HER4-mediated Biological and Biochemical Properties in NIH 3T3 Cells

EVIDENCE FOR HER1-HER4 HETERODIMERS*

(Received for publication, June 20, 1995, and in revised form, October 27, 1995)

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The human epidermal growth factor receptor (HER) family of type I receptor tyrosine kinases has been linked to the progression of certain human adenocarcinomas. The mechanism by which these receptors function with respect to tumor development is thought to involve overproduction of the gene product resulting from an increase in gene copy number or expression (1–12). Receptor overexpression often correlates with a high level of constitutive tyrosine kinase activity, and this activity is thought to promote signals leading to uncontrolled cell growth (13).

Several ligands that bind to and stimulate the kinase activity of the HER family members have been identified and are classified as EGF-like ligands. EGF, amphiregulin, betacellulin, and transforming growth factor-β are specific for HER1 (14). Heregulin and its rat homologue neu differentiation factor, are a subfamily of the EGF-like ligands that have been shown to bind to and activate both HER3 and HER4 (15–22). The molecular characterization of a HER2 ligand has not been reported; however, a number of factors have been shown to activate HER2 tyrosine kinase activity specifically (23–25).

Activation of the HER4 receptor family of tyrosine kinase receptors, using EGFR as the model, is thought to involve the binding of ligand and subsequent homodimerization of the receptor resulting in a conformational change and activation of the intrinsic tyrosine kinase activity (58). Recent advances suggest that a more complex mechanism of HER activation can occur. Heregulin has been shown to bind and stimulate both HER3 and HER4 (15, 26). Thus, the signaling repertoire of any one ligand can be multifaceted depending upon the cellular receptor expression pattern, potentially allowing the same ligand to deliver distinct downstream signals. Further, ligand-induced receptor activation in the HER family is not limited to homodimerization, for it has been demonstrated that many of the HER family members can associate with and activate each other (15, 27–30). HER2 has been shown to heterodimerize with HER1, HER3, and HER4, and EGF stimulation of HER1 has been shown to activate HER3 signaling (31). Therefore, the signaling of receptors by multifunctional ligands on homoreceptor and heteroreceptor complexes could result in a multiplicity of downstream signaling events.

Activation of growth factor receptors results in the transmission of stimulatory signals from the outside to the inside of the cell. This process makes use of intracellular proteins, or targets, that contain Src homology region 2 (SH2) domains that recognize and bind to the activated receptor (32, 33). A number of these proteins have been identified and shown to interact with membrane-associated and cytoplasmic tyrosine kinases (32, 34–36). The specificity of their binding is dictated by receptor sequences that flank a specific phosphotyrosine residue (33, 37). Since the HER family members are similar but not identical in their amino acid sequence, an expected diversity of associations with different SH2-containing proteins has been demonstrated. HER1 has been shown to associate with phospholipase C-γ, SHC, and Grb2 (13, 38–42). HER2 can associate with phospholipase C-γ, SHC, and Ras-GTPase activating protein (43–45). HER3 has recently been shown to activate PI 3-kinase (31, 41, 46).

We have focused on characterizing the newest member of the HER family, HER4, with regard to its biological activity and activation of downstream targets. To this end, a collection of HER4-expressing 3T3 cell lines has been generated and used to assay the biological and enzymatic activities of this receptor.

EXPERIMENTAL PROCEDURES

Cell Culture—NIH 3T3 clone 7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum (Life Technologies, Inc.) at 37°C in 5% CO2 (47). Cells expressing receptors...
were produced by pooling G418 resistant colonies from cultures that had been cotransfected with the pSV2neo plasmid and maintained in 500 μg/ml G418. Transfections were done using Lipofectamine (Life Technologies, Inc.). HER2 and HER4 plasmid construction have been described previously (15). Cell lines expressing both HER1 and HER4 were produced by transfection of HER1-expressing plasmid into the pEGX-2T vector and selecting by histidinol selection. Clonal lines were isolated and lines expressing similar amounts of HER4 were used.

Ligands—Herregulin β2 used in all assays was synthesized as described previously (48). Briefly, the EGF domain of heregulin β2 was fused to the Fc portion of a human IgG1 gene, and this construct was transfected and expressed in COS cells. The herregulin portion of the purified IgG1 fusion was cleaved away from the Fc domain with thrombin and purified.

Focus-forming Assay—Transfected NIH 3T3 clone 7 cells were trypsinized, and approximately 500 cells were mixed with 10² progenitor NIH 3T3 clone 7 cells. The following day, EGF or heregulin β2 was added at 20 μg/ml, and the cells were fed on a 2-day schedule. Cells were stained after 7–10 days with methylene blue/carbaryl fuschia. Construction, Expression, and Purification of PI 3-Kinase SH2-GST and SHC-GST Fusion Proteins—Two glutathione S-transferase (GST) fusion proteins containing each of the two SH2 domains of the p85 subunit of PI 3-kinase was used in the construction using the pGEX-2T plasmid vector (Pharmacia Biotech Inc.) with some modifications in the multiple cloning sites (49).

**RESULTS**

HER2 and HER4 Expression and Focal Transforming Activity—To compare the signaling properties of HER4 with that of HER1 and HER2, NIH 3T3 clone 7 cells were transfected with plasmids encoding HER1, HER2, or HER4, resulting in the selection of clonal cell lines. NIH 3T3 clone 7 cells, initially derived from the NIH 3T3 ATCC cell line, are useful in biological assays due to their flatter morphology and ability to remain contact-inhibited when grown to confluence. Immunoprecipitation of cell extracts using receptor-specific antibodies demonstrated that HER1 (47), HER2, and HER4 were overexpressed in the appropriate cell lines (data not shown). HER1, HER2, and HER4 transfectants were seeded onto a monolayer of parental cells and grown in the presence or absence of specific ligand (Fig. 1). Used as a positive control, the HER1-expressing 3T3 clone 7 cell line was able to form foci only when stimulated with EGF. The HER2-expressing cell line was able to form foci in the absence of any added ligand and did not respond to either EGF or heregulin β2 (50–52). HER4-expressing cells were able to form foci in the absence of ligand; however, this activity was further stimulated by the addition of recombinant heregulin β2. These results demonstrate that HER4 is able to induce cellular transformation similar to that shown for HER1 and HER2.

**HER4 Tyrosine Phosphorylation**—To determine whether heregulin-induced focal transforming activity in HER4 transfectants correlated with an increase in receptor tyrosine phosphorylation, extracts from ligand-stimulated and unstimulated HER cell lines were denatured, and the levels of tyrosine phosphorylation were analyzed by immunoblot using an antiphosphotyrosine antibody (Fig. 2). Analysis of extracts in this manner minimizes the impact of protease, phosphatase,
the absence of Mg$^{2+}$

**Fig. 1.** HER4 autokinase activity. Since HER4 is tyrosine-phosphorylated in response to ligand we set out to determine whether this change in modification correlated with HER4 in vitro autokinase activity. Antibody to HER4 immunoprecipitates from HER4 transfectants demonstrated a heregulin β2-dependent activation of a 180-kDa protein (Fig. 3). This band is HER4 (Fig. 2A). Depending upon the extraction conditions, immunoprecipitation of HER4 resulted in the in vitro activation of receptor kinase activity. Lysis buffer containing Mg$^{2+}$ promoted the in vitro activation of HER4, whereas, in the absence of Mg$^{2+}$, HER4 activation was ligand-dependent. The presence of Mg$^{2+}$ on HER4 kinase activity is unique to HER4 and has no effect on HER1, HER2, or HER3 (data not shown).

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**HER4 Association with PI-3-Kinase and SHC—** The signaling through growth factor receptors results in the phosphorylation of downstream targets. We set out to identify HER4-associated targets and to determine if there were differences in signaling between HER4 and that of other HER family members. In vitro and potential autokinase activities that might occur during precipitation. In the HER2 transfectants, the HER2 receptor was constitutively tyrosine-phosphorylated and unresponsive to ligand. The HER4-expressing line demonstrated an elevated level of tyrosine phosphorylation of a 180-kDa protein in the absence of ligand when compared with control 3T3–7 cells; however, phosphorylation could further be induced by exposure to heregulin β2. Immunoprecipitation of HER4 from extracts with a monoclonal antibody to HER4 (6-4-11) confirmed that HER4 and has no effect on HER1, HER2, or HER3 (data not shown).

**HER4 and Potential Autokinase Activities that Might Occur during**

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**IGF-1—**IGF-1 is a growth factor that can activate several tyrosine phosphorylation events. In 3T3-HER4 cells, IGF-1 stimulates the phosphorylation of SHC and PI-3-kinase (data not shown). The untransfected control cell line, stimulation with PDGF, but not EGF and heregulin β2, resulted in the activation of 85-kDa PI-3-kinase, reflecting the activation of the endogenous mouse PDGF receptor. There was a detectable activation of SHC by IGF-1 in the parental cell line, possibly due to low level activation of the endogenous mouse PDGF receptor.

**Identification of PI-3-Kinase and SHC Binding Sites—** HER4 contains a single putative binding site for PI-3-kinase (YTPM) at amino acid 1056 and three potential SHC binding sites (NPXY) at amino acids 1188, 1242, and 1284. To investigate whether or not these sites mediate interaction with these targets, phosphospecific-containing peptides correlating to these sequences were synthesized and used to inhibit the in vitro binding of PI-3-kinase and SHC to HER4 (Fig. 5A). GST was fused to the N- and C-terminal SH2 domains of PI-3-kinase and full-length SHC and used to precipitate HER4. Peptide 1056, which includes the PI-3-kinase binding motif, was able to inhibit the binding of both PI-3-kinase SH2 fusions to HER4 (Fig. 5B). No inhibition was seen when the unphosphorylated control peptide (1056Y) was used, nor with other peptides containing NPXY motifs. The GST-SH2 fusion was also able to associate with HER4, and this binding was inhibited 74% by peptide 1188 and 91% by peptide 1242. These data suggest that there may be two SHC binding sites on HER4 and that 1242 is the primary site. In addition, peptide 1284, which also contains an NPXY motif, did not compete. This demonstrates that sequences adjacent to NPXY must be important for SHC recognition of the receptor.

**Heterodimerization between HER4 and HER1—** It has previously been demonstrated that certain members of the HER family of receptors can heterodimerize with one another (15, 27–31). We set out to determine whether or not HER4 was able to heterodimerize with HER1. The first indication that HER1 and HER4 could functionally interact was demonstrated in a growth in soft agar assay. 3T3- and 3T3/HER4-transfected cell

**Fig. 3.** HER4 in vitro kinase activity. Antibody to HER4 immunoprecipitates of control and ligand-stimulated cell lines were assayed in an in vitro kinase reaction. 32P-Labeled receptor complexes were analyzed on SDS-PAGE and visualized by exposure to a PhosphorImager screen. The arrow points to radiolabeled receptor.
lines were plated in agar in the presence of heregulin β2 or EGF. The control 3T3 cells did not form colonies under any condition, whereas the HER4 expressing cells formed colonies in response to both heregulin β2 and EGF (Fig. 6).

If HER1 and HER4 can functionally interact and elicit a growth response, then stimulation with the appropriate ligand might result in cross-phosphorylation of one receptor by the other. Stimulation of parental 3T3 cells resulted in increased tyrosine phosphorylation of mouse EGFR in response to EGF but not heregulin (Fig. 7A). However, in cells transfected with HER4, tyrosine phosphorylation of the endogenous EGFR was elevated in the absence of ligand and further increased in response to both EGF and heregulin β2. Increasing the level of EGFR expression also increased the level of heregulin β2-induced EGFR tyrosine phosphorylation. In contrast, there was no detectable increase in HER4 phosphorylation in response to EGF (Fig. 7B). These results suggest that HER1 and HER4 can exist in a heterodimer complex and that transphosphorylation can occur in one direction, in that activation of HER4 results in the phosphorylation of HER1 but HER4 is not a substrate for HER1.

**DISCUSSION**

The activation of HER family members, as well as their respective signaling through downstream targets is different for each receptor. Further, the complexity of these signaling pathways becomes even more diverse due to receptor heterodimerization, which may alter the specificity or strength of response to a given ligand. It has been demonstrated that HER1, HER2, and HER3 can interact with one another (15, 27–31). It has also been shown that HER4 activation can result in the phosphorylation of HER2. In this paper, we describe some of the properties of HER4 that are similar and different from those of other family members. We were able to demonstrate that HER4 can also complex with HER1, resulting in an enhanced growth signal in agar in response to EGF and the transphosphorylation of EGFR by HER4 in response to heregulin β2. The endogenous level of EGFR tyrosine phosphorylation was elevated in cells co-expressing HER4, suggesting that the heterodimer may exist at low levels in the absence of ligand. Increasing the level of EGFR by transfecting HER1 resulted in a higher level of both ligand-independent and heregulin-stimulated HER1 phosphorylation, suggesting that the stoichiometry of receptor heterodimers is dependent upon receptor expression levels. We did not observe an EGF-dependent phosphorylation of HER4, which suggests that HER4 is not a substrate for HER1. Nevertheless, EGF could induce colony formation in cells expressing HER4, which suggests that a HER1-HER4 complex is being formed. It remains to be investigated whether or not HER4 and HER3 can form a complex that would complete the possible combinations of currently known HER family member heterodimerizations.

Biologically, we have shown that HER4 can stimulate 3T3 cells to grow and overcome cell-to-cell contact inhibition at a low level in the absence of exogenous ligand and that this
activity can be further induced by the addition of heregulin β2 but not EGF. Conversely, both heregulin β2 and EGF were able to induce the growth of colonies in soft agar in HER4-expressing cells but not the control NIH 3T3 cells. It is unclear why there is a difference in EGF responsiveness between the focal transformation assay and the agar assay. Perhaps the heterodimer complex is providing the necessary downstream signals needed for growth in soft agar but not the signals needed to overcome contact inhibition.

In analyzing HER4 tyrosine phosphorylation in rapidly de-natured extracts, HER4 is slightly phosphorylated in the absence of ligand and can be further induced by the addition of heregulin β2. Immunoprecipitation of HER4 in the presence of Mg²⁺ results in the in vitro stimulation of HER4 tyrosine phosphorylation and stimulation of HER4 in vitro kinase activity. This activation, in the absence of ligand, is likely a result of concentrating the receptor by immunoprecipitation and is unique to HER4, for it does not occur with HER1, HER2, and HER3. Removal of Mg²⁺ from the lysis buffer restores ligand responsiveness to our biochemical analysis. While HER1 and HER4 are both ligand-responsive, HER2 is constitutively hyperphosphorylated.

The in vitro kinase activity associated with these receptors provided a means to identify downstream targets activated by these receptors. HER4 was able to induce the phosphorylation of both SHC and 85-kDa PI 3-kinase. HER1 could activate SHC but not 85-kDa PI 3-kinase, which identifies a divergence of activities between HER1 and HER4. HER2 kinase activity and subsequent association with downstream targets could not be determined due to the lack of in vitro kinase activity associated with the receptor. HER2 is constitutively tyrosine-phosphorylated and appears to associate with downstream targets in other types of assays (43–45, 53, 54). The lack of HER2 in vitro kinase activity remains to be investigated.

The association of SH2-containing proteins with growth factor receptors is integral in transmitting growth-stimulatory signals. The elements required for the association of SH2-containing proteins to tyrosine kinases involve a phosphorylated tyrosine residue flanked by specific sequences. Specifically, YXXM (34) and NPXY (37) are essential motifs for the binding of PI 3-kinase and SHC, respectively, to their target kinases. The C-terminal domain of HER4 contains one YXXM motif (YTMP). Peptide 1056, corresponding to this motif, was able to inhibit binding of both N- and C-terminal PI 3-kinase SH2 domains. HER1 does not contain a YYXY motif in its C terminus, which correlates with the inability to phosphorylate the 85-kDa subunit of PI 3-kinase in our assay. These data confirm the essential nature of the YYXY motif in HER4 for binding PI 3-kinase and that each of the PI 3-kinase SH2 domains alone is sufficient for association with HER4.

HER4 contains three potential SHC binding sites (NPXY) within its C-terminal coding sequence located at amino acids 1188, 1242, and 1284. Full-length SHC was able to form a complex with HER4, and this interaction was inhibited by peptides 1188 and 1242. This suggests that there are two potential SHC binding sites on HER4. Recent observations have identified a second motif in the N-terminal portion of SHC, distinct from the SH2, that is able to associate with tyrrosine-phosphorylated EGFR (55) and a SHC-associated protein, p145 (56). Identification of the domain within SHC required to associate with HER4 is under investigation.

The relationship between the HER family of receptors and their ability to respond to similar ligands resulting in both homo- and heterodimerized complexes lends itself to a very complex system of cell signaling. Since multiple HER members are often expressed concomitantly, the specific combination and relative level of expression of different receptors will determine the response to a given ligand. This is evident in human tumor cell lines in which heregulin can induce either a mitogenic (21, 57) or a terminally differentiating signal (17), depending upon the cell type. Thus, altering the pattern of expression of HER family members may offer a selective growth advantage during tumor progression in the presence of HER family ligands. It has been shown that overexpression of several EGF-like growth factors, such as transforming growth factor α, amphiregulin, and cripto-1, represents a hallmark feature of many solid tumors. A better understanding of the downstream signals produced from both receptor homo- and heterodimers, as well as the ligands involved, is critical to a better understanding of the biology of HER family signal transduction and its role in tumor progression.
