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Heregulin-dependent regulation of HER2/neu oncogenic signaling by heterodimerization with HER3

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Amplification and/or overexpression of HER2/neu and HER3 genes have been implicated in the development of cancer in humans. The fact that these receptor tyrosine kinases (RTKs) are frequently coexpressed in tumor-derived cell lines and that heterodimers form high affinity binding sites for heregulin (HRG) suggests a novel mechanism for signal definition, diversification or amplification. In cells expressing HER2 and HER3, tyrosine phosphorylation of HER3 is markedly increased upon exposure to recombinant HRG. ATP binding site mutants of HER2 and HER3 demonstrate transphosphorylation of HER3 by HER2, but not vice versa. HRG-induced transphosphorylation of HER3 results in a substrate phosphorylation pattern distinct from HER2 cells and enhances association of the receptor with SHC and phosphoinositol 3-kinase in transfected 293 and mammary carcinoma-derived MCF-7 cells. The physiological relevance of HER2/HER3 heterodimerization is demonstrated by HRG-dependent transformation of NIH 3T3 cells co-expressing the two receptors. These findings demonstrate the acquisition of expanded signaling capacities for HER2 by HRG-induced heterodimerization with HER3 and provide a molecular basis for the involvement of receptor heteroactivation in the development of human malignancies.

Keywords: HER2/neu/HER3/oncogene/receptor heteroactivation/signal transduction

Introduction

The HER3 transmembrane glycoprotein exhibits close structural homology to the epidermal growth factor receptor (EGFR) related subclass of receptor tyrosine kinases (RTKs) (Plowman *et al.*, 1990). Members of this family, which includes EGFR, HER2, HER3 and HER4 (also designated erbB1–4) (Yarden and Ullrich, 1988a,b; Plowman *et al.*, 1990, 1993a; Ullrich and Schlessinger, 1990; Prigent and Lemoine, 1992) have been implicated in the development and progression of human neoplasia (Ullrich *et al.*, 1984; Libermann *et al.*, 1985; Di Marco *et al.*, 1989; Lemoine *et al.*, 1992). HER2 overexpression caused by gene amplification and/or transcriptional up-regulation occurs in a significant fraction of human breast and ovarian carcinomas, where it has been correlated with reduced patient survival (King *et al.*, 1985; Kraus *et al.*, 1987; Slamon *et al.*, 1987, 1989). Constitutive activation of the intrinsic kinase activity of HER2 either by a point mutation as in neu, the rat homolog of HER2 (Bargmann and Weinberg, 1988), or by receptor overexpression (DiFiore *et al.*, 1987; Hudziak *et al.*, 1987) appears to be a crucial step in the oncogenic process (Guy *et al.*, 1992). Similarly, HER3 has been found to exhibit elevated levels of expression and constitutive tyrosine phosphorylation in breast cancer cell lines (Kraus *et al.*, 1989; Lemoine *et al.*, 1992), implicating this EGFR family member in the pathogenesis of human malignancies.

In an effort to identify specific ligands for the members of group I RTKs, human heregulin (HRG) was isolated as a putative ligand for HER2/neu on the basis of its ability to induce tyrosine phosphorylation of this receptor in MCF-7 human breast cancer cells (Holmes *et al.*, 1992). Independently, 'neu differentiation factor' (NDF), the rat homologue of HRG, was purified and cloned from ras-transformed fibroblasts (Peles *et al.*, 1992b; Wen *et al.*, 1992) by virtue of its ability to activate HER2 in MDA-MB-453 cells and to induce differentiation of mammary tumor cells to growth-arrested, milk protein-producing cells. A comparative study by Peles *et al.* (1993) with tumor cell lines of different origins showed that the activation of HER2 by HRG is cell type-specific, indicating that other, as yet unknown cellular components might be involved in this process. As a possible explanation of these results, heterodimerization between different members of the type I RTK family was suggested (Peles *et al.*, 1993).

Such promiscuous receptor interactions between members of the EGFR-related family were first described for the EGFR and HER2 by Stern and Kamps (1988) and by Wada *et al.* (1990). Kokai *et al.* (1989) observed synergy in the transforming activities of coexpressed human EGFR and rodent neu, suggesting that this receptor crosstalk is biologically relevant. A recent report by Soltoff *et al.* (1994) presents evidence that heterodimerization and transactivation can also occur between EGFR and HER3 upon EGF stimulation of A431 cells, an epidermoid carcinoma cell line highly overexpressing EGFR. Furthermore, Sliwkowski *et al.* (1994) recently described high affinity binding of HRG by HER2/HER3 heterodimers as well as tyrosine phosphorylation of both receptors in response to HRG. Finally, HRG has been shown to be a ligand also for HER4 (Plowman *et al.*, 1993a) and receptor crosstalk has been shown to occur between HER2 and HER4 (Plowman *et al.*, 1993b). In this regard it is noteworthy that the above mentioned HRG-stimulated increase of the tyrosine phosphorylation signal in HER2 immunoprecipitates is only observed in cells in which HER2

is coexpressed with either HER3 or HER4 (Plowman *et al.*, 1993b).

We report here that activation of the signaling potential of the HER2/HER3 heterodimer occurs by unidirectional transphosphorylation of HER3 by the HER2 kinase and is regulated by the heregulin ligand. This results in apparent HER2 signal modulation, which may be physiologically relevant for cell-characteristic signal definition and is likely to play an important role in oncogenesis. Evidence for this is provided by the demonstration of enhanced signaling factor recruitment, leading to proliferation and transformation of stimulated HER2/HER3 coexpressing cells.

Results

HER2-mediated transactivation of HER3

Activation through heterodimerization is thought to play a role in the definition of signals by subclass I RTKs. So far, evidence to this effect has been provided by receptor cross-linking, ligand binding and receptor tyrosine phosphorylation experiments (Stern and Kamps, 1988; Wada *et al.*, 1990; Qian *et al.*, 1992; Sliwkowski *et al.*, 1994). To substantiate these biochemical findings and, most importantly, to demonstrate their significance in the context of a biological response, we investigated the functional relationships between the RTKs HER2 and HER3 in conjunction with the α-heregulin ligand.

The *Escherichia coli*-produced heregulin α form, representing the EGF-like region of the growth factor precursor, has been demonstrated to be biologically active (Holmes *et al.*, 1992). We therefore used a heregulin cDNA fragment isolated by polymerase chain reaction (PCR) amplification from MDA-MB231 cDNA and the pGEX vector system to construct an expression vector that upon transformation of *E.coli* yielded a 32 kDa fusion protein with amino-terminal glutathione S-transferase (GST) sequences and C-terminal α heregulin. The purified GST–α-HRG fusion protein, hereinafter designated α-HRG, was tested for its ability to induce tyrosine phosphorylation of HER2 and HER3 either during transient expression in 293 cells or in stable NIH 3T3 cell transfectants. Western blot analysis revealed little or no effect of α-HRG on tyrosine phosphorylation in 293 cells expressing HER2 or HER3 alone (Figure 1A, lanes 1–4). As observed previously, overexpressed HER2 was constitutively tyrosine phosphorylated (Figure 1A, lanes 1 and 2). In the case of HER3, low level tyrosine phosphorylation was detectable only in the presence of α-HRG (Figure 1A, lane 4). HER2 and HER3 coexpression, however, enhanced the HER3 tyrosine phosphorylation state, which could be further stimulated by addition of the α-HRG fusion protein to the culture medium (Figure 1A, lanes 7 and 8). Interestingly, the pattern of tyrosine-phosphorylated substrates coprecipitating with HER2 appeared to be different from those associated with HER3. The cell substrate phosphotyrosine signal in anti-HER2 immunoprecipitates was also significantly increased in α-HRG-stimulated cells coexpressing HER2 and HER3 (Figure 1A, lane 6 versus 5). The fact that in cells coexpressing both receptors tyrosine phosphorylation of HER2 and HER3 occurred even in the absence of ligand (Figure 1A, lanes 5 and 7) suggests that constitutive heterodimerization and activation

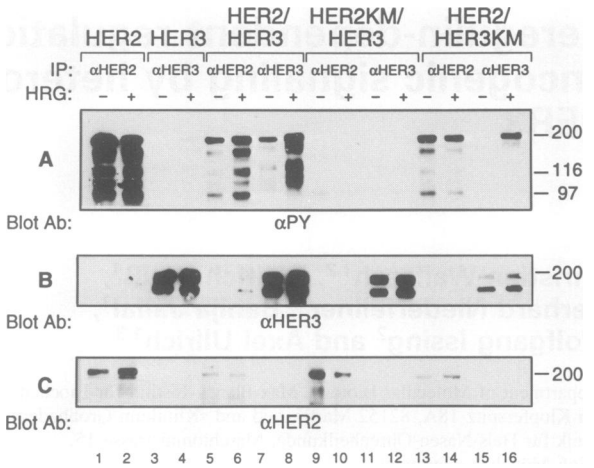

Fig. 1. α-HRG-activated HER2-mediated cross phosphorylation of HER3 is unidirectional. (**A**) 293 cells transiently expressing indicated receptor combinations were lysed after incubation with or without recombinant α-HRG (6 μg/ml) for 5 min at 37°C. Anti-HER2 and anti-HER3 immune complexes were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. Upon incubation with anti-phosphotyrosine antibodies, immunoprecipitates were detected using the ECL system (Amersham). After stripping as described in Materials and methods, receptor expression was analyzed by reprobing the same membrane with HER3- (**B**) and HER2-specific antibodies (**C**).

of HER2 and HER3 occurs to some extent, and can be further enhanced by α-HRG stimulation. This interpretation is supported by detection of small amounts of HER3 in HER2 precipitates of unstimulated cells expressing both receptors and an increase in the amount of HER3 coprecipitating with HER2 upon α-HRG stimulation (Figure 1B, lanes 5 and 6). When a kinase-inactive form of HER2 (HER2KM) carrying a mutation of a critical lysine within the ATP-binding site was coexpressed with HER3, both receptors were not tyrosine phosphorylated, even in the presence of α-HRG (Figure 1A, lanes 9–12). In the reverse experiment, however, in which HER2 was coexpressed with the kinase-inactive HER3KM receptor mutant, HER2/HER3 transphosphorylation was stimulated by α-HRG (Figure 1A, lane 16), while both receptor and substrate phosphorylation was inhibited in anti-HER2 immunoprecipitates (Figure 1A, lane 14 versus 13). This indicated that HER3 was a substrate of the HER2 kinase and may be transactivated by α-HRG-induced heterodimerization. Interestingly, in this process α-HRG appears to regulate both the HER2 kinase and the tyrosine phosphorylation state of HER3.

Receptor heteroactivation in mammary carcinoma cell lines

To substantiate further the physiological and/or pathological relevance of our findings, we investigated α-HRG-induced receptor tyrosine kinase phosphorylation in human carcinoma cell lines that endogenously express HER2 and/or HER3 to different extents. Peles *et al.* (1993a) had demonstrated that in MCF-7 mammary adenocarcinoma cells, NDF, the rat homologue of HRG, stimulated tyrosine phosphorylation of endogenous HER2, whereas this was not the case in HER2-overexpressing SKOV-3 human ovarian adenocarcinoma cells. Our experiments shown in

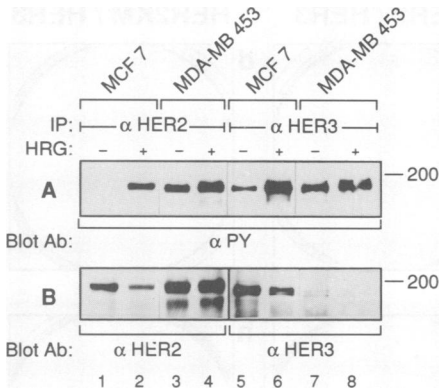

Fig. 2. α-HRG-induced tyrosine phosphorylation of HER2 and HER3 in MCF7 and MDA-MB-453 mammary carcinoma cells. (**A**) Cells were lysed after incubation with or without recombinant α-HRG (6 μg/ml) for 5 min at 37°C. Anti-HER2 and anti-HER3 immunoprecipitates were separated on 7.5% SDS–polyacrylamide gels and after transfer to nitrocellulose membranes probed with anti-phosphotyrosine antibodies. Immune complexes were detected with horseradish–peroxidase conjugated goat anti-rabbit antiserum (Bio-Rad) followed by ECL (Amersham). (**B**) Immunoblot analysis of the same membrane with HER2- or HER3-specific antibodies.

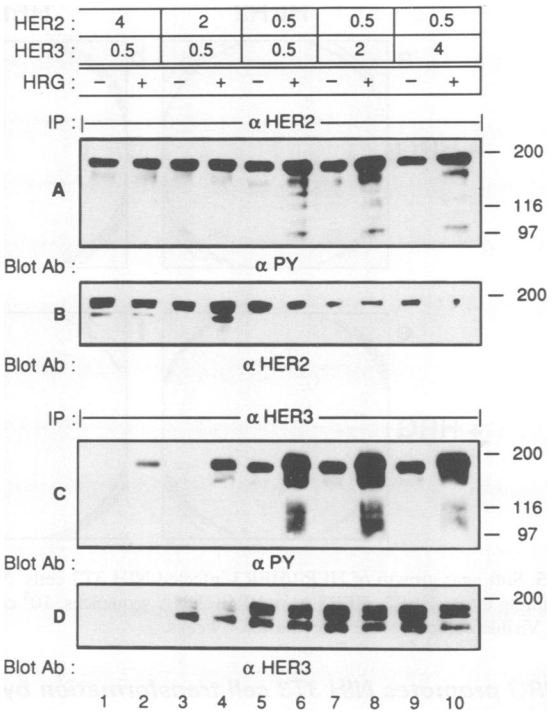

Fig. 3. Effects of quantitative parameters on HER2/HER3 activation. 293 cells were transfected with indicated amounts (μg) of HER2 and HER3 expression plasmids. Upon α-HRG stimulation (6 μg/ml, 5 min, 37°C) cells were lysed and anti-HER2 and anti-HER3 immunoprecipitates were separated on 7.5 % SDS–polyacrylamide gels and transferred to nitrocellulose membranes. After incubation with either anti-phosphotyrosine antibodies (**A** and **C**) or anti-HER2 (**B**)/anti-HER3 (**D**) antibodies, immune complexes were detected using the ECL system.

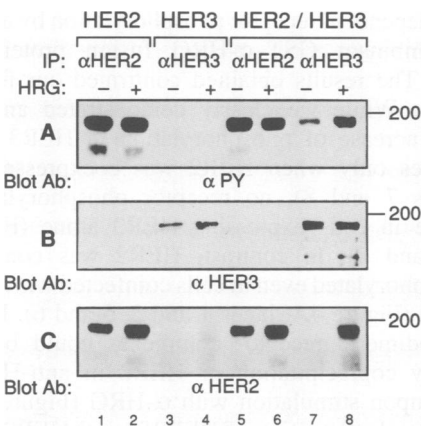

Fig. 4. α-HRG stimulates transphosphorylation of HER3 in HER2/HER3-expressing NIH 3T3 cells. (**A**) NIH 3T3 cells, infected with retroviruses carrying the cDNAs for HER2 or HER3 or coinfected with both, were lysed upon incubation with or without recombinant α-HRG (6 μg/ml, 5 min at 37°C). Anti-HER2 and anti-HER3 immune complexes were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose. Upon incubation with anti-phosphotyrosine antibodies, immunoprecipitates were detected using the ECL system (Amersham). Receptor expression was analyzed by reprobing the same membrane with HER3- (**B**) or HER2- (**C**) specific antibodies.

Figure 2 extend previous observations (Peles *et al.*, 1993) and provide information regarding the mechanistic basis of these phenomena. In MCF-7 cells, high levels of HER3 and low levels of HER2 expression both correlated with an α-HRG-inducible phosphotyrosine (PY) signal (Figure 2B and A, lanes 1 and 2, and 5 and 6). In contrast, MDA-MB453 breast carcinoma cells, which overexpress HER2 and contain low HER3 levels (Figure 2B, lanes 3 and 4, and 7 and 8), even after extensive starvation exhibited constitutive, yet still α-HRG-inducible phosphorylation of both receptors (Figure 2A, lanes 4 and 8). Moreover, the specific PY levels were found to be significantly higher for HER3 (Figure 2A, lanes 7 and 8, and Figure 2B, lanes 7 and 8). Since these cells have been reported also to express HER4, the observed α-HRG-dependent increase in tyrosine phosphorylation of HER2 and HER3 may include effects resulting from additional heterointeractions with this RTK (Plowman *et al.*, 1993b).

To examine the effects of variable quantitative parameters on α-HRG-induced transactivation, we transiently transfected HER2 and HER3 cDNA expression vectors into 293 cells at ratios of 8:1, 4:1, 1:1, 1:4 and 1:8 (w/w) and determined the consequences on ligand regulation of receptor phosphorylation. As shown in Figure 3, phosphorylation of HER3 was α-HRG inducible at all relative expression levels (Figure 3C, lanes 2, 4, 6, 8 and 10). Interestingly, as long as HER2 was expressed in excess, basal phosphorylation of HER3 was absent or low (Figure 3C, lanes 1 and 3), while with increasing HER3 expression, ligand-independent tyrosine phosphorylation was observed in anti-HER3 immunoprecipitates, which could be stimulated three to five times by addition of α-HRG to the culture medium (Figure 3C, lanes 5, 7 and 9). At a 1:1 transfection ratio, where α-HRG-stimulated HER3 tyrosine phosphorylation leveled off at a maximum level, the ligand-induced phosphotyrosine signal in anti-HER2 immunoprecipitates increased (Figure 3A, lanes 5 and 6). These results suggested that receptor transactivation between HER2 and HER3 is regulated by α-HRG and modulated by relative expression levels of the two receptors, possibly due to differential formation of homo- or heterodimeric receptor complexes.

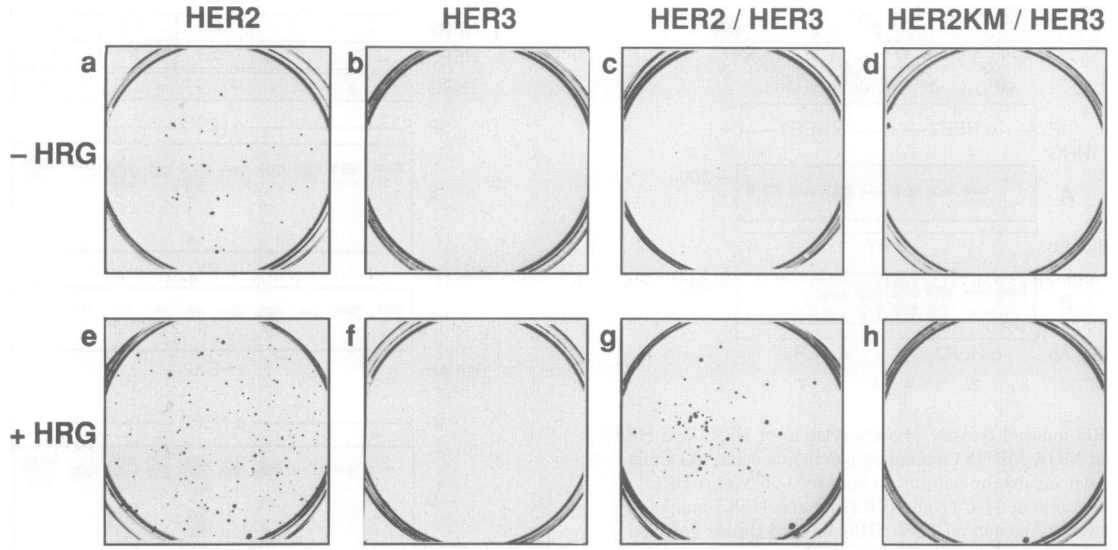

Fig. 5. Soft agar growth of HER2/HER3-infected NIH 3T3 cells. NIH 3T3 cells were infected/coinfected as indicated with recombinant retrovirus containing either HER2, HER3 or α-HRG cDNA sequences. 10^4 cells were seeded in 6 cm dishes on 0.4% bottom agar and covered with 0.2% top agar. Visible colonies were scored after 4 weeks.

α-HRG promotes NIH 3T3 cell transformation by HER2/HER3 transactivation

The biological relevance of our observations was further emphasized by investigations of the transforming capacities of HER2 and HER3 in NIH 3T3 cells in the absence or presence of α-HRG. NIH 3T3 cells at comparable multiplicities of infection (m.o.i.s) were exposed to retroviruses conferring expression of the HER2 kinase-negative HER2KM or HER3. Cells expressing HER2 or HER3 alone, as well as cells coexpressing HER2 and HER3 or HER2KM and HER3, were first analyzed for α-HRG-dependent receptor phosphorylation by addition of the recombinant GST–α-HRG fusion protein to the medium. The results obtained confirmed our findings in 293 cells. While we clearly demonstrated an α-HRG-induced increase of phosphorylation in HER3 immunoprecipitates only when HER2 was coexpressed (Figure 4A, lanes 7 and 8), no receptor phosphorylation was detectable in cells expressing HER3 alone (Figure 4A, lanes 3 and 4). In contrast, HER2 was constitutively autophosphorylated even in cells coinfected with the HER3 retrovirus (Figure 4A, lanes 1 and 2, 5 and 6). Formation of heterodimeric receptor complexes could be demonstrated by coprecipitation of HER2 in anti-HER3 precipitates upon stimulation with α-HRG (Figure 4C, lane 8). Conversely, detection of HER3 in anti-HER2 immunoprecipitates was not possible due to interference of mAb 2C4 with HER2/HER3 heterodimerization, as reported earlier (Sliwkowski *et al.*, 1994).

As shown in Figure 5, transformation of HER2/HER3 cells, as determined by anchorage-independent growth, was dependent on superinfection with an α-HRG retrovirus (Figure 5c and g). As previously reported (Di Fiore *et al.*, 1987; Hudziak *et al.*, 1987), infection of NIH 3T3 cells with HER2 virus alone induced transformation, as determined by colony formation in semisolid media. Surprisingly, this response was somewhat enhanced by superinfection with α-HRG virus (Figure 5a and e), possibly due to the involvement of another endogenous NIH 3T3 component such as HER3 or HER4. In contrast, infection with HER3 virus had no effect with or without α-HRG coexpression. On the other hand, coexpression with HER3 after double infection suppressed the HER2 transformation that was achieved with the same m.o.i. of virus as in Figure 5a and rendered this activity α-HRG dependent (Figure 5g). When instead of wild-type (wt) HER2 a kinase-inactive HER2 mutant was coexpressed with HER3, superinfection with α-HRG virus did not induce cell transformation (Figure 5, h versus d), supporting our model of a unidirectional mechanism for α-HRG-induced receptor transactivation.

Signaling potential of HER2/HER3 heterodimers

To investigate the signaling capacity of activated HER2/HER3 heterodimers, we compared substrate binding patterns of stimulated or unstimulated receptors that were precipitated from 293 cells coexpressing HER2 and HER3 or either of the receptors alone. For this approach we initially expressed the signal-transducing proteins PLCγ, GAP, SHC, GRB2 and the non-catalytic subunit of PI3-kinase individually in 293 cells and, after metabolic labeling with [^{35}S]methionine, lysates of these cells were used in *in vitro* association experiments with receptor immunoprecipitates from unlabeled, α-HRG-stimulated or unstimulated cells (Herbst *et al.*, 1992). This experiment indicated stimulation of SHC and p85 binding to HER3 immunoprecipitates from α-HRG-stimulated cells coexpressing HER2 (data not shown). In confirmation of this initial observation, we detected phosphorylated SHC in anti-HER3 precipitates from HER2/HER3-transfected 293 cells (Figure 6A, lanes 11 and 12; Figure 6B, lanes 11 and 12). Moreover, anti-SHC immunoprecipitates of the same cell lysates contained a phosphorylated 180 kDa band, representing comigrating HER3 and HER2 (Figure 6C, lanes 7 and 8; Figure 6D, lanes 7 and 8). Our failure to detect any endogenous SHC protein coprecipitating with HER2 from HER2-expressing 293 cells (Figure 6B, lanes 1 and 2) suggested that this signal-transducing adaptor protein preferentially associated with HER2/HER3 heterodimers.

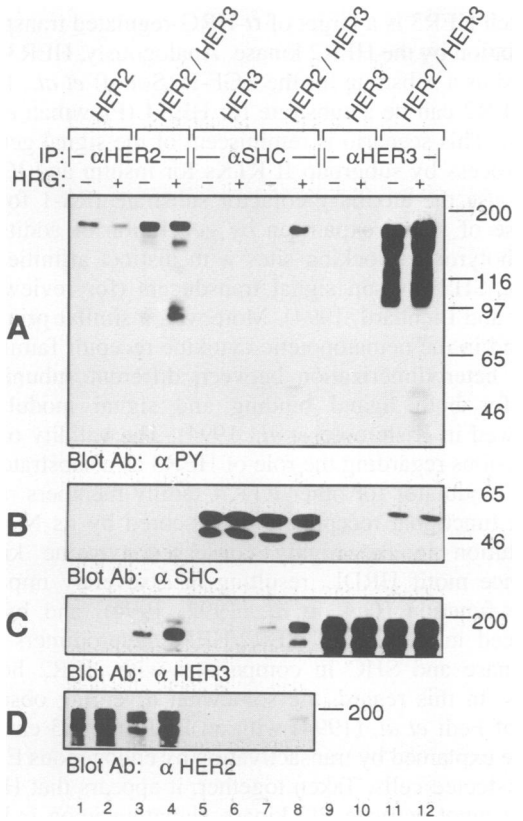

Fig. 6. Association of SHC with HER3 upon α-HRG stimulation in HER2/HER3-transfected 293 cells. (**A**) 293 cells transiently expressing either HER2 or HER3 alone, or coexpressing both receptors were incubated with or without recombinant α-HRG (6 μg/ml) for 5 min at 37°C. Cell lysates were divided and immunoprecipitation was performed on one half of the lysate with anti-receptor antibodies (αHER2, αHER3), on the other half with anti-SHC (αSHC) antibodies. Immune complexes were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose membranes. After incubation with anti-phosphotyrosine antibodies, immunoprecipitates were detected using the ECL system (Amersham). Expression of receptors and of SHC as well as coprecipitation of these proteins with each other was analyzed by reprobing the same membrane first with anti-SHC antiserum (**B**) and then with anti-receptor antibodies [anti-HER3 (**C**), anti-HER2 (**D**)].

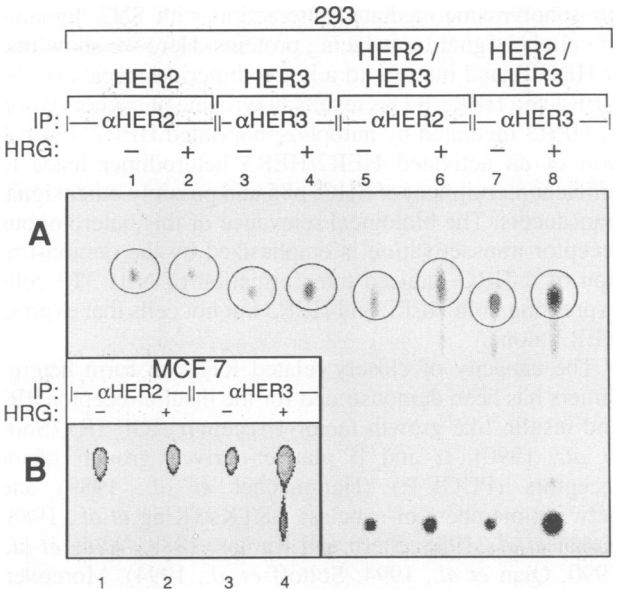

Fig. 7. α-HRG stimulation-dependent HER3/PI3-kinase association. (**A**) 293 cells transiently expressing either HER2 or HER3 alone, or coexpressing both receptors were incubated with or without recombinant α-HRG (6 μg/ml) for 5 min at 37°C. Anti-HER2 and anti-HER3 immune complexes from lysates of these cells were used in a PI-kinase assay, as described in Materials and methods. Chromatography plates were exposed to imaging plates and quantitated by phosphoimage analysis (Fuji, Ray-test). (**B**) A similar PI3-kinase assay was performed with anti-HER2 and anti-HER3 immune-complexes from MCF 7 cells, which had been incubated with or without α-HRG (6 μg/ml) for 5 min at 37°C.

In similar experiments we analyzed PI3-kinase involvement in HER2/HER3 signal transduction. PI-kinase assays were performed with receptor immunoprecipitates from α-HRG-stimulated 293 cells transfected with HER2, HER3 or HER2/HER3 (Figure 7A). In addition, enzyme activity was determined in receptor immunoprecipitates from MCF-7 cells (Figure 7B). From lysates of 293 cells expressing HER2 alone, little PI-kinase activity coprecipitated with HER2 and this activity was not enhanced by α-HRG stimulation (Figure 7A, lanes 1 and 2). Incubation of control samples with 10^{-7} M of the PI3-kinase inhibitor, Wortmannin, during immunoprecipitation did not decrease the HER2-coprecipitating activity, suggesting that the detected PI kinase activity did not result from PI3-kinase, but probably PI4-kinase (data not shown). The low PI-kinase activity coprecipitating with HER3 from unstimulated cells also could not be significantly increased upon α-HRG stimulation (Figure 7A, lanes 3 and 4). HER2 immunoprecipitates from HER2/HER3-cotransfected 293 cells revealed a similar amount of coprecipitating PI-kinase activity to those from cells expressing HER2 alone (Figure 7A, compare lanes 5 and 1), which was increased ~2-fold upon α-HRG stimulation. This minor increase (Figure 7A, compare lanes 5 and 6), however, was not inhibited by Wortmannin. In contrast, PI-kinase activity in anti-HER3 immunoprecipitates from lysates of the same HER2/HER3 cells was already higher in the absence of α-HRG than the activity coprecipitating from α-HRG-stimulated cells expressing HER3 alone (Figure 7A, lane 7 versus 3). Addition of α-HRG further enhanced the activity ~2.5-fold (Figure 7A, lane 8 versus 7). In these samples ~90% of the coprecipitating enzyme activity was inhibited by 10^{-7} M Wortmannin, indicating that it actually represented PI3-kinase activity (data not shown). Taken together, these data suggested that HER2 by itself did not efficiently activate PI3-kinase, but, when coexpressed with HER3, formation of HER2/HER3 heterodimers and transphosphorylation of HER3 by HER2 led to efficient recruitment of PI3-kinase.

Similarly, in MCF-7 mammary carcinoma cells, HER2-associated PI-kinase activity was constitutively high (Figure 7B, lanes 1 and 2). In HER3 immunoprecipitates, the ligand caused a 2- to 3-fold enhancement of receptor-associated enzyme activity (Figure 7B, lanes 3 and 4), indicating an important role for PI3-kinase in HER2/HER3-mediated signal transduction in this cell line.

Discussion

Selective formation of homodimers between specific ligand-bound receptor tyrosine kinases is a mechanism which is thought to activate their kinase function, followed by intermolecular transphosphorylation and subsequent

phosphotyrosine-mediated interaction with SH2 domains of cellular signal-transducing proteins. Here we show that α-HRG ligand binding to a heterodimeric complex of the HER2 and HER3 RTKs results in tyrosine phosphorylation of HER3 mediated by autophosphorylated HER2. Formation of an activated HER2/HER3 heterodimer leads to efficient recruitment of SHC, p85 and possibly other signal transducers. The biological relevance of this heterologous receptor transactivation is emphasized by the demonstration of α-HRG-induced transformation of NIH 3T3 cells expressing both HER2 and HER3 but not cells that express HER3 alone.

The capacity of closely related RTKs to form heterodimers has been demonstrated for the insulin receptor (IR) and insulin-like growth factor-1 receptor (IGF-1R) (Soos *et al.*, 1990), α and β platelet-derived growth factor receptors (PDGF-R) (Hammacher *et al.*, 1989) and between members of subclass I RTKs (King *et al.*, 1988; Kokai *et al.*, 1988; Stern and Kamps, 1988; Wada *et al.*, 1990; Qian *et al.*, 1994; Soltoff *et al.*, 1994). Moreover, Lammers *et al.* (1990) even observed transphosphorylation between the EGFR and IR kinases in an artificial system involving receptor chimeras. So far, however, the question of whether these biochemical phenomena represent a natural mechanism for the generation of expanded signaling diversity has not been answered satisfactorily. For the EGF-R subfamily of RTKs, the significance of heterodimerization has been brought into focus because of the unexpected and initially controversial observations regarding the biological activity of a novel EGF-related growth factor, 'heregulin' or 'neu differentiation factor', which had been isolated as a ligand for HER2/neu using tumor-derived cell lines as reporter systems (Holmes *et al.*, 1992; Peles *et al.*, 1992a). Surprisingly, however, this putative ligand did not bind to cells that only overexpressed HER2, yet it was able to bind to and activate HER2 in mammary carcinoma cells, indicating involvement of an additional component. Recently a number of studies have begun to shed light on the multiple binding activities of HRG (Plowman *et al.*, 1993b; Carraway *et al.*, 1994; Sliwkowski *et al.*, 1994). Ligand-binding experiments and biochemical analysis have demonstrated that HRG binds weakly to HER3 and HER4 but with high affinity to HER2/HER3 heterodimers (Plowman *et al.*, 1993b; Sliwkowski *et al.*, 1994). In the case of HER4, transphosphorylation of and/or heterodimerization with HER2 has been suggested (Plowman *et al.*, 1993b). These previous observations and our findings suggest promiscuous receptor interactions as a mechanism of activation and signal diversification for the subfamily of EGFR-related RTKs and possibly other RTK subtypes.

Evidence supporting this hypothesis was derived from quantity-controlled transfection experiments that yielded cells transiently coexpressing different relative amounts of HER2 and HER3 (Figure 3). Interestingly, phosphorylation of HER3 could be ligand-stimulated at all HER2/HER3 expression ratios, while HER2 was constitutively activated when expressed in excess over HER3 but appeared to become regulated to some extent by α-HRG at approximately equimolar expression levels. These observations in conjunction with results of coexpression experiments involving HER2, HER3 and the ATP-binding site mutants HER3KM and HER2KM, support the concept in which HER3 is a target of α-HRG-regulated transphosphorylation by the HER2 kinase. Analogously, HER3 may be used as a substrate for the EGF-R (Soltoff *et al.*, 1994) and HER2 can be a substrate for HER4 (Plowman *et al.*, 1993b). This scenario is reminiscent of the signal generation process by subgroup II RTKs for insulin and IGF-1, which use the auxiliary cofactor substrate IRS-1 for the purpose of signal expansion by generation of additional phosphotyrosine docking sites with distinct affinities for diverse SH2 domain signal transducers (for review see Keller and Lienhard, 1994). Moreover, a similar principle is found in the hematopoietic cytokine receptor family, in which heterodimerization between different subunits is used for both ligand binding and signal modulation (reviewed in Kishimoto *et al.*, 1994). The validity of our conclusions regarding the role of HER3 as a substrate and signal modulator for other RTK I family members rather than a functional receptor is underscored by its N for E substitution in the highly conserved tyrosine kinase sequence motif HRDL, resulting in a severely impaired kinase function (Guy *et al.*, 1992, 1994), and by the enhanced interaction of HER2/HER3 heterodimers with PI3-kinase and SHC in comparison with HER2 homodimers. In this regard, the somewhat diverging observations of Fedi *et al.* (1994) with an EGFR/HER3 chimera may be explained by transactivation by endogenous EGFR in transfected cells. Taken together, it appears that HER3 alone cannot activate PI3-kinase signaling upon α-HRG stimulation, and only very poorly the SHC–GRB2 pathway. On the other hand, HER2 by itself couples to some extent to PI3-kinase and to the SHC–GRB2 pathway (Peles *et al.*, 1992b; Segatto *et al.*, 1993). A dramatic increase of PI-kinase activation and of SHC association and tyrosine phosphorylation, however, is observed in α-HRG-stimulated cells coexpressing HER2 and HER3.

The pathophysiological relevance of these heteromolecular interactions is suggested by the fact that many cancer cell lines coexpress members of this RTK family to different extents and in various combinations (Kraus *et al.*, 1993; Plowman *et al.*, 1993a). As an extension of our experiments with transfected cell lines, we therefore investigated receptor transactivation in the mammary carcinoma cell lines MCF-7 and MDA-MB453, which express HER2 and HER3 in different relative amounts. While in MCF-7 cells expressing high levels of HER3 and little HER2, α-HRG regulates HER2 as well as HER3 activity, this ligand has little effect on the same receptors in MDA-MB453 cells, which exhibit an excess of HER2 over HER3. In these cells basal tyrosine phosphorylation of both receptors was already high, which, consistent with our 293 transfection experiments, is likely to be caused by constitutive autophosphorylation of HER2 homodimers and transphosphorylation of HER3. Most importantly, the significance of a pathophysiological function of HER3 as a signal modifying and enhancing substrate of other subclass I RTKs is demonstrated by the dependence of α-HRG-stimulated NIH 3T3 cell transformation on coexpression of active HER2 and HER3, while a kinase-defective HER2 mutant abolished this effect. Oncogenic signal transduction by subclass I RTKs may therefore involve either autocrine or paracrine activation of overexpressed EGFR homodimers, ligand-independent HER2 homodimerization or ligand-induced heterodimerization

between kinase-competent EGFR and HER2 and the signaling 'cofactor' HER3. One may assume that the cancer cells resulting from these oncogenic events differ in their pathophysiological properties and clinical manifestations, but no data supporting this prediction are currently available.

In summary, the diversity of possible interactions between different members of the EGFR-related family and the existence of multiple possible ligands with different affinities for different homo- and heterodimeric receptor complexes reveal an enormous potential for the regulation of complex cellular responses. Disruption of the finely tuned molecular interactions underlying this mechanism, however, may have severe pathological consequences, as suggested by the frequent involvement of subclass I RTKs in cancer.

Materials and methods

Construction and expression of human HRG–GST fusion proteins

Total RNA was isolated from the human melanoma cell line MDA-MB 231 with the Roti® Quick-Kit for purification of RNA from cells and tissue (Roth) according to the manufacturer's protocol. 1 μg of RNA was primed with specific antisense oligonucleotide HRG 244 (5′-GGAATTCCAGCACTCTCTTCTGGTA-3′) and reverse transcribed to generate first strand cDNA with a cDNA synthesis kit (GIBCO BRL). 2 μl of this reaction mixture were used for PCR amplification with the primers HRG 244 and HRG 177 (5′-ATGGATCCCCAGCCATC-TTGTAAAATGT-3′). The underlined sequences add an *Eco*RI or a *Bam*HI restriction site to the 5′ ends of the primers. PCRs were performed in a final volume of 50 μl with Perkin Elmer-Cetus GeneAmp PCR kits. Twenty-five cycles in a Biotron thermocycler amplified the desired product, which included the EGF-like domain of the HRG precursor, shown previously to be active in the form of a fusion protein (Holmes *et al.*, 1992). Each cycle included 30 s at 94°C, 30 s at 54°C and 30 s at 72°C. 10 μl of each reaction mixture were analyzed by electrophoresis in 1.5% agarose gels followed by ethidium bromide staining. DNA molecular weight standards were purchased from Boehringer Mannheim. PCR products were subcloned after *Eco*RI and *Bam*HI digestion and gel elution into the pGEX 3X expression vector (Pharmacia) and transformed into *E.coli* (F′298) according to standard protocols. Plasmid DNA from transformed colonies was analyzed by DNA sequencing.

Recombinant human GST–HRG fusion proteins (designated HRG below) were produced in *E.coli* and purified according to the purification protocol for pGEX fusion proteins (Pharmacia). The purified recombinant proteins were dialyzed overnight against PBS and 10% glycerol.

Generation of recombinant retroviruses

The retroviral expression vector pLXSN has been described previously (Miller and Rosman, 1989). The vector pLEN was derived from pLAEN (Adam *et al.*, 1991) by removing the adenosine deaminase (ADA) sequences (T.von Rüden, personal communication). The *Eco*RI site of pLEN was used to generate pLEN SL, in which a PCR fragment consisting of the multiple cloning site of pSL1190 (Pharmacia) was inserted. Using standard methods, pLEN SL HER3 and pLEN SL HER3KM were generated by cloning a *Xba*I–*Xho*I fragment from pBS II SK carrying the cDNAs of wt HER3 and mutated HER3, respectively, into pLEN SL. The *Xho*I site of pLXSN was used to insert an *Xho*I fragment containing the entire HER2 coding sequence from pBS SK HER2. A mutated form of the receptor cDNA, HER2 K753M (HER2KM) was subcloned in the same way. Stable GP+E 86 producer lines were generated using a modified infection protocol (Miller and Buttimore, 1986). Low titer amphotrophic virus, which was generated by transient transfection of retrovirus expression plasmids into the helper virus free packaging cell line PA317 (Miller *et al.*, 1985), was used to infect GP+E 86 secondary packaging cells, followed by selection of GP+E 86 producer line clones in G418 (1 mg/ml). The virus titer was determined by infecting NIH 3T3 cells with serial dilutions of retrovirus containing cell-free GP+E 86 supernatants and determining the number of G418-resistant colonies. Titers were ~1 x 10^7 c.f.u./ml for HRG, wt HER3 and mutant receptor virus supernatant.

Retrovirus-mediated gene transfer

Subconfluent NIH 3T3 cell (10^5 cells per 6 cm dish) were incubated with supernatants of GP+E 86 cells releasing high titer pLXSN–HER2, pLEN–HER3, pLXSN–HER2KM and pLEN–HER3KM virus (>1×10^6 G418 c.f.u./ml; m.o.i. of 10) for 4–12 h in the presence of Polybrene (4 mg/ml; Aldrich). Double infections (HER2/HER3, HER2KM/HER3, HER2/HER3KM) were achieved by repeatedly applying the same regimen with the corresponding supernatants. The level of receptor expression was raised by multiple rounds of infection (Bordignon *et al.*, 1989).

Transient overexpression of HER2, HER2KM, HER3 and HER3KM proteins in eukaryotic cells

The 293 cell system (ATCC CRL 1573) was used for transient expression of different receptors. 293 cells were maintained in DMEM supplemented with 10% FCS, penicillin and streptomycin (100 IU/ml) at 5% CO_2 and 37°C. Transfections were carried out using a modified calcium phosphate method (Chen and Okayama, 1987). 8×10^5 cells/6 cm dish were incubated overnight in 4 ml of growth medium. 9 μg of supercoiled DNA was mixed with 0.25 M $CaCl_2$ in a final volume of 150 ml. The mixture was combined with the same volume of 2× transfection buffer (50 mM BES, pH 6.95, 280 mM NaCl, 1.5 mM Na_2HPO_4) and incubated for 10 min at room temperature before it was added dropwise to the cells. After incubation for 16 h at 37°C under 3% CO_2, the medium was removed, cells were washed twice with PBS and were then starved for 24 h in DMEM supplemented with 0.1% FCS.

Receptor phosphorylation analysis

Transfected 293 cells or infected NIH 3T3 cells were stimulated with either 6 μg/ml recombinant human α-HRG or, as a control, with GST for 5 min at 37°C. Following HRG stimulation, the cells were lysed on ice with 0.4 ml lysis buffer [50 mM HEPES pH 7.5, containing 150 mM NaCl, 1 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 10 mg/ml aprotinin]. Crude lysates were transferred to microfuge tubes and centrifuged at 12 500 *g* for 20 min at 4°C.

For immunoprecipitations, the appropriate antiserum and 30 μl of protein A–Sepharose (Pharmacia) were added to the cleared lysate and incubated for 3 h at 4°C. Immunoprecipitates were washed three times with 1 ml washing buffer [20 mM HEPES pH 7.5, containing 150 mM NaCl, 10% (v/v) glycerol, 1% (v/v) Triton X-100]. Sample buffer containing SDS and 2-mercaptoethanol was added and the samples denatured by heating at 95°C for 5 min.

Immunoblotting

Proteins were fractionated by 7.5% SDS–PAGE and electrophoretically transferred to nitrocellulose filters. For immunoblot analysis, nitrocellulose filters were first incubated with mouse monoclonal or rabbit polyclonal primary antibodies for 3 h at 4°C, then as secondary antibody a peroxidase-coupled goat anti-mouse or goat anti-rabbit antibody (Bio-Rad) was added, followed by an enhanced chemoluminescence (ECL) substrate (Amersham) reaction. The substrate reaction was detected on Kodak X-Omat film. Filters, which were used more than once with different antibodies, were stripped according to the manufacturer's protocol, blocked and reprobed.

Phosphatidylinositol 3-kinase assay

PI3-kinase assays were performed as described previously (Morgan *et al.*, 1990) with some modifications. Briefly, HER2 and HER3 protein was precipitated from α-HRG-treated or untreated cells as described above. The immunoprecipitates were washed four times with 1 ml of the following buffer: 50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5 mM EGTA, 0.2 mM Na-ortho VO_4, 10% glycerol. After the last wash, 40 μl aliquots of a master mix consisting of 10 μl 5× buffer (100 mM Tris–Cl, pH 7.5, 500 mM NaCl, 25 mM MgAcetate, 25 mM $MnCl_2$, 2.5 mM EGTA), 10 μl phosphatidylinositol + phosphatidylserine (0.5 mg/ml each in 20 mM HEPES, pH 7.5), 5 μl 0.2 mM ATP, 5 μl [γ-^{32}P]ATP solution (@ 10 μCi per sample) and 5 μl H_2O were added to the immunoprecipitates and the kinase reaction was stopped after incubation at 30°C for 30 min by the addition of 100 μl of 1 M HCl and 100 μl of methanol-chloroform [1:1 (v/v) mixture]. The lipid-containing organic phase was resolved on oxalate-coated, thin-layer chromatography plates (Silica Gel 60; Merck) developed in chloroform:methanol:water:glacial acetic acid:acetone (40:13:7:12:15 v/v). The plates were then dried and exposed for autoradiography and/or phosphoimager (Fuji) analysis.

Soft agar assay

To examine the ability of NIH 3T3 cells to form colonies in soft agar, subconfluent NIH 3T3 cells (10^5 cells per 6 cm dish) were infected with pLXSN–HER2KM, pLXSN–HER2 or pLEN–HER3 virus. Stable HER2KM-expressing NIH 3T3 cells were infected with pLEN HER3 virus, and stable HER3-expressing NIH 3T3 cells were infected with pLXSN HER2 virus. In cases in which an autocrine stimulus was to be created, cells were superinfected with pLEN α-HRG virus (m.o.i. of 5). Cells were plated in 6 cm dishes in a top layer of 3 ml of DMEM containing 10% FCS and 0.2% agar (GIBCO). The bottom layer contained DMEM, 10% FCS and 0.4% agar. Visible colonies were scored after 4 weeks.

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