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HER2 cytoplasmic domain generates normal mitogenic and transforming signals in a chimeric receptor

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We have investigated the biological function of an unidentified human growth factor, the ligand of the putative HER2 receptor, by characterizing the signalling properties of its receptor. HER2 (or c-*erb*B-2), the human homolog of the rat *neu* proto-oncogene, encodes a transmembrane glycoprotein of the tyrosine kinase family that appears to play an important role in human breast carcinoma. Since a potential ligand for HER2 has not yet been identified, it has been difficult to analyze the biochemical properties and biological function of this cell surface protein. For this reason, we replaced the HER2 extracellular domain with the closely related ligand binding domain sequences of the epidermal growth factor (EGF) receptor, and examined the ligand-induced biological signalling potential of this chimeric HER1–2 protein. This HER1–2 receptor is targetted to the cell surface of transfected NIH 3T3 cells, forms high and low affinity binding sites, and generates normal mitogenic and cell transforming signals upon interaction with EGF or TGFα. The constitutive activation of wild-type HER2 in transfected NIH 3T3 cells suggests the possibility that these cells synthesize the as yet unidentified HER2 ligand and activate HER2 by an autocrine mechanism.

Key words: EGF/HER2 receptor/HER1–2 chimera/human growth factor/NIH 3T3 cells/TGFα

Introduction

The cell surface glycoprotein termed HER2 (Coussens *et al.*, 1985; Yamamoto *et al.*, 1986) is the human homolog of the rat proto-oncogene product *neu* (Bargmann *et al.*, 1986a) and is closely related to the EGF receptor in its overall structural organization and primary sequence (Coussens *et al.*, 1985). Both the HER2 gene product and the EGF receptor contain cytoplasmic tyrosine kinase domains which are connected via hydrophobic transmembrane sequences with two cysteine-rich repeat sequences in their extracellular ligand binding domains (for review see Yarden and Ullrich, 1988). While the EGF receptor is known to bind EGF, transforming growth factor alpha (TGFα), and the vaccinia virus genome encoded polypeptide p19, the ligand of the receptor-like HER2 gene product is still unknown, and its normal biological role is not yet clear. An involvement of the HER2 receptor in cellular growth control mechanisms has been suggested by the recent findings that overexpression of unaltered HER2 leads to transformation of mouse NIH 3T3 fibroblasts, and may also be linked to the genesis of a substantial portion of human mammary carcinomas (Hudziak *et al.*, 1987; Di Fiore *et al.*, 1987a; Slamon *et al.*, 1987), 30% of which contain an amplified HER2 gene.

We have constructed receptor chimerae to examine the biochemical and biological properties of HER2 before its own specific ligand has been identified. Using the related, but distinct EGF receptor (HER1) ligand binding domain fused to the transmembrane and cytoplasmic portions of HER2, we were able to test the signalling function of the HER2 cytoplasmic domain by activation with either EGF or TGFα. When the chimeric molecule HER1–2 is expressed in mouse fibroblasts, we find that the receptor is transported to the cell surface, where it forms typical high and low affinity EGF binding sites. Moreover, the high affinity state of EGF binding was abolished by treatment with phorbol ester (PMA), and EGF binding to HER1–2 results in activation of the heterologous cytoplasmic kinase domain *in vitro* and *in vivo*. In intact cells receptor activation results in a mitogenic response and under certain circumstances in transformation. These data demonstrate that HER2 has the potential to generate a normal growth-promoting as well as an abnormal transforming signal only when stimulated by its ligand. Furthermore, our findings suggest the possibility that NIH 3T3 cells produce the as yet unidentified HER2 ligand.

Results

Construction and expression of HER1–2 chimeric receptor

To examine the biochemical properties and biological signalling potential of the growth factor receptor-like cell surface glycoprotein HER2 prior to the identification of its cognate ligand, we replaced its extracellular domain with the binding domain of the human EGF receptor (HER1). The fusion was carried out using cloned cDNAs and a synthetic DNA linker that connected *Xba*I–*Xho*II and *Bsm*I–*Dra*I fragments of HER1 and HER2, respectively, and linked amino acids 621 of HER1 (Ullrich *et al.*, 1984) and 654 of HER2 (Coussens *et al.*, 1985) at the extracellular border of the HER2 transmembrane sequence (Figure 1 and Materials and methods). The hybrid cDNA was inserted into a mammalian expression vector that contained an expression module for mouse dihydrofolate reductase under SV40 early promoter control (Riedel *et al.*, 1987). This enabled us to amplify transfected DNA sequences by exerting selective pressure with increasing concentrations of methotrexate in the growth medium. Using Ca^{2+} precipitation for transfection of cells (Graham and van der Eb, 1973), neomycin (G418) selection and subsequent amplification, mouse NIH 3T3 fibroblasts stably expressing either the HER1–2 chimera or control wild-type HER1 and HER2 were generated. Immunoprecipitation from lysates of

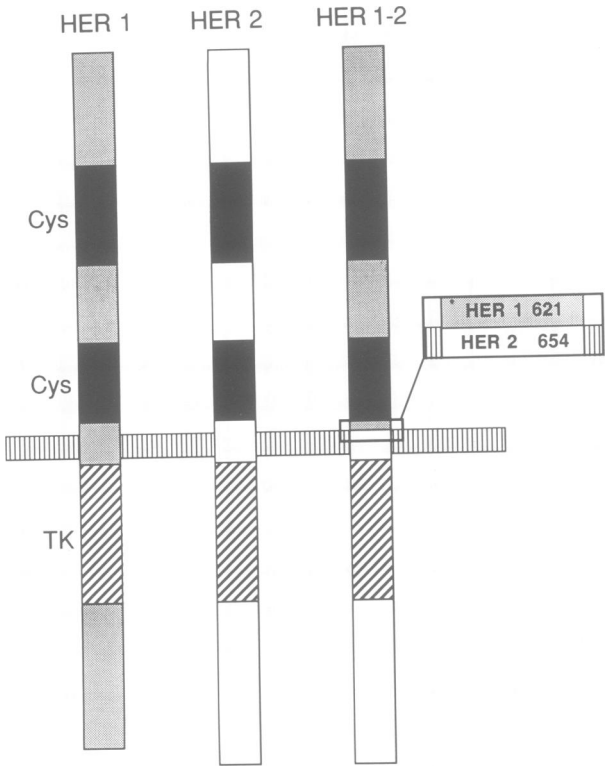

Fig. 1. Schematic receptor structures. Receptor models are shown inserted into the plasma membrane bilayer with their extracellular domains on top of their intracellular portions below. Black areas represent cysteine-rich regions; hatched areas indicate tyrosine kinase domains. HER1, Human EGF receptor. HER2, EGF-receptor-like putative receptor. HER1−2, chimeric receptor composed of extracellular HER1 sequences fused to the transmembrane and cytoplasmic HER2 sequences. Amino acid residues of HER1 and HER2 that were connected in the sequence fusion are shown in the blow-up.

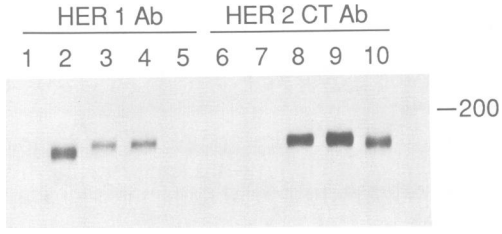

Fig. 2. Detection and characterization of chimeric and wild-type receptors. Stably expressing clonal cell lines were [^{35}S]methionine-labeled overnight, lysed and after adjustment for similar receptor amounts in each sample immunoprecipitated as described in Materials and methods. Receptors were immunoprecipitated with either human EGF receptor extracellular domain-specific RI antibody (HER1Ab, **lanes 1−5**) or C-terminal peptide specific HER2 antiserum (HER2 CT Ab, **lanes 6−10**). **Lanes 1** and **6**: control NIH 3T3 cells transfected with expression vector only (3TCVN). **Lanes 2** and **7**: NIH 3T3 cells transfected with HER1 expression vector (3TH1/300). **Lanes 3** and **8**: RatI cells (line #6) transfected with HER1−2 receptor sequences (CVH12R/6). **Lanes 4** and **9**: NIH 3T3 cells transfected with HER1−2 expression construct (3TH12/1600). **Lanes 5** and **10**: NIH 3T3 cells transfected with HER2 receptor expression construct (3TH2). Mol. wt markers are indicated in kilodaltons (kd).

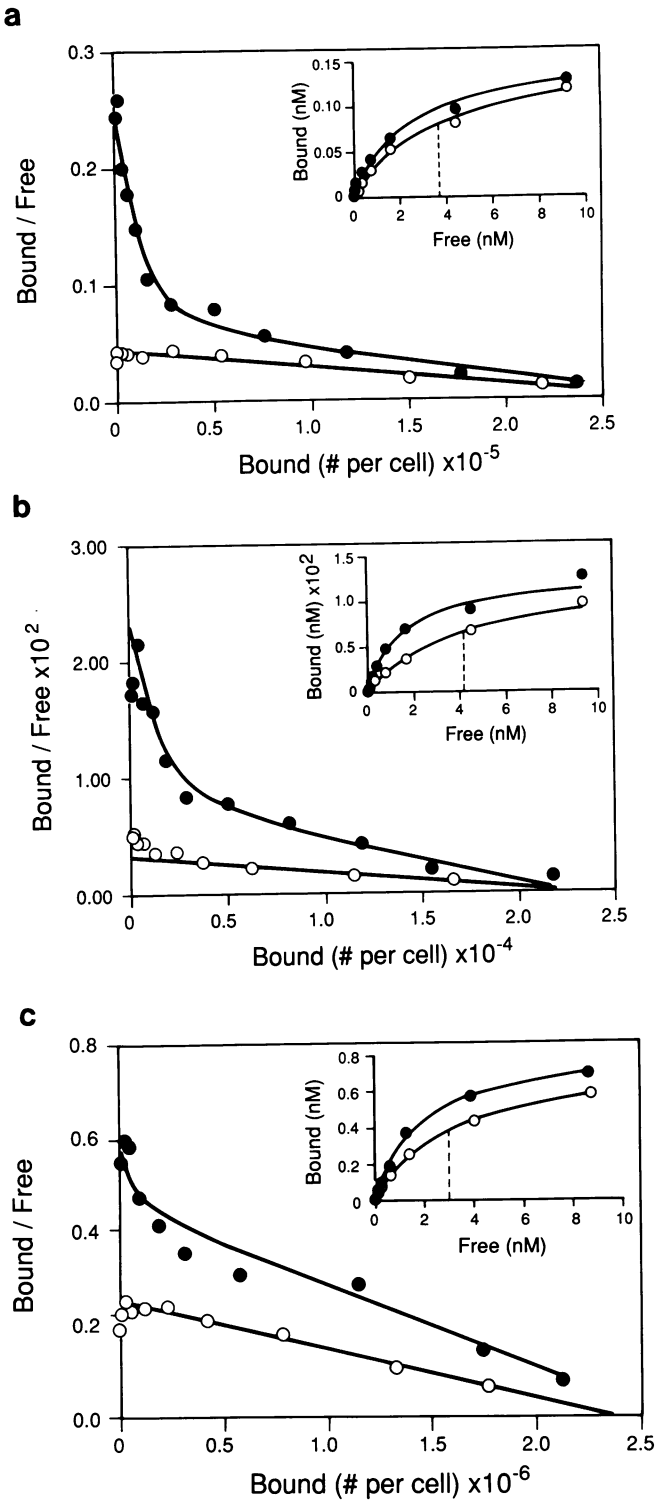

Fig. 3. Scatchard analysis of [^{125}I]EGF binding. [^{125}I]EGF binding was determined over a range of 0.1−300 ng/ml EGF by incubation at room temperature for 1 h in the presence (○) or absence (●) of 100 nM phorbol ester. (**a**) 3TH1/300 cells expressing HER1 (3×10^5 receptors/cell). (**b**) 3TH12/22 cells expressing HER1−2 chimera (2.2×10^4 receptors/cell). (**c**) 3TH12/2600 cells expressing HER1−2 chimera (2.6×10^6 receptor/cell).

[^{35}S]methionine-labeled cells, using either a monoclonal antibody against the extracellular domain of HER1 or a polyclonal antibody against the carboxy-terminal heptadecamer of HER2 (Hudziak *et al.*, 1987), demonstrated that the chimeric molecule synthesized in transfected NIH 3T3 cells contained both HER1 and HER2 sequences (Figure 2). Despite the fact that the calculated molecular weight (mol. wt) of HER1−2 (137.036 kd) is lower than

Table I. Receptor expression and binding characteristics

Cell line	Receptor	Number of EGF binding sites	kd −PMA	kd +PMA
3TH1/300	HER1	1.2×10^4	3.3×10^{-11}	
		2.9×10^5	2.7×10^{-9}	3.7×10^{-9}
3TH12/22L	HER1−2	1.2×10^3	4.1×10^{-11}	
		2.2×10^4	1.7×10^{-9}	4.2×10^{-9}
3TH12/2600H	HER1−2	1.1×10^4	2.5×10^{-11}	
		2.6×10^6	1.8×10^{-9}	3.0×10^{-9}

Data were derived from Scatchard analysis of saturation binding experiments. High (upper line) and low (lower line) affinity values are shown. Values determined in separate experiment varied by maximally 20%.

that of HER2 (137.870 kd), the synthesized chimeric glycoprotein migrated more slowly in SDS gels (mol. wt 188 versus 180 kd), suggesting more extensive glycosylation of the EGF receptor extracellular domain of HER1−2.

Ligand binding and affinity modulation

To determine whether binding properties of the EGF receptor extracellular domain were preserved when connected to heterologous transmembrane and cytoplasmic domains, we carried out Scatchard analyses of [^{125}I]EGF binding experiments to NIH 3T3 cells expressing either HER1−2 or HER1 control receptors. Figure 3a shows data obtained with a cell line expressing 3×10^5 human EGF receptors over a background of $\sim 5 \times 10^3$ mouse receptors. As expected, a curvilinear Scatchard plot was obtained, reflecting the presence of high and low affinity states of the EGF receptors (Brown *et al.*, 1979; Lee and Weinstein, 1979; Shoyab *et al.*, 1979; Salomon, 1981). Similar results were obtained when two independently isolated NIH 3T3 cell lines expressing 2.3×10^4 (Figure 3b) or 2.6×10^6 (Figure 3c) HER1−2 chimeric receptors per cell were analyzed. Both the binding constants and the extent of high and low affinity binding states of the EGF receptor were preserved in the chimeric receptor molecules (Table I and Figure 3b and c).

The EGF receptor is known to be phosphorylated on serine and threonine residues by protein kinase C (PKC) when cells are exposed to phorbol esters. This causes abolition of high affinity binding sites resulting in a single low affinity binding state, and concomitant reduction of the receptor tyrosine kinase activity (Hunter *et al.*, 1985; Davis and Czech, 1985). To date, this type of modulation has only been described for the EGF receptor. The HER1−2 receptor chimera allowed us to ask whether HER2 cytoplasmic sequences are targets for PKC phosphorylation and whether such phosphorylation could modulate the heterologous HER1 binding domain. The Scatchard plots of [^{125}I]EGF binding experiments presented in Figure 3 show that, as is true for HER1, PMA treatment of transfected cells leads to a specific loss of HER1−2 high affinity states and to the presence of low (*K*d 3−4.2 nM) affinity chimeric receptors only (Table I). Thus, PMA activation of PKC changes the affinity of the HER1−2 extracellular domain for EGF, most likely by receptor phosphorylation on serine and threonine residues. One PKC phosphorylation site of the EGF receptor, Thr 654, is conserved in the HER2 sequence, but does not appear to be the only residue involved in the regulation of EGF receptor ligand affinity (Livneh *et al.*, 1988; Davis, 1988; Lin *et al.*, 1985). Other candidate target serines within the kinase domain are present in homologous positions of both molecules, and are possibly involved in the modulation of HER2 affinity for its unidentified ligand.

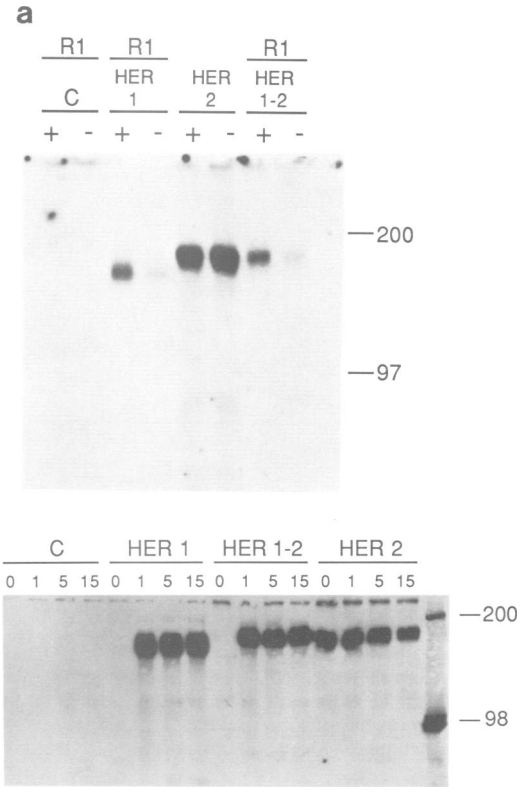

Fig. 4. Receptor autophosphorylation *in vitro* and in living cells. (**a**) Lysates from stably transfected NIH 3T3 lines were incubated in the presence (+) or absence (−) of 1 μM EGF, immunoprecipitated with the indicated antibodies, and kinase reactions were carried out as described in Materials and methods. Monoclonal antibody RI was used to precipitate HER1 and HER1−2, and H2-CT antibody was used for HER2. Mol. wt markers are indicated in kd. (**b**) Confluent quiescent cells were stimulated for the indicated times with 100 nM EGF, and receptor autophosphorylation was determined by immunoblotting with a phosphotyrosine antibody. No EGF was added to (O) time points. Control cells (C) were stably transfected with CVN plasmid. Mol. wt markers are indicated in kd.

In vitro and in vivo autophosphorylation

To further examine the functional integrity of the HER1−2 chimera, we tested the ability of the EGF receptor ligand binding domain to activate the kinase activity of the HER2 cytoplasmic portion *in vitro*. Figure 4a shows that like HER1, HER1−2 displays EGF-inducible autophosphorylation *in vitro*, while HER2 undergoes autophosphorylation even in the apparent absence of its specific ligand.

The autophosphorylation of HER1−2 was further explored by experiments in living cells utilizing phosphotyrosine antibodies. NIH 3T3 cells expressing HER1, HER2, or HER1−2 were exposed to EGF for various lengths of time, and then the reactions were stopped by cell lysis with hot SDS sample buffer. The samples were electrophoresed, transferred onto a nitrocellulose membrane and tyrosine-phosphorylated bands were localized with phosphotyrosine antibodies [^{125}I]protein A. As shown in Figure 4b, tyrosine phosphorylation was strongly induced

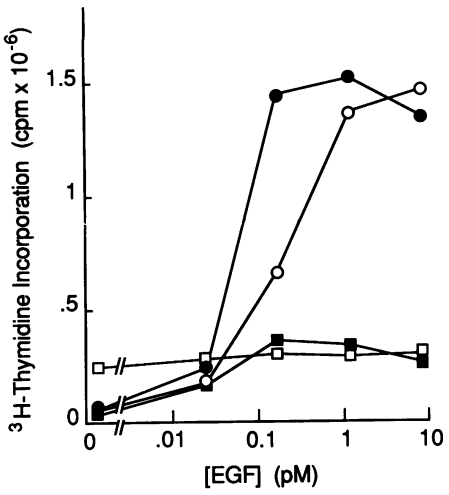

Fig. 5. EGF-stimulated [^{3}H]thymidine incorporation by transfected NIH 3T3 cells. Monolayers of quiescent transfected NIH 3T3 cells were stimulated with various concentrations of EGF as described in Materials and methods. The mitogenic response was measured by determining [^{3}H]thymidine incorporation into DNA 20 h after addition of EGF. (○), HER1 expressing cells (3TH1/300). (●), HER1−2 expressing cells (3TH12/2600). (□), HER2 expressing cells (3TH2). (■), control cells transfected with expression plasmid (CVN) containing no receptor sequences (3TCVN). Each point represents the average of two measurements, which differed by ~10%.

by EGF in HER1- and HER1−2-expressing cells, and both receptors underwent EGF-induced autophosphorylation.

In HER2-expressing cells, the 185-kd band was found to be phosphorylated even in the absence of EGF, which had no further stimulating effect. These *in vitro* and *in vivo* results further suggest that the HER2 kinase is constitutively activated in NIH 3T3 cells. However, when the same HER2 kinase is under the control of the EGF receptor ligand binding domain in HER1−2, the kinase is quiescent unless activated by EGF stimulation. An intriguing interpretation of these findings is that NIH 3T3 cells normally secrete the ligand for HER2, which results in autocrine stimulation, activation of the intrinsic kinase and autophosphorylation of this receptor.

Mitogenic and transforming activity of HER1−2 chimera

Growth factor receptors mediate the mitogenic activities of their ligands, and the receptor cytoplasmic domains are thought to be fully responsible for generating an intracellular signal that leads to the mitogenic response (Yarden and Ullrich, 1988). To investigate whether the structurally intact and biochemically functional HER1−2 chimera is able to generate a mitogenic signal, we stimulated serum-starved quiescent cell monolayers with EGF and measured the induction of [^{3}H]thymidine incorporation into DNA. Figure 5 shows that EGF strongly stimulated a mitogenic response in both HER1- and HER1−2-expressing NIH 3T3 cells. The maximal response measured for both cell lines was ~4 times that obtained with vector-transfected control NIH 3T3 cells. Whether the consistently observed lower ED_{50} value for HER1−2 in comparison with HER1 reflects a stronger mitogenic signalling potential of the HER2 cytoplasmic domain cannot be stated with certainty. HER2-expressing NIH 3T3 cells did not respond to EGF stimulation; however, consistent with the earlier described constitutive activation of the HER2 kinase, these cells could not be grown to quiescence.

While overexpressed human EGF receptors need to be activated by autocrine or externally added EGF or TGFα to induce transformed phenotype in NIH 3T3 cells, overexpression of unaltered HER2 is sufficient to lead to transformation of this cell type (Hudziak *et al.*, 1987; Di Fiore *et al.*, 1987a). Like the EGF receptor (Di Fiore *et al.*, 1987b; Velu *et al.*, 1987; Riedel *et al.*, 1988), activation of the HER1−2 chimera by EGF addition or TGFα cDNA cotransfection was essential to induce colony formation in soft agar or focus formation in cell monolayers, respectively (Figure 6, Table II). Interestingly, while HER2-expressing cells form colonies in soft agar, focus formation was not induced by transfection of our HER2 expression construct (Table II, Figure 6, Table III). These results confirm the transforming potential of HER2, but demonstrate that ligand activation is necessary to achieve this effect, analogous to the EGF receptor. Furthermore, our experiments suggest that NIH 3T3 cells may secrete the ligand for HER2, but that the levels produced may not be sufficient to induce loss of cell contact inhibition, necessary for focus formation. Alternatively, the SV40 early promoter based expression vector may not generate high enough HER2 levels necessary for this effect, as the findings of Di Fiore *et al.* (1987a) suggest.

Substrate phosphorylation

Upon activation by their ligands, receptors of the tyrosine kinase family induce similar primary responses within cells (see Yarden and Ullrich, 1988, for review). However, their distinct primary structures suggest that despite similarities in their biological effects, there may also be receptor-specific signals generated within a single cell type. Phosphorylation of cellular protein substrates on tyrosine residues may play an important role in the process of signal transduction by receptor tyrosine kinases (RTKs). To investigate whether such closely related receptors as HER1 and HER2 show any distinct substrate phosphorylation specificity, we labeled cells with inorganic ^{32}Pi, stimulated with EGF, and immunoprecipitated phosphotyrosine-containing proteins with an anti-phosphotyrosine antibody. SDS−PAGE analysis, shown in Figure 4, demonstrates that among substrates that are shared between HER1, HER2 and HER1−2, a protein substrate of 37 kd mol. wt is unique to cells expressing HER2 and in EGF-stimulated HER1−2 cells. This finding clearly demonstrates that closely related RTKs share certain cellular substrates, but in addition phosphorylate specific target proteins, and may trigger distinct signalling pathways. Furthermore, this experiment shows that substrate specificity is defined by the cytoplasmic domain of RTKs and that HER2 substrate phosphorylation is constitutively activated in NIH 3T3 cells.

Discussion

The rat *neu* proto-oncogene and its human homolog HER2 (or c-*erb*B-2) were obtained independently either by isolation of the transforming gene from chemically induced neuroblastomas (Padhy *et al.*, 1982) or by accidental cross-hybridization with the avian oncogene v-*erb*B (Coussens *et al.*, 1985; Yamamoto *et al.*, 1986). Comparison of its cDNA deduced primary structure revealed that it contains

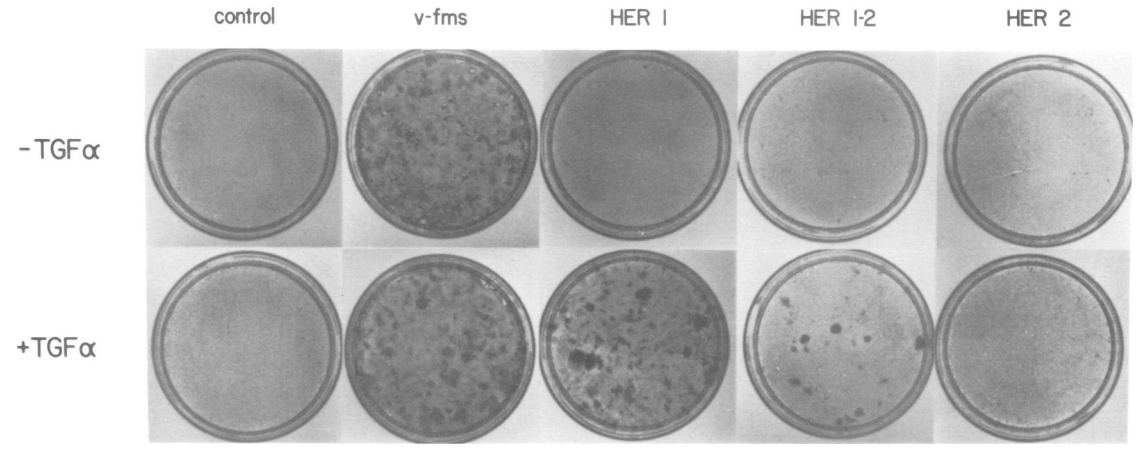

Fig. 6. Focus formation assays. NIH 3T3 cells were transfected either with expression plasmids for v-*fms*, HER1, HER1−2, or HER2, alone or in combination with a TGFα expression construct. Monolayers were scored 2 weeks after transfection and stained 1 week later.

all major structural features of a cell surface receptor with tyrosine kinase activity. Yet designation of HER2 as a receptor was formally not possible since a ligand for the HER2 protein is unknown. Furthermore, since the family of receptor tyrosine kinases includes molecules that are involved in diverse biological functions (reviewed in Yarden and Ullrich, 1988), such as regulation of metabolic processes (insulin receptor), cell type-specific differentiation (CSF-I receptor, EGF receptor), *Drosophila* eye development (sevenless gene product), and mitogenesis (EGF receptor, PDGF receptor, IGF-I receptor), it has not yet been possible to assign a function-related designation to HER2.

We constructed a receptor chimera to begin to characterize the biochemical and biological functions of HER2, and thereby its yet unidentified ligand. This approach had previously proved successful in creating functional insulin−EGF receptors, EGF receptor−v-*erb*B chimerae, and insulin receptor−v-*ros* hybrids. Using the EGF receptor ligand binding domain fused to HER2 transmembrane and cytoplasmic domains, we were able to investigate various aspects of biological signalling activities normally induced through the HER2 molecule by this currently unknown ligand. The HER1−2 chimera is correctly synthesized, processed and transported to the cell surface, where it forms EGF low and high affinity binding sites with characteristics identical to wild-type EGF receptor (Brown *et al.*, 1979; Lee and Weinstein, 1979; Shoyab *et al.*, 1979; Salomon, 1981). This demonstrates that heterologous cytoplasmic and transmembrane domains do not disturb the formation of an authentic binding site conformation by EGF receptor extracellular sequences. Furthermore, these data suggest that the HER2 extracellular domain may also exist in two affinity states for its own ligand. As is true for the EGF receptor, the binding affinity of HER2 for its own ligand may also be regulated by PMA treatment of cells, because PMA-induced affinity modulation for EGF was perfectly preserved in the HER1−2 chimera. This down modulation phenomenon appears to be characteristic for subclass I RTKs, since the insulin receptor does not change its affinity after PMA treatment of expressing cells.

Ligand-induced autophosphorylation is thought to be a crucial step in the process of receptor-mediated signalling. Defects in this process either lead to a loss of biological activity or constitutive activation and loss of control. In

Table II. Colony formation of soft agar

	Colonies/dish	
	−EGF	+EGF
3TCVN	0	0
3TH1/300	0	1480
3TH2	148	112
3TH12/2600	0	844

Stably expressing NIH 3T3-CL7 cell lines were plated (2×10^5 cells/dish) in the presence or absence of 50 ng/ml EGF. Colonies were scored after 2 weeks. Numbers shown are from one representative experiment.

Table III. Expression plasmid-mediated focus formation

	Foci/μg DNA	
Expression plasmid	−TGFα	+TGFα
CVN	0	0
CVN/HER1	0	2520
CVN/HER2	0	0
CVN/HER1−2	0	720
c-*fms*	3900	3600

Transfection experiments with (+TGFα) or without (−TGFα) cotransfection with a TGFα expression plasmid were carried out as described in Materials and methods. The numbers represent the average of three experiments in which the relative focus formation efficiencies were consistent.

previous work, we have shown that insulin binding to an insulin−EGF receptor chimera results in activation of the heterologous EGF receptor kinase and autophosphorylation *in vitro* (Riedel *et al.*, 1986). This reaction is also functional in the HER1−2 receptor hybrid, both *in vitro* as well as in intact cells. Besides retaining full kinase activity, HER1−2 is also able to generate an apparently HER2-specific signal which results in phosphorylation of cellular protein substrates, including a specific 37-kd protein, and a mitogenic response comparable to that of the activated human EGF receptor in NIH 3T3 cells.

Furthermore, HER1−2 expression leads to transformation of NIH 3T3 cells in a strictly ligand-dependent fashion. The transforming potential of HER2 and its rat homolog *neu* has been demonstrated previously (Hudziak *et al.*, 1987; Di

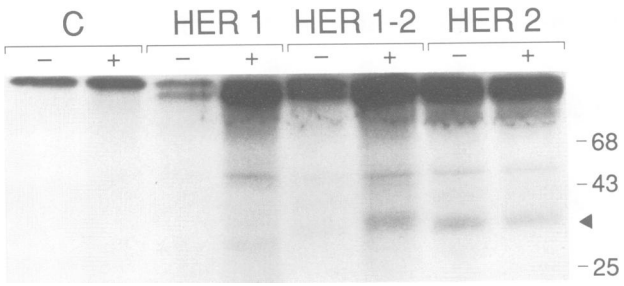

Fig. 7. EGF-stimulated *in vivo* phosphorylation of receptor substrates. Confluent monolayers of stably expressing transfected cells were labeled with radioactive inorganic phosphates as described in Materials and methods, and then incubated in the presence (+) or absence (−) of 100 ng/ml EGF. Following stimulation, cells were lysed and immunoprecipitated with antiphosphotyrosine antibody. As illustrated, the first two lanes (**C**) from the left are immunoprecipitates from control cells transfected with the CVN expression vector containing no receptor sequences. The next two lanes (**HER1**) are from lines expressing wild-type EGF receptor. The lanes labeled **HER1−2** are from cells expressing the HER1−2 chimeric receptor and the lanes labeled **HER2** are from cells expressing HER2 receptors. The arrowhead indicates a band of mol. wt ~37 kd, which appears in the last three lanes. Mol. wt markers indicated are in kd.

Fiore *et al.*, 1987a; Padhy *et al.*, 1982). While a single point mutation in the transmembrane domain of *neu* appears to activate its transforming potential (Bargmann *et al.*, 1986b), resulting in rat neuroblastoma (Padhy *et al.*, 1982) and mouse breast carcinoma (Muller *et al.*, 1988), overexpression of the intact HER2 protein appears to be sufficient to transform NIH 3T3 cells (Hudziak *et al.*, 1987; Di Fiore *et al.*, 1987a). Analogous experiments with the EGF receptor also demonstrate a transforming potential when overexpressed, but only when activated by EGF or TGFα (Di Fiore *et al.*, 1987b; Riedel *et al.*, 1988; Velu *et al.*, 1987). Our data demonstrate that the HER2 cytoplasmic domain in HER1−2 needs to be activated by EGF to generate a transforming signal in NIH 3T3 cells, while wild-type HER2 independently induces a transformed phenotype in these cells, yet only when determined by the colony formation assay. This, in conjunction with our finding of constitutive tyrosine phosphorylation of HER2 and a specific 37-kd substrate in NIH 3T3 cells in comparison with ligand dependence for these activities of HER1−2 in intact cells and *in vitro*, strongly support the interpretation that NIH 3T3 cells produce the ligand for HER2. Expression levels, however, appear to be insufficient to transform these fibroblasts by autocrine activation of their endogenous mouse HER2 homolog as well as for focus formation after transfection with an SV40 promoter-driven HER2 expression construct.

Identification of the apparently HER2-specific endogenous 37-kd substrate among others that are shared with the EGF receptor suggests that these two ligand−receptor systems play closely related but distinct biological roles. Detection of this substrate specificity opens new avenues towards further dissection of structure−function relationships in growth factor receptor-mediated signalling.

Materials and methods

Reagents

Receptor-grade EGF was obtained from Collaborative Research Inc. ^{125}I-labeled EGF (100 μCi/μg), [^{35}S]methionine (1000 Ci/mmol), methyl-[^{3}H]thymidine (84 Ci/mmol), and [^{125}I]protein A were from Amersham, Inc. Protein A−Sepharose was from Pharmacia, Inc. Cell culture reagents, including Geneticin (G418 sulfate), were supplied by Gibco Labs. Insulin, bovine serum albumin, PMSF, aprotinin and bacitracin were from Sigma Chemical Co., and Enhance and Aquasol-2 scintillation cocktail were from New England Nuclear. Purified tissue culture agar was from Difco. (+) Amethopterin (methotrexate) was obtained from Sigma. MabRI recognizing the extracellular domain of the human EGF receptor (Waterfield *et al.*, 1982) was from Amersham. Rabbit antibody RK-II recognizes residues 984−996 of the human EGF receptor (Kris *et al.*, 1985); H2-CT antibody was raised against the peptide CGTPTAENPEYLGLDVPV (residues 1239−1255 of the HER2 receptor). An IgG fraction of mouse monoclonal antibody 5E2 that recognizes phosphotyrosine was used in antiphosphotyrosine immunoblotting experiments.

Expression plasmid construction

Expression plasmid CVN/HER2, containing the entire HER2 receptor gene, was digested with enzymes *Bsm*I−*Dra*I to remove the extracellular domain-encoding sequences. A 2.0-kb *Xba*I−*Xho*II fragment, containing the extracellular domain-encoding sequences of HER1 isolated from the plasmid CVN/HER1, was added, together with the two-part synthetic *Xho*II−*Bsm*I linker (5′ GATCCCGTCCATCGTCTCTGCGGTGGTTGG 3′ and 5′ AACCACCGCAGAGACGATGGACGG 3′) and joined to yield the above CVN/HER2 plasmid in a three-factor ligation. The resulting plasmid contained the extracellular domain encoding sequences of HER1 fused to the transmembrane and cytoplasmic encoding sequences of HER2 under the control of the SV40 E promoter and hepatitis B surface antigen polyadenylation signal. In addition, the expression plasmid contained the wild-type DHFR and neomycin resistance genes, also under the control of an identical promoter and signal.

Cell culture

Mouse NIH 3T3-CL7 fibroblasts were cultured in DMEM containing 1g/l of glucose, 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Stable lines were cultured in the above medium plus 400 μg/ml G418-sulfate. RatI fibroblasts were cultured in F12/DMEM (50:50) containing 10% fetal bovine serum, 2 mM L-glutamine, and antibiotics. Stable transfectants were cultured in the same medium as their parental lines supplemented with 400 μg/ml G418-sulfate.

Generation of transfected cell lines

Expression vectors were introduced into NIH 3T3-CL7 or RatI cells by a variation of the method of Graham and van der Eb (1973). Two days following transfection, cells were split into two 10-cm dishes with DMEM (NIH 3T3-CL7) or F12/DMEM (RatI) containing 10% fetal bovine serum and 400 μg/ml G418-sulfate. Cells were refed every 5−7 days.

Amplification of transfected DNA sequences was achieved by selection in DMEM (NIH 3T3-CL7) or F12/DMEM (RatI) containing 7% extensively dialyzed fetal bovine serum and 100−500 nM methotrexate. Medium was changed every 5−7 days. Cells were cloned at various stages of selection and screened for expression by [^{35}S]methionine labeling and immunoprecipitation.

Characterization of receptor protein synthesis and phosphorylation

For metabolic labeling of proteins, subconfluent stably transfected cell lines were grown in 6-cm tissue culture dishes. The cells were washed twice with phosphate buffered saline (PBS). 3 ml of methionine-free medium prepared from an MEM select-amine kit (Gibco) and 300 μCi [^{35}S]methionine was added and the incubation continued for 16 h. To detect and characterize receptor proteins, [^{35}S]Met-labeled cell monolayers were washed twice with PBS and solubilized in 500 μl of 1% Triton X-100 buffer as described by Kris *et al.* (1985). The 500-μl aliquot was then incubated with 2 μl of antiserum for 2 h shaking at 4°C. 80 μl protein A−Sepharose slurry (1:1 swollen in dH_2O) was added for 1 h shaking at 4°C. The immunoprecipitates were washed five times in 1 ml HNTG buffer (20 mM Hepes pH 7.5, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100). The pellet was then resuspended in 30 μl of 2 × SDS gel sample buffer. Samples were boiled for 5 min, centrifuged and analyzed on 7% SDS polyacrylamide gels. Gels were fixed for 1 h in 10% acetic acid/10% TCA/30% MeOH, incubated for 1 h in Enhance, precipitated for 1 h in H_2O and dried under vacuum at 60°C.

For immunoblot analysis of phosphotyrosine-containing proteins, confluent stably transfected cells plated in 35 mm tissue culture dishes were first starved for 18 h in serum-free media. Following addition of 100 nM EGF for 1, 5, and 15 min, media was drained and cells were harvested by addition of hot sample buffer to the dishes. Samples were mixed, boiled for 5 min, and electrophoresed on 7% SDS−polyacrylamide gels, then transferred to

nitrocellulose (Schleicher and Schuell) using a standard Western blotting protocol. Filters were treated 6 h with monoclonal antiphosphotyrosine antibody, 5E2 (100 ng/ml), washed three times for 15 min, incubated in [^{125}I]protein A (400 000 c.p.m./ml) 2 h at room temperature, then washed three times, dried, and exposed to X-ray film.

To determine receptor autophosphorylation activity, confluent 6-cm dishes of stably transfected cells were washed three times with PBS, then lysed in 0.5 ml lysis buffer containing 1% Triton X-100. After 5 min incubation at 4°C, lysates were collected and centrifuged for 10 min at 4°C in an Eppendorf centrifuge. Lysates were diluted to a final concentration of 0.3% Triton X-100 and where indicated EGF was added (1 μM) for a 30 min incubation at 4°C. Protein A–Sepharose and antibody were added to lysates, incubated for 90 min at 4°C, then washed five times with HNTG [150 mM NaCl/20 mM (pH 8.0)/0.1% Triton X-100/10% glycerol]. 10 μl of HNTG containing 10 mM $MnCl_2$, 10 μCi of [γ-^{32}P]ATP and 15 μM of unlabeled ATP were added to the immunoprecipitate and incubated 60 s on ice. 50 μl of hot sample buffer was added to terminate the reaction. Samples were then electrophoresed on 7% SDS–polyacrylamide gels. Dried gels were exposed on Kodak X-Omat AR film.

Ligand binding experiments and analysis

Subconfluent stably transfected cells were plated in 2.2 cm, 12-well tissue culture dishes (Costar). The following day, wells were washed twice with DMEM/0.1% BSA/20 mM Hepes pH 7.5 and incubated in the same medium for 60 min at 24°C in the presence of various amounts of [^{125}I]EGF. Nonbound radioactivity was removed by three washes with the above media and cells were lysed in 0.2 N NaOH. The radioactivity of the lysates was determined in a gamma counter. To determine the effect of phorbol esters on ligand binding, cells were incubated with 100 nM PMA for 60 min at 37°C before binding [^{125}I]EGF at 24°C. Scatchard analysis was carried out using a computer program developed by S.Felder (Rorer Biotechnology) for fitting of the data against a one- or two-site model of ligand binding and for determination of receptor numbers and kd values.

Transformation assays

2×10^5 cells (NIH 3T3-CL7) were plated in 35-mm dishes. The medium was changed the following day and 4 h later they were transfected by calcium phosphate coprecipitation (Graham and van der Eb, 1973) with 5 μg NIH 3T3 high mol. wt carrier DNA, and 100 ng of a control (pCVN) expression construct, a v-*fms* expression construct (Roussel *et al.*, 1987) or the human chimeric EGF receptor expression plasmids. In parallel, cells were cotransfected with 100 ng of a TGFα expression plasmid (Rosenthal *et al.*, 1986). The following day, cells from each dish were split into three 6-cm dishes with DMEM containing 5% FCS. Medium was changed every 3–4 days and foci were scored on day 14. Plates were stained with crystal violet on day 21.

To determine the ability of cell lines to form colonies in soft agar, 5×10^4 or 2×10^5 cells were plated in a 6-cm dish in the presence or absence of 50 ng/ml EGF in a top layer of 4 ml of MEM containing 10% FCS and 0.2% agar. The bottom layer was 4 ml of MEM containing 10% FCS and 0.4% agar +/− EGF. Visible colonies were scored after 21 days.

[^{3}H]Thymidine incorporation assay

Confluent cell monolayers in 24-well culture dishes were brought to quiescence for 24 h in medium containing 0.5% fetal bovine serum. Stimulation of DNA synthesis by addition of various concentrations of receptor-grade EGF was monitored 18 h later by 4-h pulse labeling with 0.5 μCi methyl-[^{3}H]thymidine. Cells were then washed three times with PBS and soluble radioactivity was extracted with 1 ml of 5% TCA for 30 min at 4°C. Subsequently, the cells were washed twice with PBS and solubilized in 500 μl of 0.2 N NaOH and the incorporated radioactivity was determined.

Endogenous substrate phosphorylation analysis

Confluent 6-well tissue culture dishes (Costar) of stably transfected cells were washed with phosphate-free media and then labeled 16 h (Gibco: DMEM without phosphate salts) with 400 μCi/ml [^{32}P]orthophosphate (Amersham). Cells were incubated with and without 100 ng/ml EGF in DMEM for 5 min at room temperature, then lysed in buffer containing 1% Triton X-100 as described by Kris *et al.* (1985) supplemented with 10 μM sodium orthovanadate and 10 μM $ZnCl_2$. Immunoprecipitations were performed as described above, using an antiphosphotyrosine antibody. After washing, the precipitated pellet was treated with a solution containing 100 mM glycine (pH 2.5) for 5 min at room temperature to remove the precipitated proteins from the protein A beads. The solution was removed, neutralized, added to sample buffer, boiled and run in 15% SDS polyacrylamide gels. Gels were dried and exposed to film.

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