Signal transduction by epidermal growth factor and heregulin via the kinase-deficient ErbB3 protein

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The role of protein tyrosine kinase activity in ErbB3-mediated signal transduction was investigated. ErbB3 was phosphorylated *in vivo* in response to either heregulin (HRG) in cells expressing both ErbB3 and ErbB2, or epidermal growth factor (EGF) in cells expressing both ErbB3 and EGF receptor. A recombinant receptor protein (ErbB3-K/M, in which K/M stands for Lys → Met amino acid substitution) containing an inactivating mutation in the putative ATP-binding site was also phosphorylated in response to HRG and EGF. Both the wild-type ErbB3 and

mutant ErbB3-K/M proteins transduced signals to phosphatidylinositol 3-kinase, Shc and mitogen-activated protein kinases. Separate kinase-inactivating mutations in the EGF receptor and ErbB2 proteins abolished ErbB3 phosphorylation and signal transduction activated by EGF and HRG respectively. Hence the protein tyrosine kinase activity necessary for growth factor signalling via the ErbB3 protein seems to be provided by coexpressed EGF and ErbB2 receptor proteins.

INTRODUCTION

The ErbB/HER subfamily of polypeptide growth factor receptors [1,2] comprises the well-characterized epidermal growth factor (EGF) receptor (ErbB1/HER1), the *neu* oncogene product (ErbB2/HER2), and the more recently identified ErbB3/HER3 and ErbB4/HER4 receptor proteins. Each of these receptors is predicted to consist of an extracellular ligand-binding domain, a membrane-spanning domain, a cytosolic protein tyrosine kinase (PTK) domain and a C-terminal phosphorylation domain. The occurrence of heterodimerization among ErbB/HER family receptors seems to enhance the complexity of their cellular signalling mechanisms (see [3]). For example, EGF challenge of cells coexpressing the EGF receptor (EGFR) and either ErbB2, ErbB3 or ErbB4 leads to the phosphorylation of both the EGFR and the associated ErbB family member [3–11]. Various polypeptides in the heregulin (HRG or neuregulin) family have been shown to bind with high affinity to the ErbB4 receptor protein [12,13], but they apparently also bind to ErbB3 [13,14] and an ErbB2/ErbB3 [15] co-receptor complex. Whereas HRG has been shown to activate the PTK activity of the ErbB4 protein [12], it does not seem to activate PTK activity in cells expressing the ErbB3 protein alone [15]. ErbB2 does not itself bind HRG but coexpression of ErbB2 and ErbB3 yields a high-affinity HRG co-receptor, the PTK of which is activated in response to HRG binding [15,16].

Experiments *in vitro* have indicated that the PTK activity of the ErbB3 protein is attenuated significantly relative to that of other ErbB/HER family members; this has been attributed to the occurrence of non-conservative amino acid substitutions in the predicted catalytic domain of ErbB3 [17,18]. However, the ErbB3 protein is known to be strongly phosphorylated in a variety of cellular contexts. For example, ErbB3 is constitutively phosphorylated on tyrosine residues in a subset of human breast cancer cell lines overexpressing this protein [19], and is phosphorylated in response to either EGF or HRG in cells coexpressing ErbB3 and the EGFR [7,8] or ErbB2 [15] respectively. If the ErbB3 protein is devoid of intrinsic PTK activity, its

phosphorylation in the cellular context would be presumed to result from the action of an associated PTK, for example via a transphosphorylation by either the EGFR or ErbB2 PTK. However, it is also possible that the PTK of ErbB3 is masked under the conditions of previous experiments *in vitro* or is expressed only in the context of an activated co-receptor complex. Indeed, Kraus et al. [19] observed a ligand-stimulated PTK activity in the ErbB3 protein.

The ErbB3 receptor is unique among receptor PTKs in that the amino acid sequence of the C-terminal phosphorylation domain [20,21] contains six repeats of the motif Tyr-Xaa-Xaa-Met (YXXM), known to serve, when phosphorylated, as a binding site for the Src homology 2 (SH2) domains of the p85 subunit of phosphatidylinositol (PtdIns) 3-kinase [22,23]. In previous studies, chimaeric proteins composed of the extracellular ligand-binding domain of EGFR and the cytoplasmic domain of ErbB3 were used to examine the cellular proteins involved in ErbB3 signal transduction [24,25]. EGF stimulation of NIH-3T3 cells expressing these chimaeric proteins resulted in their increased phosphorylation on tyrosine residues and triggered their association with PtdIns 3-kinase. Subsequent investigations of cancer cells endogenously expressing both the EGFR and ErbB3 [7,8] and transfected fibroblasts ectopically expressing these proteins [26] showed EGF-dependent ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association. As the YXXM motif is not present in the C-terminal phosphorylation domain of the EGFR, it seems that ErbB3 might co-operate with EGFR in the activation of a mitogenic signalling pathway not otherwise directly engaged by the EGFR.

ErbB3, like other ErbB family receptor proteins [27], also incorporates a consensus motif, Asn-Pro-Xaa-Tyr (NPXY), for binding the PTB (phosphotyrosine-binding) domain of the Shc protein [28–32]. This motif has been implicated in the binding of Shc to EGFR and ErbB2 [28,33,34] and to the insulin receptor [35]. Phosphopeptide competition experiments have indicated that the sequence Asn-Pro-Asp-Tyr¹³⁰⁹ (NPDY) in human ErbB3, when phosphorylated, mediates the association of Shc with this receptor [25], a finding confirmed by our recent site-

Abbreviations used: EGF, epidermal growth factor; EGFR, EGF receptor; GST, glutathione S-transferase; HRG, heregulin; K/M, Lys → Met amino acid substitution; MAPK, mitogen-activated protein kinase; PTK, protein tyrosine kinase.

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directed mutagenesis experiments (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). The Shc protein is rapidly phosphorylated on activation of various receptor or non-receptor PTKs and is involved in the translocation of the Grb2/Sos complex to the plasma membrane [36,37]. Sos, a Ras guanine nucleotide exchange factor, then activates the Ras protein [38,39], which in turn stimulates the phosphorylation and activation of mitogen-activated protein kinases (MAPKs or ERKs) through a protein kinase cascade [40–42].

The present study examined the role of receptor PTK activity in growth factor signal transduction mediated by the ErbB3 protein in conjunction with other ErbB family members. To this end, EGFR, ErbB2 and ErbB3 proteins, either alone or in combination, were expressed ectopically in cultured cell models. The importance of the intrinsic PTK activity of individual ErbB family members was assessed by the use of recombinant EGFR, ErbB2 and ErbB3 proteins incorporating kinase-inactivating mutations.

EXPERIMENTAL

Cell lines and reagents

NIH-3T3 and COS7 cells were purchased from the American Type Culture Collection and cultured as recommended. Recombinant HRG- β 1 and antibodies recognizing ErbB2 (Ab-1) and ErbB3 (2F12, 2C3) were purchased from NeoMarkers. Phosphotyrosine-specific monoclonal antibody PY20 was obtained from Leinco Technologies and Transduction Laboratories. Shc, Grb2 and p85 antibodies and recombinant PY20 conjugated with horseradish peroxidase (RC20) were also acquired from Transduction Laboratories. EGFR-specific monoclonal antibody Ab4 and an MAPK-specific antibody were obtained from Oncogene Science and Zymed Laboratories respectively. Horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence reagents were purchased from Amersham. The expression plasmid containing wild-type rat ErbB3 cDNA, pcDNA3-ErbB3, is detailed elsewhere [43]. cDNA species encoding wild-type EGFR and the kinase-deficient EGFR-K/M (in which K/M stands for Lys $\rightarrow$ Met amino acid substitution) mutant protein were kindly provided by Dr. Gordon Gill (University of California, San Diego, CA, U.S.A.). The rat ErbB2 (Neu) expression plasmid was a gift from Dr. Robert Weinberg (Whitehead Institute, Cambridge, MA, U.S.A.).

Generation of ErbB3 and ErbB2 Lys $\rightarrow$ Met mutant proteins

Rat ErbB3 cDNA was mutated with the Transformer Site-directed Mutagenesis Kit (Clontech). A Met codon was introduced at amino acid position 740 to replace the Lys codon by using a 24 bp mutagenic primer, 5'-CAGTCTGCATTATGGTCATGGAGG-3'. Lys⁷⁵⁸ of rat ErbB2 cDNA was mutated by use of the Ex-Site Mutagenesis kit (Stratagene) and a 24 bp mutagenic primer, 5'-CATCATGGTGTGAGAGAAAACAC-3'. The affected regions of the ErbB2 and ErbB3 cDNA species were sequenced to verify that the mutant expression vectors had been constructed successfully.

Production of recombinant HRG- β 1^{177–241}

The cDNA encoding the EGF-like domain of the β 1 isoform of HRG (HRG- β 1^{177–241}) was generated by reverse transcriptase-mediated PCR with the oligonucleotides 5'-GCGAATTCCC-ATCTTGTAATAATGTGCG-3' (forward primer, corresponding to bp 623–641 of human HRG- β 1 cDNA) and 5'-CTCGGCC-GCTACTCCGCCTCCATAAATCAATC-3' (reverse primer, corresponding to bp 794–815). The first-strand cDNA was

transcribed from human brain mRNA (Clontech) with the reverse primer and SuperscriptII reverse transcriptase (Gibco-BRL). PCR (30 cycles) was performed after addition of the forward primer and *Pfu* polymerase (Stratagene). The authenticity of the PCR-amplified HRG cDNA was verified by DNA sequencing, and the HRG cDNA was subcloned downstream of the glutathione S-transferase (GST) coding sequence in the expression vector pGEX-KG [44]. The GST–HRG fusion protein was purified from cultures of *Escherichia coli* by glutathione–agarose affinity chromatography, dialysed and then digested with thrombin for 30 min at room temperature. The majority of GST and undigested GST–HRG was removed by running the mixture through a second glutathione–agarose column. HRG was freeze-dried and further purified by Superose Fast Performance Liquid Chromatography (Pharmacia). Fractions containing HRG were identified by SDS/PAGE [45], pooled and stored at -85°C . These manipulations resulted in the inclusion of nine amino acid residues, Gly-Ser-Pro-Gly-Asp-Pro-Pro-Ala-Asn, upstream of the authentic HRG peptide sequence. In some experiments a 30 kDa HRG- β 1 protein (NeoMarkers) was employed, which corresponded to the secreted form of the growth factor. Although the potency of this protein was significantly greater than that of the HRG- β 1^{177–241} peptide, qualitatively similar results were obtained with the two HRG preparations.

Gene transfection, immunoprecipitation and immunoblotting

COS7 cells (10^7) were suspended in Dulbecco's modified Eagle's medium, electroporated at 250 V and 1180 μF (Cell-Porator; Gibco-BRL) in the presence of the indicated plasmids, and used for experiments at 36–60 h after transfection. NIH-3T3 cells were transfected by a calcium phosphate method [46], and clones expressing the ectopic genes were selected in the presence of geneticin (G418).

Dishes of cells (80–90% confluent) were serum-starved for 16–20 h with cell culture medium containing 0.1% (v/v) fetal calf serum. Cells were washed twice with serum-free medium and incubated with EGF or HRG diluted in culture medium containing 0.1% BSA or the dilution vehicle for 5–7 min at 37°C . Cells were washed twice with PBS and lysed with NP40 lysis buffer [1% (v/v) Nonidet P40/50 mM Hepes/Na/150 mM NaCl/2 mM EDTA/3 mM EGTA/2 mM sodium orthovanadate/10 mM sodium pyrophosphate/50 mM NaF/2 $\mu\text{g}/\text{ml}$ pepstatin A/10 $\mu\text{g}/\text{ml}$ aprotinin/10 $\mu\text{g}/\text{ml}$ leupeptin/2 mM PMSF (pH 7.4)]. The total cell lysate was centrifuged for 10 min at 13000 g and the supernatant was collected. Appropriate antibodies were added and incubated for 1 h on ice. Protein A–agarose or protein G–agarose was added and the suspensions were rocked for 1–3 h at 4°C . Immunoprecipitates were washed with a buffer containing 1% (v/v) Nonidet P40, 50 mM Hepes/Na, 150 mM NaCl, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate and 50 mM NaF, pH 7.4, and subjected to SDS/PAGE. Resolved proteins were transferred to a PVDF membrane and detected with the indicated antibodies by enhanced chemiluminescence luminography (Amersham). For the gel-shift assay of MAPK activation, the amount of bisacrylamide was reduced [acrylamide/bisacrylamide, 30:0.04 (w/v)] and the electrode buffer was used at double concentration.

RESULTS

EGF-dependent phosphorylation of ErbB3 in transfected cells expressing EGFR and ErbB3 proteins

Our previous studies have shown that the ErbB3 protein is an excellent substrate for the PTK activity of the EGFR [18].

Figure 1 EGF-dependent ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in cells co-transfected with wild-type and mutant EGFR and ErbB3 cDNA species

COS7 cells were transfected by electroporation with wild-type (WT) EGFR, WT ErbB3, Lys⁷²¹ → Met (K/M) mutant EGFR, or Lys⁷⁴⁰ → Met (K/M) mutant ErbB3 cDNA species as indicated. At 24 h after transfection, cells were serum-starved for 11 h and stimulated with 50 nM EGF or vehicle for 5 min at 37 °C. Detergent lysates of the cells were immunoprecipitated with ErbB3-specific antibody. (A) Expression of the recombinant ErbB3 protein in transfected cells was demonstrated by subjecting each immunoprecipitate to SDS/PAGE and immunoblotting with ErbB3-specific antibody (α-B3). EGFR expression was shown by immunoblotting an equivalent portion of each cell lysate with EGFR-specific antibody (α-ER). (B) Each immunoprecipitate was immunoblotted with anti-phosphotyrosine (α-P-tyr) and an antibody recognizing the p85 subunit of PtdIns 3-kinase (α-p85).

Evidence suggesting that the ErbB3 protein is a physiological substrate for EGFR has also been presented [7,8]. In these studies, cultured cells expressing high levels of both EGFR and ErbB3 were stimulated with EGF; phosphorylation of ErbB3 on tyrosine residues was detected by immunoblotting. To demonstrate more directly the cross-phosphorylation of ErbB3 by the EGFR, COS7 cells were co-transfected with EGFR and ErbB3 expression vectors. Both wild-type and mutant receptor proteins containing a kinase-inactivating Lys → Met substitution in their putative ATP-binding sites (EGFR-K/M [47] and ErbB3-K/M) were employed in these experiments.

Immunoblotting with receptor-specific antibodies indicated that each of the wild-type and K/M mutant receptors was successfully expressed in COS7 cells (Figure 1A). Cell lysates were subsequently immunoprecipitated with ErbB3 antibody; the immunoprecipitates were subjected to immunoblotting with anti-phosphotyrosine. In cells expressing wild-type receptors, EGF stimulated the phosphorylation of the ErbB3 protein (Figure 1B). Significantly, the mutant ErbB3-K/M protein was

phosphorylated on tyrosine residues to a similar extent to that of the wild-type protein. The ErbB3 immunoprecipitates showed no presence of EGFR when probed with an EGFR-specific antibody (results not shown). These results demonstrated that intrinsic ErbB3 PTK activity was not necessary for EGF-dependent ErbB3 phosphorylation. Activation of the EGFR also promoted the co-precipitation of p85 with both the wild-type and the mutant ErbB3 proteins (Figure 1B). In contrast, stimulation of the kinase-deficient form of the EGFR (EGFR-K/M) did not induce ErbB3 phosphorylation or ErbB3/p85 association. Therefore the activation of ErbB3 phosphorylation and signal transduction by EGF depended on the PTK activity of the EGFR but did not require that of ErbB3.

The apparent cross-phosphorylation of ErbB3 by the EGFR was not detected in parental COS7 cells, which indicated that the possible presence of endogenous EGFR and ErbB3 proteins in this cell line did not compromise the interpretation of these experiments (Figure 1B). Also, transfected COS7 cells over-expressing the ErbB3 proteins alone showed no phosphorylation of ErbB3 on stimulation with EGF (results not shown). These results were consistent with previous findings that the EGFR-mediated phosphorylation of ErbB3 occurs only in cells expressing a high level of EGFR [7,8].

HRG-dependent ErbB3 phosphorylation and ErbB3 signal transduction in cells expressing wild-type and mutant ErbB3 proteins

To extend our understanding of signal transduction mediated by the HRG-stimulated ErbB3 receptor, we generated stable NIH-3T3 cell lines that expressed a high level of wild-type ErbB3 and ErbB3-K/M (Figure 2A). Cells expressing the wild-type protein showed a constitutive phosphorylation of ErbB3 on tyrosine residues. However, as we have previously observed [43], phosphorylation of ErbB3 was clearly enhanced on stimulation with HRG-β1¹⁷⁷⁻²⁴¹, a recombinant protein comprising the EGF-like domain of the HRG-β1 molecule. The mutant protein ErbB3-K/M was phosphorylated in response to HRG to a similar extent to the wild-type protein, which showed that intrinsic ErbB3 kinase activity was not required for HRG-dependent phosphorylation of the receptor. Treatment of mock-transfected cells with HRG did not elicit any phosphorylation response (Figure 2A). Also, ErbB3 immunoprecipitates showed no presence of the ErbB2 protein when probed with an ErbB2 antibody (results not shown). Given that ErbB3 seems not to possess intrinsic kinase activity [17,18], it was presumed that ErbB2, present endogenously in the NIH-3T3 fibroblasts, mediated the HRG-dependent phosphorylation of ErbB3. Our recent immunoblotting experiments have indicated that NIH-3T3 fibroblasts express the ErbB2 protein (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work), and when anti-phosphotyrosine immunoprecipitates were subjected to immunoblotting with ErbB2 antibody, HRG was seen to augment the phosphorylation of the endogenous ErbB2 protein (Figure 2A). These results suggested that the intrinsic kinase activity of ErbB2 might be responsible for the HRG-dependent phosphorylation of both ErbB2 and ErbB3, which was consistent with the notion that ErbB2 and ErbB3 functioned as HRG co-receptors.

To examine further the signalling potentials of the wild-type and mutant ErbB3 proteins, ErbB3 immunoprecipitates from transfected cells were probed with an antibody recognizing the p85 subunit of PtdIns 3-kinase. An HRG-dependent ErbB3/p85 association was seen with cells expressing either the wild-type or the mutant protein (Figure 2B). Apparently, mutation of the

Figure 2 HRG-mediated phosphorylation and signal transduction of wild-type and mutant ErbB3 proteins in stably transfected NIH-3T3 cells

NIH-3T3 cells were transfected with either the parent pcDNA3 expression vector (—) or pcDNA3 vectors incorporating ErbB3-WT or ErbB3-K/M cDNA species as indicated. Cloned cells expressing comparable levels of wild-type (WT) and mutant proteins were selected and propagated. Mock-, ErbB3-WT- and ErbB3-K/M-transfected cells were serum-starved for 16 h and stimulated with either 30 nM HRG- β 1^{177–241} or vehicle for 5 min at 37 °C. **(A)** HRG-stimulated phosphorylation of ErbB3-WT, ErbB3-K/M, and endogenous ErbB2 proteins. HRG-stimulated or control cells were dispersed in NP40 lysis buffer. The lysates were immunoprecipitated (IP) with either ErbB3-specific antibody (α -B3) or anti-phosphotyrosine (α -P-tyr), and the immunoprecipitates were resolved by SDS/PAGE. ErbB3 immunoprecipitates were immunoblotted (IB) with either ErbB3 antibody or anti-phosphotyrosine as indicated. Anti-phosphotyrosine immunoprecipitates were immunoblotted with ErbB2-specific antibody (α -B2). **(B)** HRG-stimulated association of the p85 subunit of PtdIns 3-kinase with wild-type and mutant ErbB3 proteins. Lysates of HRG-stimulated or control cells were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS/PAGE. Immunoprecipitation of the ErbB3 protein and HRG-stimulated co-immunoprecipitation of p85 were detected by immunoblotting with anti-ErbB3 (α -B3) and anti-p85 (α -p85) respectively. **(C)** Stimulation of Shc phosphorylation and Shc/Grb2 association by HRG. Cells treated as in **(A)** were lysed and immunoprecipitated with Shc-specific antibody. Immune complexes were immunoblotted with either the anti-phosphotyrosine-horseradish peroxidase conjugate RC20 (α -P-tyr) or a Grb2-specific antibody (α -Grb2). HRG-stimulated phosphorylation of the 52 kDa isoform of the Shc protein is indicated in the anti-phosphotyrosine blot. **(D)** HRG-stimulated activation of MAPK mediated by wild-type and mutant ErbB3 proteins. Cells were challenged with HRG or vehicle, then lysed as described above. Each cell lysate (30 μ g of protein) was subjected to the gel mobility-shift assay of MAPK activation (see the Experimental section). Both the p42 and p44 forms of MAPK were observed in non-stimulated cells. The appearance of more slowly migrating bands indicated the activation of both MAPK forms in response to HRG.

ATP binding site of ErbB3, which would abolish any intrinsic kinase activity, did not affect its ability to associate with the p85 protein, an event presumably mediated by the HRG-dependent phosphorylation of the ErbB3 C-terminus.

The signal-transducing protein Shc has been implicated in the actions of a number of receptor PTKs [30,48–50]. In our recent studies (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work), Shc was found to associate with the ErbB3 protein and was phosphorylated on tyrosine residues in response to HRG. We sought to determine whether the HRG-stimulated kinase-inactive ErbB3-K/M would similarly involve Shc. Cells expressing either wild-type ErbB3 or the ErbB3-K/M mutant protein were stimulated with HRG- β 1^{177–241}, and Shc immunoprecipitates were blotted with phosphotyrosine antibody. Shc was seen to be phosphorylated in response to HRG in cells

expressing either protein (Figure 2C). Negligible Shc phosphorylation was detected in the mock-transfected NIH-3T3 cells. Among the three isoforms of Shc, phosphorylation of p52 was more evident than that of p61 or p46. Anti-Shc immunoprecipitates from the NIH-3T3 transfectants were also probed with a Grb2-specific antibody. HRG- β 1^{177–241} seemed to elicit a strong association of Grb2 and Shc in cells expressing ErbB3 (Figure 2C). Although the response of the mutant receptor protein was apparently weaker, ErbB3-K/M also mediated an HRG-dependent Shc/Grb2 association.

In the context of NIH-3T3 cells expressing wild-type ErbB3, MAPK isoforms undergo an HRG-dependent gel mobility shift (presumably owing to phosphorylation by MAPK kinase [51]), which corresponds to the activation of MAPK activity as indicated by immune complex kinase assays (U. Vijapurkar, K.

Figure 3 HRG-stimulated ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in COS7 cells transfected with ErbB2 and ErbB3 cDNA species

(A) Coexpression of ErbB2 with wild-type (WT) and kinase-deficient ErbB3 proteins. COS7 cells were transiently transfected with the parent expression vector (—), or vectors incorporating cDNA species encoding ErbB2-WT, ErbB3-WT or ErbB3-K/M proteins as indicated. Transfected cells were serum-starved and then stimulated with 30 nM HRG- $\beta 1^{177-241}$ or vehicle for 7 min at 37 °C. Detergent lysates (1 mg of protein) were immunoprecipitated with ErbB3-specific antibody, and the immunoprecipitates were subjected to SDS/PAGE and immunoblotting with ErbB3 antibody (α -B3). The expression of ErbB2 was shown by immunoblotting with 10 μ g of cell lysate protein with ErbB2-specific antibody (α -B2). (B) HRG-stimulated ErbB3 phosphorylation and ErbB3/p85 association in cells expressing wild-type or mutant ErbB2 and ErbB3 proteins. The immunoprecipitates described in (A) were resolved by SDS/PAGE, and the phosphorylated forms of wild-type and mutant ErbB3 were detected by immunoblotting with anti-phosphotyrosine (α -P-tyr). Association of the p85 subunit of PtdIns 3-kinase with the ErbB3 proteins was detected by immunoblotting with p85 antibody (α -p85).

Cheng and J. G. Koland, unpublished work). The gel mobility-shift assay [52] was used to determine whether HRG could stimulate MAPK phosphorylation via the ErbB3-K/M receptor protein. In the present study, when transfected cells were stimulated with HRG- $\beta 1^{177-241}$, both p42 (Erk2) and p44 (Erk1) forms of MAPK showed retarded migration on SDS/polyacrylamide gels (Figure 2D). With respect to this MAPK gel mobility shift, wild-type and mutant ErbB3 proteins were indistinguishable. The mock-transfected NIH-3T3 cells showed no HRG-dependent MAPK shift. These results confirmed that the Shc/Grb2/MAPK pathway, as well as the PtdIns 3-kinase pathway, was involved in HRG-stimulated signal transduction by ErbB3. Importantly, each of the signal transduction events mediated by the wild-type ErbB3 protein was also mediated by the ErbB3-K/M mutant. These results demonstrated that any intrinsic PTK activity of ErbB3 was not required for these signalling functions.

Figure 4 HRG-stimulated ErbB3 phosphorylation in cells co-transfected with wild-type and mutant ErbB2 and ErbB3 cDNA species

(A) COS7 cells were transiently transfected with vectors incorporating cDNA species of either wild-type (WT) or kinase-deficient Lys⁷⁵⁸ → Met (K/M) ErbB2 and either ErbB3 or ErbB3-K/M proteins as indicated. Cells were serum-starved and then stimulated with 1 nM recombinant HRG- $\beta 1$ (NeoMarkers) for 5 min at 37 °C. Detergent lysates (1.5 mg of protein) were immunoprecipitated with ErbB3 antibody, and each immunoprecipitate was subjected to SDS/PAGE and immunoblotting with ErbB3-specific antibody (α -B3). The expression of ErbB2 was shown by immunoblotting 10 μ g of cell lysate protein with ErbB2-specific antibody (α -B2). (B) HRG-stimulated ErbB3 phosphorylation in cells coexpressing either ErbB2 or ErbB2-K/M and either ErbB3 or ErbB3-K/M. ErbB3 immunoprecipitates described in (A) were resolved by SDS/PAGE and immunoblotted with anti-phosphotyrosine (α -P-tyr).

ErbB3 phosphorylation and ErbB3/PtdIns 3-kinase association in COS7 cells expressing ErbB2 and ErbB3 receptors

To investigate more directly the role of the ErbB2 receptor PTK in the HRG-stimulated phosphorylation of the ErbB3 protein, wild-type ErbB3 and ErbB3-K/M receptors were expressed alone or together with ErbB2 in COS7 cells (Figure 3A). When the transfected cells were stimulated with HRG, both wild-type and mutant ErbB3 proteins were phosphorylated on tyrosine residues (Figure 3B). ErbB3 phosphorylation was greatly enhanced by co-transfection of ErbB2, which indicated that ErbB3 was phosphorylated by ErbB2 and/or an ErbB2-associated PTK. Again, negligible ErbB2 protein was detected in the ErbB3 immunoprecipitates (results not shown). The weak phosphorylation of the ErbB3 proteins in the cells transfected with ErbB3 cDNA species alone (Figure 3B) might have reflected the activity of a low level of endogenous ErbB2 present in these cells (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). Both in cells transfected with ErbB3 cDNA alone and in cells co-transfected with ErbB2 cDNA, the phosphorylation of the ErbB3-K/M mutant protein was slightly less than that of the wild-type protein (see the Discussion section). ErbB3 immunoprecipitates from the transfected COS7 cells were also probed by

anti-p85 immunoblotting (Figure 3B). Association of the p85 subunit of PtdIns 3-kinase with both wild-type and mutant ErbB3 was found to be dependent on HRG-stimulation and was enhanced by the coexpression of ErbB2.

To underscore further the role of ErbB2 PTK activity in the HRG-stimulated phosphorylation of the ErbB3 protein, ErbB3 and ErbB3-K/M receptors were coexpressed with wild-type or Lys⁷⁵⁸ → Met (K/M) mutant ErbB2 receptor (Figure 4A). When coexpressed with the wild-type ErbB2 protein, both wild-type ErbB3 and ErbB3-K/M were phosphorylated on tyrosine residues in response to HRG (Figure 4B). This phosphorylation was almost completely abolished when the wild-type or mutant ErbB3 protein was coexpressed with the ErbB2-K/M protein, which indicated that this phosphorylation was dependent on a functional ErbB2 PTK. The weak HRG-stimulated phosphorylation of ErbB3 and ErbB3-K/M in cells coexpressing the ErbB2-K/M protein was similar to that seen in COS7 cells transfected with ErbB3 cDNA species alone (compare Figure 3B).

DISCUSSION

The EGF-stimulated phosphorylation of ErbB3 has been demonstrated in studies of human cancer cell lines endogenously expressing EGFR and ErbB3 [7,8] and cultured cells ectopically expressing these receptors [11]. This apparent receptor cross-phosphorylation was confirmed here in studies of COS7 cells co-transfected with EGFR and ErbB3 cDNA species (Figure 1). Although it is possible that this phosphorylation was mediated by another protein kinase activated by the EGFR, the following observations suggest that the ErbB3 protein was phosphorylated directly by the EGFR. First, the kinase-deficient EGFR protein could not deliver this effect (Figure 1). Secondly, the cytoplasmic domain and C-terminal sequences of ErbB3 have previously been found to be excellent substrates for the EGFR [18,53]. Thirdly, evidence for cross-phosphorylation between other ErbB family receptors has accumulated [3–11,15,54–56].

The potential ErbB2-mediated phosphorylation of the ErbB3 protein was also investigated in the present study. The HRG-stimulated phosphorylation of ErbB3 expressed in COS7 cells was greatly enhanced with the coexpression of ErbB2 (Figure 3; see also [15]), and this effect was also observed with the ErbB3-K/M mutant protein. Coexpression of the kinase-deficient ErbB2-K/M protein with ErbB3 did not, however, significantly enhance HRG-dependent ErbB3 phosphorylation. These results indicated that ErbB3 kinase activity was not required for HRG-dependent ErbB3 phosphorylation, and that the PTK activity of ErbB2 was responsible for this phosphorylation. ErbB2/ErbB3 cross-phosphorylation would provide a mechanism by which both receptors could have important roles in HRG signalling. Whereas ErbB2 has intrinsic kinase activity, it cannot bind HRG in the absence of ErbB3 [15]. In contrast, ErbB3 binds HRG, but apparently does not possess intrinsic enzymic activity [17,18].

The phosphorylation of the ErbB3-K/M mutant protein on tyrosine residues was slightly lower than that of the wild-type protein when transfected COS7 cells were stimulated with HRG (Figures 3 and 4). This might suggest that ErbB3 does have intrinsic kinase activity and that the observed phosphorylation of ErbB3 was the sum of autophosphorylation and cross-phosphorylation by ErbB2. However, as we and others have not detected PTK activity in the ErbB3 protein [17,18], this possibility seems unlikely. Even if the ErbB3 receptor possessed protein kinase activity, the cross-phosphorylating activity of ErbB2 would seem to predominate over this activity, as the presence of ErbB2 greatly enhanced the phosphorylation of ErbB3. An

alternative explanation for the weaker HRG-dependent phosphorylation of the ErbB3-K/M mutant is the possibility that the Lys → Met substitution altered the conformation of the mutant protein such that it was less efficiently phosphorylated by ErbB2. Interestingly, no difference between the phosphorylation of ErbB3 wild-type and ErbB3-K/M was detected when the EGFR PTK mediated the cross-phosphorylation process (Figure 1).

The recruitment by ErbB3 of PtdIns 3-kinase to both EGF and HRG signalling pathways was also investigated here (Figures 1, 2 and 3). Both wild-type ErbB3 and the ErbB3-K/M mutant protein were shown to associate with the p85 subunit of PtdIns 3-kinase in a ligand-dependent manner. The presence of several YXXM motifs for p85 binding is unique to ErbB3, *Drosophila* insulin receptor [57] and the IRS-1 protein [58]; like IRS-1, ErbB3 seemed to function as a bridge between a receptor PTK and PtdIns 3-kinase. Previous studies have shown the HRG-dependent activation of PtdIns 3-kinase in NIH-3T3 cells transfected with both ErbB2 and ErbB3 cDNA species [26] and in cultured breast cancer cells endogenously expressing ErbB2 and ErbB3 [59].

At least one NPXY motif for Shc binding is present in each ErbB family protein. Graus-Porta et al. [60] reported that HRG induced the recruitment of Shc to ErbB family receptors and stimulated the phosphorylation of Shc on tyrosine residues in cells overexpressing both ErbB3 and ErbB4. HRG-stimulated phosphorylation of Shc has also been shown in cells overexpressing ErbB4 alone [61]. We have recently shown that ErbB3 can recruit the Shc protein on stimulation by HRG. This recruitment leads to the phosphorylation of Shc on tyrosine residues, the strong association of Shc and Grb2, and the activation of MAPKs (U. Vijapurkar, K. Cheng and J. G. Koland, unpublished work). Here we have demonstrated that the ErbB3-K/M mutant protein was also capable of mediating these signal transduction events. These results therefore suggest that any intrinsic PTK activity of ErbB3 is not necessary for its signalling function and that ErbB3, when phosphorylated in the presence of a kinase-active ErbB2 protein, can activate at least two distinct signalling pathways.

In summary, we have examined the role of PTK activity in growth factor signal transduction mediated by the ErbB3 protein. Whereas previous studies have shown that this protein exhibits negligible intrinsic PTK activity *in vitro* ([17,18], but see also [19]), the present results indicated that ErbB3 is catalytically inactive even in the context of a co-receptor complex. The ErbB3 protein is apparently phosphorylated by either the EGFR or ErbB2 PTK on stimulation of EGFR/ErbB3 or ErbB2/ErbB3 co-receptors with EGF or HRG respectively. Although the ErbB3 protein must rely on the PTK activity of another ErbB family member, it does uniquely participate in growth factor signalling by providing a high-affinity ligand-binding site in the case of the ErbB2/ErbB3 HRG co-receptor and by recruiting diverse intracellular signalling molecules. Phosphorylation of ErbB3 by a co-receptor partner triggers ErbB3/PtdIns 3-kinase association, Shc/Grb2 association and the activation of MAPKs. Given that the ErbB3 receptor protein is, along with other ErbB family members, often abundantly expressed in human cancer cells, the mitogenic signal transduction mechanisms investigated here might be particularly relevant in this context.

We thank Dr. Robert A. Weinberg and Dr. Gordon N. Gill for generously providing cDNA species, and Dr. Barry G. Kasson for his assistance in the purification of recombinant HRG. This work was supported by National Institutes of Health (NIH) grant DK44684, NIH Pharmacological Sciences Training Grant GM07069, and United States Army Research and Development Command grant DAMD17-94-J-4185. Services were provided by the University of Iowa Diabetes and Endocrinology Research Center NIH grant DK25295.

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