added. The results (Fig. 6A, top panel) show that when cells express the s80 ICD fragment there is a more rapid accumulation of p53 following the addition of Ponasterone A, particularly at 6–12 h.

In this experiment, changes in the level of the cell cycle inhibitor p21 were also followed (Fig. 6, second panel), as p53 is a transcriptional activator of the p21 gene (32). The data show that following the addition of Ponasterone A, p21 levels are increased more rapidly (at 0–6 h) in cells that express the s80 ErbB-4 ICD fragment.

In addition, we measured the transcriptional activity of p53, using a p21 luciferase reporter. The data shown in Fig. 7 demonstrate that co-expression of Hdm2 and p53 reduces, by ~36%, the transcriptional activity of p53 and that the co-expression of the s80 ICD fragment and p53 significantly increases p53-dependent transcription, by ~100%, which likely represents an effect of the s80 fragment on endogenous Hdm2. Co-expression of p53, Hdm2, and increasing levels of the s80 fragment ICD reverse the inhibitory effect of exogenous Hdm2 on p53-dependent luciferase levels. Hence, this assay of p53 function also indicates that the ErbB-4 ICD fragment is able to modulate the Mdm2 negative regulation of p53.

It seems likely that control of Mdm2 function by growth factor activation of the intact ErbB-4 receptor may include other elements besides the s80 ICD fragment. For example, activation of Akt leads to serine phosphorylation of Mdm2 and enhanced Mdm2 stability (35). ErbB-2, a heterodimerization partner for ErbB-4, can decrease p53 levels by Akt-mediated serine phosphorylation of Mdm2 (40). In addition, certain isoforms of ErbB-4 can activate Akt (39). Therefore, the capacity of heregulin to modulate p53 levels may be influenced in opposite directions by the level of ICD fragment produced and the intensity of Akt activation.

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FIG. 5. Influence of the s80 ICD fragment on Hdm2 ubiquitination. H1299 cells were transfected with the indicated constructs. After 24 h the cells were lysed in RIPA buffer and the lysates precipitated with anti-Mdm2 and blotted with anti-FLAG. In the middle and bottom panels, equal aliquots of each lysate were blotted with anti-Mdm2 or anti-ErbB as indicated. *, nonspecific band; WCL, whole cell lysate.

FIG. 6. Influence of the s80 ICD fragment on p53 Levels. HIp53 cells were transfected with an s80 ICD construct or vector as indicated; 24 h later Ponasterone A (10 μM) was added to induce p53 expression. At the indicated times thereafter, cells were lysed in RIPA buffer, and equal aliquot amounts of each lysate were blotted for p53, p21, s80 ICD, or tubulin as indicated.

FIG. 7. s80 ICD increases the transcriptional activity of p53. H1299 cells were transfected with the indicated vectors together with a p21 luciferase construct and a Renilla luciferase construct as a control for transfection efficiency. After 24 h the amount of luciferase activity was determined. The data presented are the average of two experiments.
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