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# Role of Shc and Phosphoinositide 3-Kinase in Heregulin-Induced Mitogenic Signaling via ErbB3

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ErbB3/HER3 is a cell surface receptor which belongs to the ErbB/HER subfamily of receptor protein tyrosine kinases. When expressed in NIH/3T3 cells, ErbB3 can form heterodimeric coreceptor with endogenous ErbB2. Among known intracellular effectors of the ErbB2/ErbB3 are mitogen-activated protein kinase (MAPK) and phosphoinositide (PI) 3-kinase. In the present study, we studied relative contributions of above two distinct signaling pathways to the heregulin-induced mitogenic response via activated ErbB3. For this, clonal NIH-3T3 cell lines expressing wild-type ErbB3 and ErbB3 mutants were stimulated with heregulin $\beta_1$. While cyclin D1 level was markedly high and further increased by treatment of heregulin in cells expressing wild-type ErbB3, the elimination of either Shc binding or PI 3-kinase binding lowered both levels. This result was supported by the reduction of cyclin $D_1$ expression by preteatment with MAPK kinase inhibitor or PI 3-kinase inhibitor before stimulation with heregulin. In accordance with the cyclin $D_1$ expression, elimination of either Shc binding or PI 3-kinase binding reduced the heregulin-induced DNA synthesis and cell growth rate. Our results obtained by the comparison of wild-type and ErbB3 mutants indicate that the full induction of the cell cycle progression through $G_1$/S phase by ErbB3 activation is dependent on both Shc/MAPK and PI 3-kinase signal transduction pathways.

Key Words: ErbB3/HER3, Shc, PI 3-kinase, Cyclin D1, Mitogenesis

## INTRODUCTION

The ErbB3 is a cell surface receptor which belongs to the ErbB/HER subfamily of receptor protein tyrosine kinases (Walker, 1998; Olayioye et al, 2000). Polypeptide ligands of epidermal growth factor family bind to coreceptors formed between ErbB family members and induce receptor phosphorylation leading to activation of downstream signaling (Lemke, 1996; Riese & Stern, 1998). Heregulin, the endogenous peptide ligand to ErbB2/ErbB3 heterodimeric coreceptor, induces ErbB3 phosphorylation and binding of putative downstream signaling molecules to the receptor (Vijapurkar et al, 1998). Receptor homo- and hetero-dimerization is essential to form functional receptor for the ligands and phosphorylation of each constituents of the dimeric receptors (Pinkas-Kramarski et al, 1996). All ErbB family receptors have intrinsic protein tyrosine kinase activity, except the ErbB3 receptor that lacks kinase activity (Guy et al, 1994; Sierke et al, 1997). Despite lack of kinase activity, ErbB3 can be phosphorylated on tyrosine residues by the kinase activity of other ErbBs that form heterodimeric coreceptors with ErbB3 (Soltoff et al, 1994; Wallasch et al, 1995). ErbB coreceptors were shown to be associated with the generation of a variety of signals and resulting biological responses (Olayioye et al, 1998; Riese et al, 1998; Sundaresan et al, 1998).

The ErbB3 C-terminus incorporates several binding sites for signal transducing proteins. These signal-transducing proteins could mediate the mitogenic signal of ErbB3. One Asn-Pro-Xaa-Tyr (NPXY) sequence motif of ErbB3 is the consensus binding site for Shc and six Tyr-Xaa-Xaa-Met (YXXM) sequence

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motifs is the binding sites for phosphoinositide 3-kinase (PI 3-kinase). Shc has been shown to directly associate with the phosphorylated C-terminal NPXY sequence of ErbB3 (Vijapurkar et al, 1998). Shc is shown to activate the mitogen-activated protein kinase (MAPK) signaling pathway (Guan, 1994; Seger & Krebs, 1995; et al, 1994) resulting in increased cell proliferation in case of the EGF receptor (ErbB1) signaling (Posada & Cooper, 1992; Pages et al, 1993). Shc-mediated MAPK activation has also been implicated in mitogenic signaling by the heregulin-stimulated ErbB2/ErbB3 coreceptor (Vijapurkar et al, 1998). The binding of PI 3-kinase with ErbB3 upon ligand binding to the receptor has been shown in many reports (Kim et al, 1994; Soltoff et al, 1994; Hellyer et al, 1998). By binding with ErbB3, PI 3-kinase is activated and generates 3-phosphorylated lipid products. These 3-phosphorylated lipid products then activate Akt (Franke et al, 1997; Klippel et al, 1997). PI 3-kinase and Akt are shown to be important mediators for heregulin-stimulated mitogenesis via the ErbB2/ErbB3 heterodimeric coreceptor (Carraway et al, 1995). Until now Shc and PI 3-kinase are known to be the major transducers of heregulin signaling via the ErbB2/ErbB3 coreceptor and the activation of these two transducers seems to be associated with heregulin-induced mitogenesis.

In present study, we aimed to determine the relative contributions of these two pathways to ErbB3-mediated activation of cyclin $D_1$ expression and subsequent cell proliferation. By using stably transfected cell lines expressing wild type ErbB3 or mutant ErbB3, we dissected the roles of the MAPK and PI 3-kinase pathways in mitogenic responses to heregulin via the ErbB3. The results indicated that while both pathways contribute to heregulin-induced cyclin $D_1$ expression, DNA synthesis and cell growth, the PI 3-kinase pathway plays a more critical role in the response.

## METHODS

### *Reagents and materials*

Materials and reagents used in the present study were purchased from the following suppliers: Cell culture medium, Lipofectamine transfection reagent, Life Technologies; LY294002 and PD98059, Sigma Chemical; recombinant heregulin-$\beta_1$ and anti-cyclin $D_1$ monoclonal antibody (Ab-1), NeoMarkers; anti-mouse IgG horseradish peroxidase conjugates and enhanced chemiluminescence (ECL) reagents, Amersham; [methyl-$^3$H]thymidine (20 Ci/mmole), NEN Life Science Products.

### *Cell lines and cell culture*

NIH-3T3 cells stably expressing the ErbB3-WT (wild type ErbB3), ErbB3-Y1325F (Shc binding motif-mutated ErbB3), ErbB3-6F (PI 3-kinase binding motif-mutated) and ErbB3-7F (both Shc and PI 3-kinase binding motif-mutated ErbB3) were used. These cell lines express comparable levels of various ErbB3 that is not expressed in parent NIH/3T3 cells (unpublished observation). Cell lines were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 700 $\mu$g/ml Geneticin (G418). Before stimulating the cells with heregulin-$\beta_1$, cells were starved in growth medium containing 0.1% serum for 18 hrs. The cells were then stimulated with heregulin-$\beta_1$ at the specified concentrations and time in the presence or absence of inhibitors (LY294002, a PI 3-kinase inhibitor, and PD98059, a MAPK kinase inhibitor).

### *Western blot analysis*

Serum-starved cells were treated with 5 nM heregulin-$\beta_1$ for 12 hours and the cells were lysed with NP-40 lysis buffer (1% Nonidet P-40, 50 mM HEPES/Na, pH 7.4, 150 mM sodium chloride, 2 mM EDTA, 3 mM EGTA, 2 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 50 mM sodium fluoride, 2 $\mu$g/ml pepstatin A, 10 $\mu$g/ml aprotinin, 10 $\mu$g/ml leupeptin, 2 mM phenlymethylsulfonyl fluoride). The whole cell lysates were cleared by centrifugation. After protein concentration was assayed, proteins were resolved by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. After blocking membranes [5% (w/v) dry milk in PBS/0.1% Tween-20], membrane-bound proteins were probed with specific antibodies and detected by ECL luminography.

### *Cell growth*

Four thousand cells were seeded in 60 mm dishes and serum-starved for 18 hours. Heregulin was added for 48 hours at the specified concentrations and the number of cells was counted using hemocytometer.

*DNA synthesis measurement*

DNA synthesis rate was determined by the [$^3$H] thymidine incorporation method. Briefly, serum-starved cells for 18 hours were stimulated with heregulin-$\beta_1$ for 18 h. Then cells were incubated for another 3 hours in medium containing 0.5 mCi/ml of [methyl-$^3$H] thymidine. Cells were washed twice with ice-cold phosphate-buffered saline, once with 5% trichloroacetic acid, and then solubilized DNA with 0.1 M sodium hydroxide. The radioactivity incorporated in DNA was measured by liquid scintillation counting.

## RESULTS

*Cyclin D1 protein expression levels in cells expressing wild type or mutant ErbB3*

Our previous studies showed that cells expressing the ErbB3-Y1325F that are defective in binding with Shc, show a decreased mitogenic response to heregulin, and that activation of PI 3-kinase might account for the residual mitogenic activity observed in these cells (Vijapurkar et al, 1998). By using ErbB3 mutants that selectively activate either the MAPK or PI 3-kinase pathway, we assessed the relative contributions of these two pathways to the mitogenic signal via activated ErbB3. As cyclin $D_1$ is the key regulator of the cell cycle, the effects of heregulin on the mitogenesis could be assessed by changes in the level of celluar cyclin $D_1$. Cyclin $D_1$ is an important regulator for $G_1$/S cell cycle progression (Baldin et al, 1993; Quelle et al, 1993). Elevated cyclin $D_1$ levels has also been reported to be associated with the transforming activities of the constitutively activated ErbB2 proein (NeuT) (Lee et al, 2000). Therefore, we examined whether cyclin $D_1$ protein level in the cells expressing wild type ErbB3 and ErbB3 mutants was increased by stimulation with heregulin. Cells expressing the ErbB3-WT showed a higher basal level of cyclin $D_1$ protein than that of the parental cells, and this level was further increased after a 12-hour stimulation with heregulin (Fig. 1). Cells expressing the mutant ErbB3 proteins showed neither an enhanced basal nor a heregulin-dependent cyclin $D_1$ expression. Apparently, both of Shc/MAPK and PI 3-kinase signaling pathways contribute to heregulin-induced cyclin $D_1$ expression in the context of signaling via ErbB3.

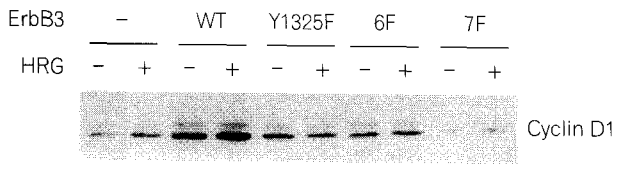


**Fig. 1.** Cyclin $D_1$ expression in NIH/3T3 cells expressing wild-type ErbB3 and mutant ErbB3s. Clonal cell lines expressing wild-type and mutant ErbB3s were serum-starved for 18 hours and treated for 12 hours with 5 nM heregulin-$\beta_1$. Cell lysates were probed with a monoclonal antibody specific to cyclin $D_1$ and detected by ECL luminography (−, parent NIH/3T3 cells; WT, wild-type ErbB3; Y1325F, Shc binding motif-mutated ErbB3; 6F, PI 3-kinase binding motif mutated-ErbB3; 7F, both Shc and PI 3-kinase binding motif-mutated ErbB3; HRG, heregulin-$\beta_1$;).

*Effect of MAPK kinase inhibitor and PI 3-kinase inhibitor on cyclin $D_1$ expression in cells expressing wild type ErbB3*

The heregulin-induced cyclin $D_1$ expression in cells expressing wild type ErbB3 was decreased by 72% by pretreatment with LY294002, a specific PI 3-kinase inhibitor, and by 44% by pretreatment with PD98059, a specific MAPK kinase inhibitor (Fig. 2). This result supports the result of the above experi-

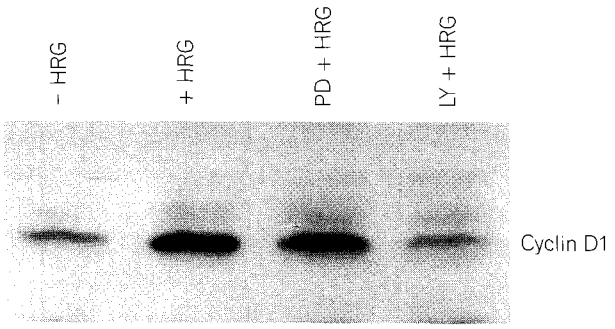


**Fig. 2.** Inhibition of cyclin $D_1$ expression by PI 3-kinase inhibitor and MAPK kinase inhibitor in NIH/3T3 cells expressing wild-type ErbB3. Serum starved cells expressing wild-type ErbB3 were stmulated with 5nM heregulin-$\beta_1$ for 12 hours in the absence or presence of LY294002 and PD98059. Cell lysates were probed with a monoclonal antibody specific to cyclin $D_1$ and detected by ECL luminography (HRG, heregulin-$\beta_1$; PD, PD98059; LY, LY294002).

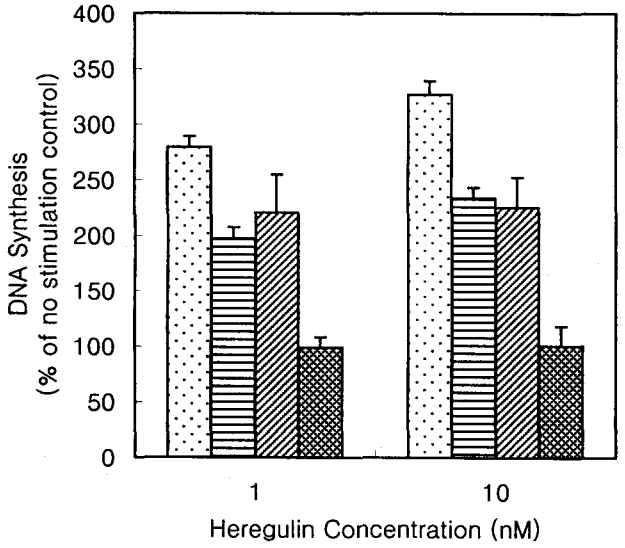


**Fig. 3.** Heregulin-stimulated [$^3$H] thymidine uptake in the NIH-3T3 cells expressing wild-type ErbB3 and mutant ErbB3. Cells expressing wild-type and mutant ErbB3 proteins were serum-starved for 18 hours followed by treatment with heregulin-$\beta_1$ for 18 h. [Methyl-$^3$H] thymidine was then added to the stimulation medium, and its incorporation into DNA was determined after 3 hours. (▨) ErbB3-WT, (▤) ErbB3-Y1325F, (▨) ErbB3-6F, (▩) ErbB3-7F. Data are shown in percent of no stimulation control. Vertical lines represent the standard deviation of triplicate assays.

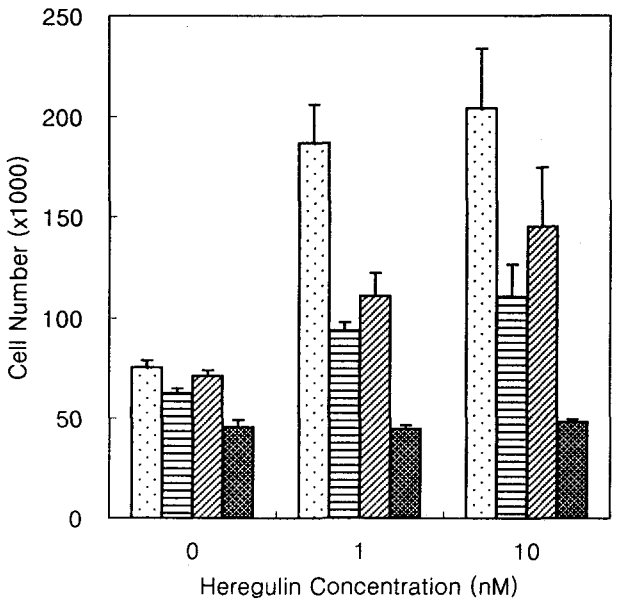


**Fig. 4.** Heregulin-stimulated cell growth in the NIH-3T3 cells expressing wild-type ErbB3 and mutant ErbB3. Cells expressing wild-type and mutant ErbB3 proteins were seeded at the density of 40,000 cells per 60 mm dish and serum-starved for 18 hours followed by treatment with heregulin-$\beta_1$ for 48 h. Cell number was counted using hemocytometer. (▨) ErbB3-WT, (▤) ErbB3-Y1325F, (▨) ErbB3-6F, (▩) ErbB3-7F. Data are means of triplicate data. Vertical lines represent the standard deviation of triplicate assays.

ment using cells expressing mutant ErbB3s. These results altogether show that both Shc/MAPK pathway and PI 3-kinase pathway are involved in heregulin-induced cyclin $D_1$ expression via activated ErbB3.

*Heregulin-stimuated DNA synthesis and cell growth in cells expressing wild type ErbB3 and mutant ErbB3s*

Heregulin-induced DNA synthesis was measured by [$^3$H]thymidine incorporation method (Fig. 3). Cells expressing ErbB3-WT incorporated more [$^3$H]thymidine indicating that heregulin stimulated DNA synthesis by activation of ErbB3. The DNA synthesis rate in cells expressing ErbB3-Y1325F and ErbB3-6F was much less than that of cells expressing ErbB3-WT. The heregulin-induced DNA synthesis in cells expressing ErbB3-7F was not present at all. Cell growth rate experiment showed basically the same result. Cells expressing ErbB3-WT proliferated faster than cells expressing ErbB3-Y1325F and ErbB3-6F. In cells expressing ErbB3-7F the heregulin-induced growth was completely abolished. Therefore, in cells expressing the ErbB3, both the MAPK and PI 3-kinase signaling pathways appeared to contribute to the DNA synthesis and cell growth in a additive manner. These results also suggest that the MAPK and PI 3-kinase pathways are the two principal transducers of the mitogenic signal of the ErbB3 heregulin receptor.

## DISCUSSION

Both MAPK and PI 3-kinase have been shown to be activated via coreceptors including ErbB2/ErbB3 coreceptor (Carraway et al, 1995; Vijapurkar et al, 1998). In the present study, the roles of the MAPK pathway and PI 3-kinase pathway in the induction of mitogenesis were examined with the aid of ErbB3 mutants which were defective in binding with either Shc or PI 3-kinase. This approach verified the experimental results generated by using chemical inhibitors

which could be somehow non-specific in their activity. Expression of the wild-type as well as the mutant ErbB3 in NIH-3T3 cells, which endogenously express ErbB2, resulted in the formation of functional heregulin coreceptors that can bind downstream signaling molecules with ErbB3 C-terminal motifs (unpublished observation). Moreover, mutation of the six tyrosine residues within the PI 3-kinase-binding 6 YXXM motifs (ErbB3-6F) did not deprive ErbB3 of the association with Shc and the ability to activate MAPK. Likewise, mutation of the tyrosine residue within the C-terminal Shc-binding NPXY motif (ErbB3-Y1325F) did not deprive ErbB3 of the ability to recruit PI 3-kinase. Heregulin is mitogenic to the cells expressing both ErbB2 and ErbB3, and separate experiments have shown MAPK (Vijapurkar et al, 1998) and PI 3-kinase (Carraway et al, 1995; Ram & Ethier, 1996) to be involved in this response. Activation of the MAPK signaling cascade is required for transmission of the mitogenic signals of several growth factors (Ricci et al, 1995; Ricketts et al, 1996; Porras et al, 1998). Experiments with a constitutively active catalytic subunit of PI 3-kinase (Hu et al, 1995) have demonstrated that activation of PI 3-kinase is sufficient to initiate cellular DNA synthesis (Frevert & Kahn, 1997; Klippel et al, 1998), but that additional signals are required for progression through the entire cell cycle (Klippel et al, 1998; Daly et al, 1999). The PI 3-kinase inhibitor wortmannin can block the activation of MAPK, showing the possible presence of cross-talk between Shc/MAPK pathway and PI 3-kinase pathway although the blockade may be cell type- or ligand-specific (Ferby et al, 1994; Duckworth & Cantley, 1997). In the present study, the heregulin-induced mitogenic response was weaker in cells expressing mutant ErbB3s defective in binding with either Shc or PI 3-kinase than in cells expressing wild type ErbB3. The level of cyclin $D_1$ protein was significantly increased in response to heregulin in cells expressing wild type ErbB3 but not in cells expressing mutant ErbB3s which are defective in binding with either Shc or PI 3-kinase. The cyclin $D_1$ protein level in cells expressing ErbB3 was much higher than in parental cells. Previous work with other systems has shown that activation of either MAPK (Lavoie et al, 1996; Cheng et al, 1998) or PI 3-kinase (Gille & Downward, 1999) can stimulate transcription of the cyclin $D_1$ gene, while activation of PI 3-kinase signaling can inhibit the ubiquitin-mediated degradation of cyclin $D_1$ protein (Diehl et al, 1998). The results of DNA synthesis and cell growth experiment were consistent with the result of cyclin $D_1$ analysis. In cells expressing mutant ErbB3s, the DNA synthesis and cell growth were lower than that in cells expressing wild-type ErbB3 showing that both Shc/MAPK pathway and PI 3-kinase pathways contribute to the heregulin-induced DNA synthesis and cell growth.

The present study indicated that activation of both the Shc/MAPK and PI 3-kinase pathways by heregulin is important for the enhancement of cyclin $D_1$ levels, the full induction of DNA synthesis and stimulation of cell growth via the ErbB3. By blocking both pathways the heregulin-stimulated mitogenic responses were completely eliminated. Thus the heregulin-stimulated mitogenic signal via the ErbB3 appeared to be transmitted by the Shc/MAPK and PI 3-kinase pathways. In addition, our result suggested that the ErbB3 protein can dominate the signal transduction of ErbB2/ErbB3 heterodimeric coreceptors which may be formed between ErbB3 and endogenous ErbB2 considering the absence of activation of MAPK pathway and PI 3-kinase pathway in cells which do not express functional ErbB3.

In summary, we have investigated the mitogenic signaling via distinct ErbB3 proteins defective in binding with Shc and PI 3-kinase. The effects of the loss of these individual ErbB3 motifs were seen in NIH/3T3 cell lines expressing mutant ErbB3s. Our results showed that cell cycle progression through $G_1$/S phase by the ErbB3 activation was dependent upon both the Shc/MAPK and PI 3-kinase signal transduction pathways. However, PI 3-kinase pathway appeared to play more critical role. It would appear that further study of the roles of ErbB3 in mitogenesis and possible cancerous transformation is warranted.

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