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Activation of HER4 by heparin-binding EGF-like growth factor stimulates chemotaxis but not proliferation

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Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is a potent mitogen and chemotactic factor for fibroblasts, smooth muscle cells and keratinocytes. It is demonstrated that HB-EGF is not only a ligand for HER1, as previously reported, but for HER4 as well. HB-EGF binds to NIH 3T3 cells overexpressing either HER1 or HER4 alone, but not HER2 or HER3 alone. Binding to HER4 is independent of HER1. The ability of HB-EGF to bind to two different receptors is in contrast to EGF which binds to HER1, but not to HER4, and heregulin-β1 which binds to HER4, but not to HER1. Besides binding, HB-EGF activates HER4. For example (i) it induces tyrosine phosphorylation of HER4 in cells overexpressing this receptor and of endogenous HER4 in MDA-MB-453 cells and astrocytes; (ii) it induces association of phosphatidylinositol 3-kinase (PI3-K) activity with HER4; and (iii) it is a potent chemotactic factor for cells overexpressing HER4. Chemotaxis is inhibited by wortmannin, a PI3-K inhibitor, suggesting a possible role for PI3-K in mediating HB-EGF-stimulated chemotaxis. On the other hand, HB-EGF is not a mitogen for cells expressing HER4, in contrast to its ability to stimulate both chemotaxis and proliferation in cells expressing HER1. It was concluded that HER4 is a newly described receptor for HB-EGF and that HB-EGF can activate two EGF receptor subtypes, HER1 and HER4, but with different biological responses.

Keywords: EGF/EGF receptors/heregulin/phosphatidylinositol 3-kinase

Introduction

The biological activities of the epidermal growth factor (EGF) family of ligands, which has at least seven members, is mediated by their interaction with receptor tyrosine kinases (RTKs), transmembrane proteins that convert extracellular growth factor signals into intracellular enzymatic activities. At least four EGF receptors have been identified that bind the EGF-like ligands. These receptors have been designated as HER1, HER2, HER3 and HER4 as a result of homologies to the originally described human EGF receptor; or erbB-1, erbB-2, erbB-3 and erbB-4 as a result of homologies to the v-*erbB* oncogene encoded by the avian erythroblastosis virus (Hynes and Stern, 1994; Earp *et al.*, 1995). Ligand binding to HER family members results in receptor homo- or heterodimerization, receptor autophosphorylation, phosphorylation of intracellular substrates and recruitment of signal transduction molecules containing SH2 domains to phosphorylated tyrosine residues at the cytoplasmic tails of the receptors (Schlessinger and Ullrich, 1992). Although several signal transduction pathways downstream from RTKs have been elucidated, it is still not completely understood how the various EGF ligands activate specific cellular responses such as proliferation, survival, differentiation, migration, adhesion or changes in gene expression by interactions with the various EGF receptors.

EGF and transforming growth factor-α (TGF-α) are ligands for HER1. They cannot bind or activate HER2, HER3 or HER4 directly. However, these ligands can activate these three receptors indirectly by binding to HER1, which in turn activates the other HER family members via heterodimer formation (Wada *et al.*, 1990; Soltoff *et al.*, 1994; Riese *et al.*, 1996b).

A number of growth factors structurally related to EGF have been described recently including amphiregulin (AR) (Shoyab *et al.*, 1989), heparin-binding EGF-like growth factor (HB-EGF) (Higashiyama *et al.*, 1991), betacellulin (βC) (Shing *et al.*, 1993) and epiregulin (Toyoda *et al.*, 1995). The biological activities of these EGF-like ligands appear to be mediated by activation of HER1, similar to EGF and TGF-α. However, ligands have been identified that do not bind to HER1, in particular, heregulin (HRG) (Holmes *et al.*, 1992), *neu* differentiation factor (NDF) (Wen *et al.*, 1992), acetylcholine receptor inducing activity (ARIA) (Falls *et al.*, 1993) and glial growth factor (GGF) (Marchionni *et al.*, 1993). These factors are alternatively spliced products of the same gene. They bind to HER3 and HER4 and activate HER2 as part of HER3–HER2 or HER4–HER2 heterodimers (Peles *et al.*, 1993; Plowman *et al.*, 1993b; Carraway and Cantley, 1994; Sliwkowski *et al.*, 1994; Karunagaran *et al.*, 1996). No direct ligand for HER2 has yet been identified.

HB-EGF is a heparin-binding member of the EGF family first identified in the conditioned medium of macrophages (Higashiyama *et al.*, 1991). As are other members of the EGF family, HB-EGF is synthesized as a transmembrane precursor that can be cleaved enzymatically to release a soluble 14–20 kDa form (Higashiyama *et al.*, 1992; Raab *et al.*, 1994). The soluble form of HB-EGF is a potent paracrine and/or autocrine mitogen for fibroblasts, smooth muscle cells (SMC) and keratinocytes (Higashiyama *et al.*, 1991; Marikovsky *et al.*, 1993). It has also been shown to be a potent chemoattractant for SMC (Higashiyama *et al.*, 1993). HB-EGF has been implicated *in vivo* as having a role in wound healing (Marikovsky *et al.*, 1993) and in processes involving SMC

hyperplasia such as atherosclerosis (Miyagawa *et al.*, 1995), pulmonary hypertension (Powell *et al.*, 1993) and uterine leiomyomas (Mangrulkar *et al.*, 1995). Given the possible physiological and pathological roles of HB-EGF, we have been interested in analyzing its receptor interactions. Like most of the HER ligands, HB-EGF binds to and activates HER1. It competes for the binding of [^{125}I]EGF to A431 cells which express a very abundant amount of HER1 (Higashiyama *et al.*, 1991); it stimulates tyrosine phosphorylation of HER1 (Higashiyama *et al.*, 1992); and it induces proliferation of cells transfected to overexpress HER1, both as a paracrine and as a juxtacrine factor (Aviezer and Yayon, 1994; Blotnick *et al.*, 1994; Higashiyama *et al.*, 1995).

However, it is possible that HB-EGF might interact with other EGF receptor subtypes. For example, our preliminary experiments have indicated that [^{125}I]HB-EGF, but not [^{125}I]EGF, can be cross-linked to MDA-MB-453 human mammary adenocarcinoma cells (Elenius *et al.*, 1996). These cells express HER2, HER3 and HER4 but not HER1 (Plowman *et al.*, 1993a). Accordingly, in order to determine whether HB-EGF could interact directly with EGF receptors other than HER1, we obtained NIH 3T3 cells expressing either HER1, HER2, HER3 or HER4 solely (Zhang *et al.*, 1996). Here, we demonstrate that HB-EGF binds to HER4, induces tyrosine phophorylation of HER4, and stimulates the association of phosphatidylinositol 3-kinase (PI3-K) activity with HER4. Furthermore, HB-EGF is a potent chemotactic factor but not a mitogen for cells expressing HER4, in contrast to the ability of HB-EGF to stimulate both these activities in cells expressing HER1. These results suggest that HB-EGF is a ligand for HER4 as well as HER1 but that these two receptors mediate different biological activities in response to this single ligand.

Results

[^{125}I]HB-EGF binds to cells expressing HER1 or HER4

Confluent NIH 3T3 cell stable transfectants expressing either human HER1, HER2, HER3 or HER4 alone (Zhang *et al.*, 1996) were incubated with 10 ng/ml of [^{125}I]HB-EGF, chemically cross-linked and lysed (Figure 1). The lysates of cell lines expressing each of the four receptors were immunoprecipitated with the corresponding specific polyclonal anti-HER antibodies. After separation on 6% SDS–PAGE gels, single cross-linked complexes were found associated only with the lysates of NIH 3T3 cells expressing HER1 or HER4 (Figure 1A, lanes 1 and 4, respectively). The [^{125}I]HB-EGF–HER4 complex, of ~190–200 kDa, was slightly larger than the [^{125}I]HB-EGF–HER1 complex, of ~180–190 kDa, consistent with the cross-linking of 14 kDa [^{125}I]HB-EGF to 180 kDa HER4 and 170 kDa HER1 in a 1:1 molar ratio. On the other hand, [^{125}I]HB-EGF did not form any high molecular weight cross-linked complexes with lysates of cells expressing HER2 or HER3 either directly (not shown) or after immunoprecipitation with the corresponding anti-HER2 or anti-HER3 antibodies, respectively (Figure 1A, lanes 2 and 3, respectively).

The cross-linking of [^{125}I]HB-EGF to A431 cell HER1 receptors has been described previously (Higashiyama

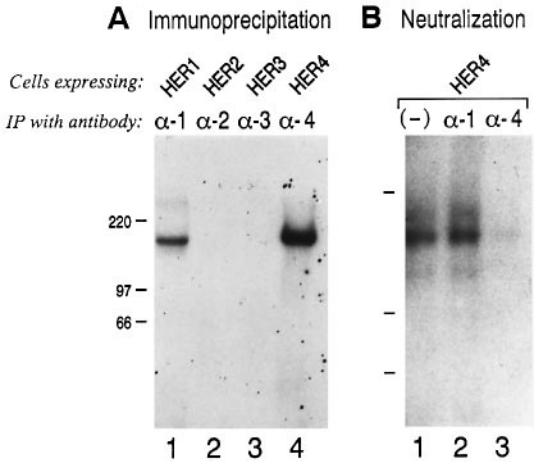

Fig. 1. Cross-linking of [^{125}I]HB-EGF to cells expressing HER1, HER2, HER3 or HER4 alone. (**A**) The NIH 3T3 cell lines expressing each of the four EGF receptor subtypes were cross-linked with DSS in the presence of 10 ng/ml (4×10^5 c.p.m./ml) of [^{125}I]HB-EGF and lysed. The lysates of cells expressing HER1 (lane 1), HER2 (lane 2), HER3 (lane 3) and HER4 (lane 4) were immunoprecipitated with anti-HER1, anti-HER2, anti-HER3 and anti-HER4 antibodies, respectively, separated on a 6% SDS–PAGE gel and the radioactive complexes were visualized by autoradiography. (**B**) HER4 cells were cross-linked with 10 ng/ml of [^{125}I]HB-EGF in the absence of neutralizing antibody (lane 1), in the presence of 5 μg/ml of neutralizing monoclonal anti-HER1 (LA1) (lane 2) or in the presence of neutralizing monoclonal anti-HER4 (H4.72.8) (lane 3) antibodies. The cells were lysed and the [^{125}I]HB-EGF–HER complexes analyzed on a 6% SDS–PAGE gel.

et al., 1994), but cross-linking to HER4 has not. Accordingly, to confirm that HB-EGF interacts with HER4, neutralizing monoclonal antibodies directed against the extracellular domain of human HER4 were analyzed for their ability to inhibit cross-linking of [^{125}I]HB-EGF to the surfaces of cells expressing HER4. Five μg/ml of anti-HER4 antibody added to the cells at the same time as 10 ng/ml of [^{125}I]HB-EGF completely blocked the formation of cross-linked complexes (Figure 1B, lane 3), when compared with a phosphate-buffered saline (PBS) control (Figure 1B, lane 1). On the other hand, a similar concentration of anti-HER1 neutralizing antibody had no effect (Figure 1B, lane 2). From the combination of cross-linking, immunoprecipitation and immunoneutralizing experiments, it was concluded that HB-EGF binds to HER4 as well as to HER1 and that HB-EGF–HER4 binding is not dependent on heterodimerization of HER4 with HER1.

Binding of EGF family ligands to cells expressing either HER1 or HER4

EGF has been shown to interact with HER1 but not HER4 (Plowman *et al.*, 1993a) while HRG interacts with HER4 but not HER1 (Plowman *et al.*, 1993b). To test the accuracy of our cross-linking experiments, cells expressing HER1 or HER4 were cross-linked with equivalent c.p.m. of [^{125}I]EGF, [^{125}I]ARIA-1 (the chicken homolog of human HRG-β1) or [^{125}I]HB-EGF. As expected from the results shown above, [^{125}I]HB-EGF formed high molecular mass cross-linked complexes with cells expressing either HER1 or HER4 (Figure 2, lanes 1 and 4, respectively). In this experiment, the relative cross-linking intensity of HB-EGF was greater for HER1 than HER4, unlike the experi-

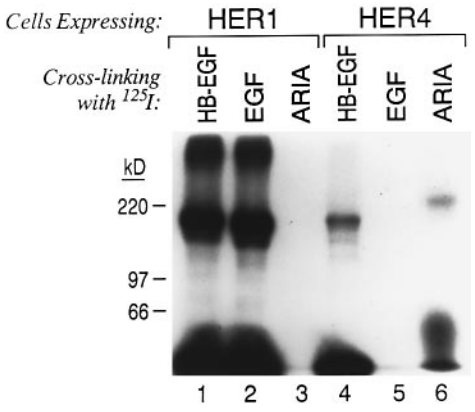

Fig. 2. Cross-linking of [^{125}I]HB-EGF, [^{125}I]EGF or [^{125}I]ARIA-1 to cells expressing HER1 or HER4. Confluent 28 cm^2 dishes of cells expressing HER1 (lanes 1–3) or HER4 (lanes 4–6) were cross-linked in the presence of 1.5×10^5 c.p.m./ml [^{125}I]HB-EGF (lanes 1 and 4), [^{125}I]EGF (lanes 2 and 5) and [^{125}I]ARIA-1 (lanes 3 and 6). Cells were lysed, and cross-linked complexes were analyzed by SDS–PAGE and autoradiography as in Figure 1A, except that no immunoprecipitation step was included.

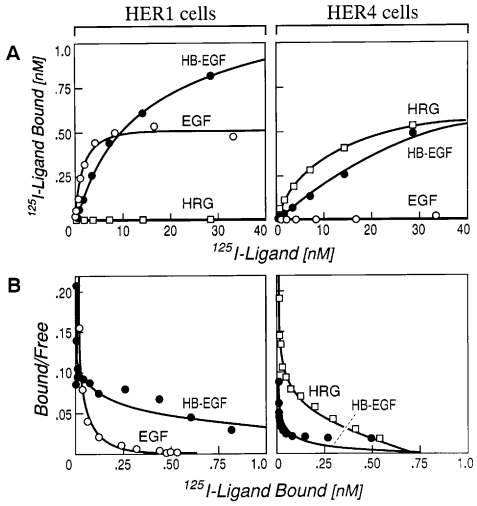

Fig. 3. Scatchard analysis of [^{125}I]EGF, [^{125}I]HB-EGF and [^{125}I]HRG-β1 binding to NIH 3T3 cells expressing HER1 or HER4. (**A**) Increasing amounts of ^{125}I-labeled ligand were added to NIH 3T3 cells expressing either HER1 or HER4. Non-specific binding was determined by competition with a 200-fold excess of unlabeled ligand and subtracted from total binding. After binding, the cells were washed, lysed, and the cell-associated radioactivity was determined using a γ-counter. Each point represents the mean of two independent measurements. (**B**) The results shown in (A), left and right, were analyzed by the method of Scatchard, and best-fit plots were obtained using the LIGAND program. The ligand-binding assays in (A) and the Scatchard plots in (B) were repeated for a total of three experiments and one representative experiment is shown. (HB-EGF, ●; EGF, ○; HRG-β1, □.)

ment shown in Figure 1. This discrepancy might be explained by the fact that in Figure 2, no immunoprecipitation was carried out after cross-linking, thus minimizing possible differential efficiencies of anti-HER1 and anti-HER4 antibodies in immunoprecipitating ligand–receptor complexes. On the other hand, [^{125}I]EGF formed a complex with cells expressing HER1 (Figure 2, lane 2) but not with cells expressing HER4 (Figure 2, lane 5). In addition, [^{125}I]EGF did not cross-link cells expressing HER2 or HER3 (not shown). In contrast, [^{125}I]ARIA-1 formed a complex with cells expressing HER4 (Figure 2, lane 6) but not HER1 (Figure 2, lane 3). The sizes of the complexes were consistent with the sizes of [^{125}I]EGF (6 kDa), [^{125}I] HB-EGF (14 kDa), [^{125}I]ARIA-1 (45 kDa), HER1 (170 kDa) and HER4 (180 kDa). Formation of the [^{125}I]HB-EGF–HER1 and [^{125}I]HB-EGF–HER4 complexes was completely abolished by addition of a 200-fold molar excess of unlabeled HB-EGF (not shown), demonstrating that radioiodination of HB-EGF did not result in altered binding properties. Taken together, these results suggest that as opposed to EGF, which binds HER1 but not HER4, and ARIA/HRG, which binds HER4 but not HER1, HB-EGF binds to both receptor subtypes.

Next, we determined the binding affinities of HB-EGF, EGF and HRG-β1 for HER1 and HER4 as well as the number of binding sites per cell for each ligand–receptor pair (Figure 3; Table I). Increasing concentrations of ^{125}I-labeled ligands were incubated with NIH 3T3 cells expressing either HER1 or HER4, and saturation binding curves (Figure 3A) and Scatchard plots (Figure 3B) were generated. Dissociation constants (K_d) and numbers of binding sites were determined using the LIGAND linear regression program (Table I). Several conclusions were drawn from the Scatchard analysis as follows. (i) The Scatchard plots were in all cases curvilinear and suggested the existence for each ligand–receptor pair of two classes of binding sites, high and low. High and low affinity binding sites have been demonstrated previously for EGF binding to HER1 (Wada *et al.*, 1990). (ii) As expected, [^{125}I]HRG-β1 did not bind to HER1, nor did [^{125}I]EGF bind to HER4. (iii) The high affinity K_d values of [^{125}I]HB-EGF and [^{125}I]EGF for HER1 were 0.14 and 0.17 nM respectively. The high affinity K_d values of [^{125}I]HB-EGF and [^{125}I]HRG-β1 for HER4 were 0.17 and 0.08 nM, respectively. The high affinity K_d values obtained for EGF–HER1 and HRG-β1–HER4 interactions are very consistent with previous reports (Wada *et al.*, 1990; Holmes *et al.*, 1992), thereby increasing our confidence in the K_d values obtained for HB-EGF–HER1 and HB-EGF–HER4 interactions. (iv) There were about four times more high affinity binding sites for [^{125}I]EGF than there were for [^{125}I]HB-EGF on cells expressing HER1, and twice as many high affinity binding sites for [^{125}I]HRG-β1 than for [^{125}I]HB-EGF on cells expressing HER4. (v) Compared with the high affinity binding values, the low affinity K_d values were ~10- to 100-fold less and the number of binding sites were 5–50 times greater.

HB-EGF stimulates tyrosine phosphorylation of HER4

To determine whether the binding of HB-EGF to the extracellular domain of HER4 activated this receptor, cells expressing HER1 or HER4 were incubated with or without 100 ng/ml of HB-EGF and, for comparison, with 100 ng/ml EGF or HRG-β1. After immunoprecipitation with anti-HER1 or anti-HER4 antibody, tyrosine phosphorylation of the receptors was analyzed by Western blotting using anti-phosphotyrosine antibodies (Figure 4A) and quantitated (Figure 4B). HB-EGF (Figure 4A, lane 2) and EGF (Figure 4A, lane 3) stimulated an 8-fold and a 9-fold increase, respectively, in HER1 tyrosine phosphorylation. HRG-β1 did not stimulate phosphorylation of HER1 above background (Figure 4A, lane 4). On the other hand, HB-

Table I. Parameters of EGF, HB-EGF and HRG-β1 binding to NIH 3T3 cells expressing either HER1 or HER4

	Cells expressing HER1		Cells expressing HER4	
Ligand	K_d (nM)	Binding sites/cell×10^4	K_d (nM)	Binding sites/cell×10^4
High affinity binding				
HB-EGF	0.14 ± 0.04	1.8 ± 0.5	0.17 ± 0.04	0.9 ± 0.3
EGF	0.17 ± 0.08	7.2 ± 1.7	–	–
HRG-β1	–	–	0.08 ± 0.03	1.9 ± 0.5
Low affinity binding				
HB-EGF	14 ± 1	98 ± 10	17 ± 4	39 ± 13
EGF	1.4 ± 0.1	36 ± 11	–	–
HRG-β1	–	–	5.3 ± 2.5	38 ± 14

Increasing concentrations of [^{125}I]EGF, [^{125}I]HB-EGF and [^{125}I] HRG-β1 were incubated with NIH 3T3 cells expressing either HER1 or HER4. Dissociation constants (K_d) and numbers of binding sites/cell were determined from three independent Scatchard analyses, one of which is shown in Figure 3. Mean K_ds, mean binding sites/cell and their standard deviations were calculated.

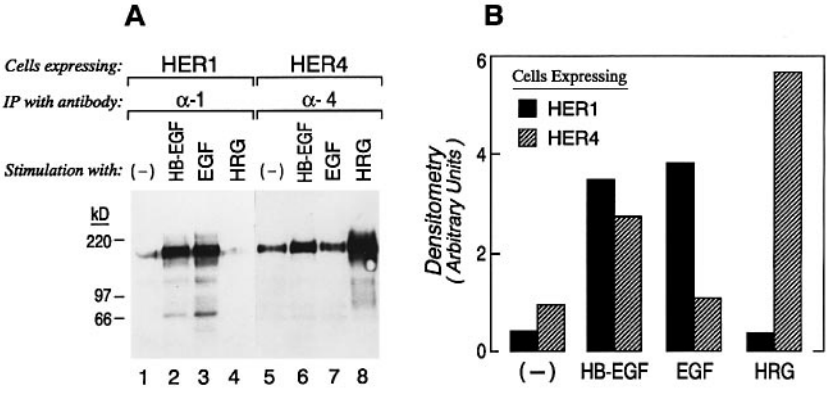

Fig. 4. Tyrosine phosphorylation of EGF receptors in cells expressing HER1 or HER4. (**A**) NIH 3T3 cells expressing HER1 (lanes 1–4) or HER4 (lanes 5–8) were starved for 24 h in a serum-free medium and incubated for 10 min on ice and then for 5 min at 37°C without ligand (lanes 1 and 5) or with 100 ng/ml of HB-EGF (lanes 2 and 6), EGF (lanes 3 and 7) or HRG-β1 (lanes 4 and 8). Cells were lysed and samples of lysates were immunoprecipitated with polyclonal anti-HER1 (lanes 1–4) or anti-HER4 (lanes 4–8) antibodies, followed by SDS–PAGE (6%) and Western blotting with anti-phosphotyrosine antibody. (**B**) The receptor-associated tyrosine phosphorylation stimulated by HB-EGF and EGF in cells expressing HER1 or HER4 shown in (A) was quantitated by densitometry.

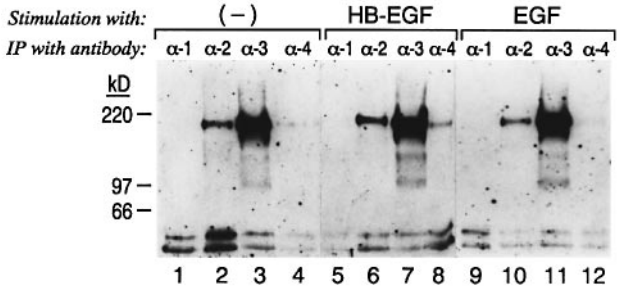

Fig. 5. Tyrosine phosphorylation of EGF receptors in MDA-MB-453 cells. MDA-MB-453 cells were incubated with HB-EGF or EGF, lysed, and lysates were immunoprecipitated with anti-HER1 (lanes 1, 5 and 9), anti-HER-2 (lanes 2, 6 and 10), anti-HER3 (lanes 3, 7 and 11) or anti-HER4 (lanes 4, 8 and 12) antibodies and analyzed for tyrosine phosphorylation as described in Figure 4A. No stimulation (lanes 1–4); 100 ng/ml HB-EGF (lanes 5–8); 100 ng/ml EGF (lanes 9–12).

EGF (Figure 4A, lane 6) and HRG-β1 (Figure 4A, lane 8) stimulated a 3-fold and a 6-fold increase, respectively, in tyrosine phosphorylation of HER4, while EGF (Figure 4A, lane 7) did not activate this receptor above background.

The ability of HB-EGF to stimulate HER4 tyrosine phosphorylation was also tested in non-engineered cells that express endogenous EGF receptors (Figure 5). MDA-MB-453 breast adenocarcinoma cells are known to express HER2, HER3 and HER4, but not HER1 (Plowman *et al.*, 1993a). Thus, these cells are useful for analyzing HER1-independent stimulation of EGF receptors by HB-EGF. Accordingly, MDA-MB-453 cells were incubated with HB-EGF or EGF, followed by immunoprecipitation of cell lysates with the various anti-HER antibodies and Western blotting using anti-phosphotyrosine antibodies. In the absence of added ligand, immunoprecipitation with the relevant antibodies demonstrated that MDA-MB-453 cells phosphorylated HER2 (Figure 5, lane 2) and HER3 (Figure 5, lane 3) constitutively, but not HER1 (Figure 5, lane 1) or HER4 (Figure 5, lane 4). In the presence of either HB-EGF or EGF, there was no induction of tyrosine phosphorylation of HER1 (Figure 5, lanes 5 and 9, respectively), HER2 (Figure 5, lanes 6 and 10, respectively) or HER3 (Figure 5, lanes 7 and 11, respectively). On the other hand, tyrosine phosphorylation of HER4 was induced by HB-EGF (Figure 5, lane 8), but not by EGF (Figure 5, lane 12).

HB-EGF-mediated tyrosine phosphorylation was also tested in non-tumor cells, namely astrocytes which are known to respond to HB-EGF by migrating and proliferating (our unpublished data). Primary rat astrocytes were incubated with either HB-EGF or EGF, and their lysates were immunoprecipitated and analyzed for tyrosine

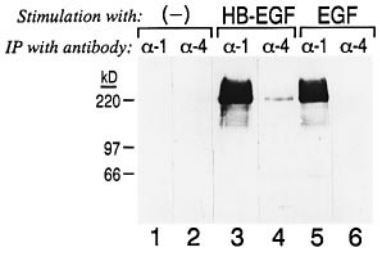

Fig. 6. Tyrosine phosphorylation of EGF receptors in rat astrocytes. Primary rat astrocytes were incubated with HB-EGF or EGF, lysed, and lysates were immunoprecipitated with anti-HER1 (lanes 1, 3 and 5) or anti-HER4 (lanes 2, 4 and 6) antibodies and analyzed for tyrosine phosphorylation as described in Figure 4A. No stimulation (lanes 1 and 2); 100 ng/ml HB-EGF (lanes 3 and 4); 100 ng/ml EGF (lanes 5 and 6).

phosphorylation of HER1 and HER4 by Western blotting (Figure 6). There was very little tyrosine phosphorylation of HER1 or HER4 in the absence of ligand (Figure 6, lanes 1 and 2). HB-EGF (Figure 6, lane 3) and EGF (Figure 6, lane 5) both strongly induced tyrosine phosphorylation of HER1. On the other hand, tyrosine phosphorylation of astrocyte HER4, although weak compared with HER1, was induced by HB-EGF (Figure 6, lane 4), but not by EGF (Figure 6, lane 6). These results are a further demonstration that HB-EGF can activate HER4 in normal non-engineered cells and also show that HER4 activation can occur even when HER1 is activated in the same cell. Whether the tyrosine phosphorylation of HER4 in astrocytes is due to a direct activation or is a result of heterodimerization with HER1 is not known at present.

HB-EGF stimulates proliferation of cells expressing HER1 but not of cells expressing HER4

HB-EGF binds to HER1 and stimulates the proliferation of cells expressing this receptor (Aviezer and Yayon, 1994; Blotnick *et al.*, 1994). Since HB-EGF binds to HER4 and stimulates tyrosine phosphorylation of this receptor, it would seem reasonable that HB-EGF might stimulate the proliferation of cells expressing HER4 as well. Accordingly, cells expressing HER1 or HER4 were treated with HB-EGF and, for comparison, with EGF, HRG-β1 or fibroblast growth factor 2 (FGF-2). Cell number was measured after a 72 h incubation in serum-free medium (Figure 7). HB-EGF increased the number of cells expressing HER1 by 3-fold in a dose-dependent manner (Figure 7A). However, it did not stimulate the proliferation of cells expressing HER4 despite its ability to activate this receptor. As expected, EGF was mitogenic for cells expressing HER1 but not mitogenic for cells expressing HER4 (Figure 7B), consistent with its lack of HER4 binding. HRG-β1, which is a ligand for HER4, but not for HER1, stimulated the proliferation of HER4 cells slightly but did not stimulate the proliferation of HER1 cells (Figure 7C). As a control to show that the cells expressing HER4 are mitogenically responsive, FGF-2, which uses a different receptor system, stimulated a dose-dependent proliferation of cells expressing HER4 or HER1 to the same degree (Figure 7D). Furthermore, the two cell types were equally responsive to 10% fetal bovine serum (FBS) with an 8- to 10-fold increase in cell number after a 72 h incubation (not shown). Comparable results were obtained when the ability of the growth factors to stimulate

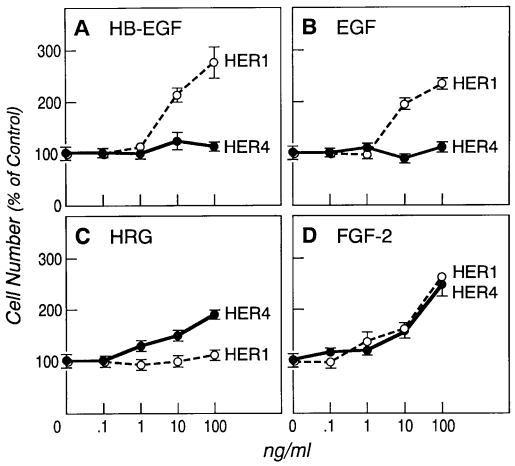

Fig. 7. Proliferation of cells expressing HER1 or HER4 in response to growth factors. Cells (5×10^4 cells/well/24-well plate) expressing HER1 (○) or HER4 (●) were plated in DMEM, 10% FCS. On the next day, the cells were incubated for 6 h in serum-free medium and various concentrations of HB-EGF (**A**), EGF (**B**), HRG-β1 (**C**) or FGF-2 (**D**) were added in fresh serum-free medium. After 72 h, the cells were trypsinized and the cell numbers in each well were counted using a Coulter counter. The cell numbers are expressed relative to the numbers of cells in control wells in which no growth factor was added. Cells in three independent wells were counted and mean cell number and standard deviations were calculated. The baseline number of untreated cells expressing HER1 or HER4 averaged $4.6\times10^4 \pm 0.5\times10^4$ and $7.3\times10^4 \pm 0.9\times10^4$ respectively.

[^{3}H]thymidine incorporation into DNA was measured (not shown). It was concluded that HER1 and HER4, while binding HB-EGF, differ in their ability to respond to HB-EGF as a mitogen.

HB-EGF stimulates chemotaxis of cells expressing HER1 and of cells expressing HER4

HB-EGF was shown previously to be a potent chemoattractant for aortic SMC (Higashiyama *et al.*, 1993). Accordingly, the ability of HB-EGF, and by comparison EGF or HRG-β1, to induce a chemotactic response in cells expressing HER1 or HER4 was analyzed using a modified Boyden chamber assay (Figure 8). Both HB-EGF and EGF stimulated chemotaxis of cells expressing HER1 in a dose-dependent manner (Figure 8A, left). EGF was more potent, with half-maximal stimulation at $<$0.1 ng/ml, while HB-EGF required ~5 ng/ml to obtain the same effect. On the other hand, when cells expressing HER4 were tested, HB-EGF was found to be a potent chemotactic factor with half-maximal stimulation at 1 ng/ml, while EGF stimulated very little if any chemotaxis (Figure 8A, right). The chemotactic effects of HB-EGF for cells expressing HER4 were inhibited by neutralizing anti-HER4 but not by neutralizing anti-HER1 antibodies (not shown), suggesting that the chemotactic effects were not due to low levels of HER1.

A checkerboard analysis (Yoshida *et al.*, 1996) demonstrating that the migratory effects of HB-EGF on cells expressing HER1 and HER4 are indeed chemotaxis, which is directed migration, rather than chemokinesis, which is increased random migration, is shown in Table II. Various concentrations of growth factor were administered to both upper and lower wells of the Boyden chamber apparatus. When no growth factor was added to the upper wells,

cells expressing HER1 and HER4 migrated towards HB-EGF in the lower wells in a dose-dependent manner, as in Figure 8. However, when equal concentrations of HB-EGF were added to both upper and lower wells to destroy the gradient, very little or no HB-EGF-enhanced migration was observed with either cell type (Table II, bold diagonal rows). Thus, HB-EGF does not stimulate migration in the absence of a gradient, characteristic of chemotaxis. Using the same checkerboard assay, the effect of EGF on stimulating the migration of HER1-transfected cells was also found to be predominantly chemotaxis (not shown).

The ability of HB-EGF to stimulate chemotaxis was also compared with that of HRG-β1, a ligand for HER4 but not HER1. HB-EGF, but not HRG-β1, stimulated chemotaxis of cells expressing HER1 (Figure 8B, left), while HB-EGF and HRG-β1 were equally effective as chemotactic factors for cells expressing HER4, with half-maximal stimulation occurring at 0.5–1.0 ng/ml (Figure 8B, right). In replicate experiments, the half-maximal chemotactic stimulation by HB-EGF for cells expressing HER4 (0.5–1.0 ng/ml) appeared to occur at reproducibly lower concentrations than for cells expressing HER1 (2.5–5.0 ng/ml).

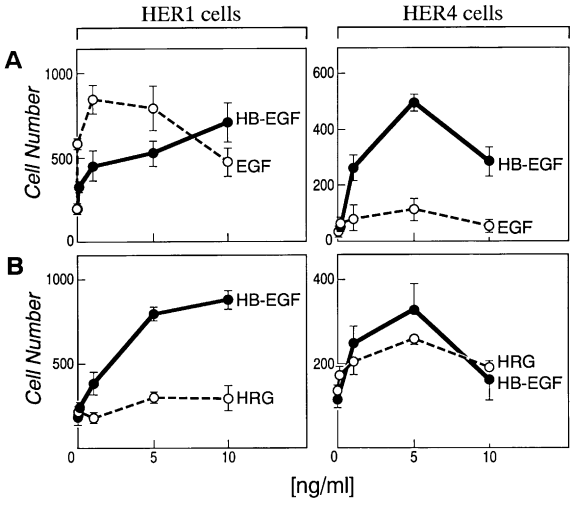

Fig. 8. Dose-dependent chemotaxis of cells expressing HER1 or HER4. The effects of increasing concentrations of growth factors on stimulating chemotaxis were measured in a Boyden chamber. (**A**) HB-EGF (●) and EGF (○) were added to cells expressing HER1 (left) or HER4 (right). (**B**) HB-EGF (●) and HRG-β1 (○) were added to cells expressing HER1 (left) or HER4 (right). Each pair of growth factors was analyzed on one cell type using wells of a single Boyden chamber. The experiments shown in (A), left and right, were carried out at the same time using the same growth factor aliquots. The same is true for the experiments shown in (B). The numbers of cells migrating through the membrane were counted under a microscope. Each point represents the mean cell number and standard deviations of four independent wells. The chemotactic response stimulated with 10% FBS showed a 6.5 ± 1.9-fold increase in cells expressing HER1 and a 4.7 ± 0.8-fold increase in cells expressing HER4.

HB-EGF stimulates association of PI3-K activity with HER4

The signal transduction pathways leading from RTKs to cell motility have been suggested to involve different forms of phosphoinositides (Cantley *et al.*, 1991). Studies with platelet-derived growth factor receptor-β (PDGFR-β) have demonstrated that PI3-K, an enzyme that phosphorylates phosphoinositides at the 3′ position of the inositol ring, is needed for the chemotactic response stimulated by PDGF-BB (Kundra *et al.*, 1994; Wennström *et al.*, 1994b). As the cytoplasmic tail of HER4 but not HER1 (Fedi *et al.*, 1994; Soltoff *et al.*, 1994) was described recently to contain a binding site for the p85 subunit of PI3-K (Cohen *et al.*, 1996), it was of interest to determine whether HB-EGF could induce the association of PI3-K activity with phosphorylated HER4. Accordingly, cells expressing HER4 were stimulated with HB-EGF or EGF and lysed, and the lysates were immunoprecipitated with a polyclonal anti-HER4 antibody. PI3-K activity co-precipitating with HER4 subsequently was measured using an *in vitro* kinase assay in which phosphorylation of PI in the presence of [γ-^{32}P]ATP to form [^{32}P]PI was analyzed by thin-layer chromatography (TLC) and autoradiography (Figure 9A). [^{32}P]PI immunoprecipitated from lysates of unstimulated cells expressing HER4 with anti-p85 antibodies was used as a standard and reflects the basal PI3-K activity of unstimulated cells (Figure 9A, lane 4). The [^{32}P]PI on the TLC membrane was quantitated using a PhosphorImager (Figure 9B). HB-EGF stimulated a 2.6-fold increase (Figure 9A, lane 2) in PI3-K activity associated with HER4 compared with non-stimulated cells (Figure 9A, lane 1). EGF (Figure 9A, lane 3), however, did not stimulate any increase in HER4-associated PI3-K activity, as expected, since it is not a ligand for HER4. There was no PI3-K activity co-precipitated directly with anti-HER1 antibody in cells expressing HER1 and stimulated with either HB-EGF or EGF (not shown), consistent with previous results (Fedi *et al.*, 1994; Soltoff *et al.*, 1994).

Wortmannin inhibits HB-EGF-stimulated chemotaxis of HER4-expressing cells

Wortmannin binds to the p110 subunit of PI3-K and inhibits PI3-K activity (Yano *et al.*, 1993). Since HB-EGF

Table II. Checkerboard analysis of HB-EGF-stimulated migration of HER1- and HER4-expressing cells

	Cells expressing HER1					Cells expressing HER4			
ng/ml	*0*	*1*	*5*	*10*	ng/ml	*0*	*1*	*5*	*10*
0	**230**	183	161	97	*0*	**238**	206	208	228
1	306	**197**	194	181	*1*	354	**242**	245	221
5	369	256	**167**	179	*5*	521	270	**280**	273
10	685	421	310	**205**	*10*	473	268	256	**265**

Concentrations of ligand (ng/ml) added to the upper wells (horizontal lines) or lower wells (vertical lines) of the Boyden chamber apparatus are shown in italics. The number of cells which migrated to the lower wells is shown as a mean of measurements made in four independent wells. Standard deviations were ≤10%. The numbers of cells which migrated in the absence of a concentration gradient (same concentration of ligand in the upper and lower wells) are shown in bold.

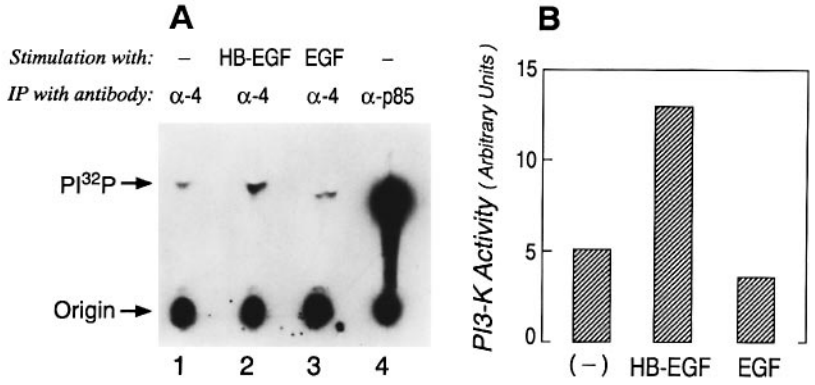

Fig. 9. Ligand-stimulated PI3-K activity. (**A**) Cells expressing HER4 were stimulated without (lanes 1 and 4) or with 100 ng/ml of HB-EGF (lane 2) or with 100 ng/ml of EGF (lane 3). The cells were lysed and aliquots from the lysates were immunoprecipitated with polyclonal anti-HER4 antibodies (lanes 1–3) or with monoclonal anti-p85 antibodies (lane 4). The co-precipitated material was analyzed for PI3-K activity using an *in vitro* kinase assay that measures incorporation of ^{32}P into [^{32}P]PI using TLC. The positions of [^{32}P]PI and the origin of the chromatography are indicated. (**B**) The amount of [^{32}P]PI associated with the TLC membrane shown in (A) was quantitated using a PhosphorImager (Molecular Dynamics).

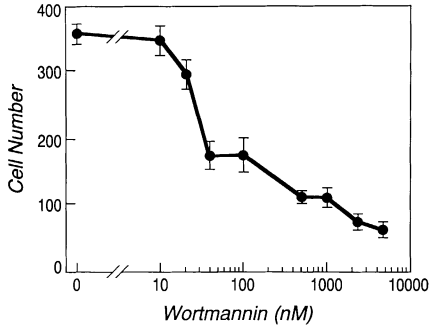

Fig. 10. Wortmannin inhibition of chemotaxis. Various concentrations of wortmannin were added to suspensions of NIH 3T3 cells expressing HER4 which were plated into the upper wells of a Boyden chamber. 5 ng/ml of HB-EGF was added to the lower wells. Chemotaxis was measured as in Figure 8. The baseline number of cells migrating in the absence of HB-EGF was 150.

induces HER4-associated PI3-K activity and stimulates chemotaxis of cells expressing HER4, wortmannin was tested for its effects on HB-EGF-induced chemotaxis of these cells (Figure 10). Wortmannin inhibited the chemotactic response of cells expressing HER4 to HB-EGF in a dose-dependent and non-toxic manner. Complete inhibition of HB-EGF-induced chemotaxis occurred at 25–100 nM. At very high concentrations, ⩾500 nM, wortmannin also inhibited background chemotaxis in the absence of added growth factor. These results suggest that PI3-K activity is involved in the signal transduction pathway of chemotaxis mediated by HER4.

Discussion

HB-EGF is capable of binding to, and activating two tyrosine kinase receptors, HER1 and HER4, but the biological responses that result from these interactions differ. Activation of HER1 by HB-EGF results in chemotaxis and in proliferation, while activation of HER4 results in chemotaxis but not in proliferation. Thus, this ligand is capable of activating differential responses by binding to different receptors.

The binding and activation of HER1 by HB-EGF confirms previous reports (Higashiyama *et al.*, 1991, 1992; Aviezer and Yayon, 1994). However, the result that HER4 is a receptor for HB-EGF is apparently a novel finding. NIH 3T3 cells expressing either HER1, HER2, HER3 or HER4 (Zhang *et al.*, 1996) were used to analyze HB-EGF–HER interactions. While HB-EGF could bind to the cells expressing HER1 or HER4, it did not bind to cells expressing HER2 or HER3. The evidence that HB-EGF interacts with HER4 in an active manner is as follows. (i) HB-EGF cross-links NIH 3T3 cells expressing solely HER4 and the binding is inhibited by neutralizing anti-HER4, but not by neutralizing anti-HER1 antibodies. (ii) HB-EGF binds to HER4 with high affinity, with a K_d of ~0.2 nM. This K_d is similar to that of EGF for HER1 (Table I; Wada *et al.*, 1990) and to that of HRG-β1 for HER4 (Table I; Holmes *et al.*, 1992). HB-EGF also has low affinity binding sites for HER4 with a K_d of ~17 nM. (iii) HB-EGF stimulates HER4 tyrosine phosphorylation and HER4 association of PI3-K activity in cells expressing solely HER4. (iv) HB-EGF is highly chemotactic, with a half-maximal stimulation at 0.5–1.0 ng/ml, for cells expressing solely HER4. Chemotaxis is inhibited by neutralizing anti-HER4, but not by neutralizing anti-HER1 antibodies.

Since HB-EGF also activates HER1, a receptor that can activate the other EGF receptors indirectly by heterodimerization (Wada *et al.*, 1990; Soltoff *et al.*, 1994; Riese *et al.*, 1996b), it was important to demonstrate that cells expressing HER4 did not express any HER1 in order to rule out the possibility that the effects of HB-EGF on cells expressing HER4 were actually results of HER1–HER4 heterodimerization. First of all, analysis of HER4 transfectants showed that these cells did not express detectable levels of HER1 when analyzed by Western blotting with anti-HER1 antibodies (Zhang *et al.*, 1996). Secondly, EGF, a well characterized ligand for HER1, did not bind to or induce any cellular responses such as tyrosine phosphorylation, chemotaxis or proliferation in cells expressing HER4, although, as expected, it stimulated all of these activities in cells expressing HER1. Moreover, EGF did not induce HER4 phosphorylation even in rat primary astrocytes which express abundant HER1 and are capable putatively of HER1–HER4 dimerization. Finally, neutralizing monoclonal antibodies directed against HER1 inhibited HB-EGF binding to, and activation of HER1 as

expected, but did not abrogate any of the activities of HB-EGF for HER4.

The results presented here, as well as in previous studies (Beerli and Hynes, 1996; Riese *et al.*, 1996a,b), suggest that EGF family ligands fall into at least three categories with respect to growth factor–receptor interactions. The first category includes EGF, TGF-α and AR which bind to and activate HER1 directly. These growth factors do not induce receptor autophosphorylation or other cellular activities directly in cells expressing HER2, HER3 or HER4 unless HER1 is expressed as well, allowing the formation of heterodimers. The second category includes the products of the HRG gene which, as we have confirmed, do not interact directly with HER1 (Peles *et al.*, 1992; Riese *et al.*, 1995). Instead, HRG is a ligand for HER3 (Carraway *et al.*, 1994) and HER4 (Plowman *et al.*, 1993b). HB-EGF falls into the third category, a ligand that can bind to and activate both HER1 and HER4 independently of each other without the necessity for heterodimerization. βC appears to be in the same category as HB-EGF since βC activates tyrosine phosphorylation of both HER1 and HER4 in the T47D breast tumor cell line which expresses all four known EGF receptor subtypes, and the activation of HER4 in these cells is only partially attributable to heterodimerization (Beerli and Hynes, 1996). Furthermore, βC induces tyrosine phosphorylation of both HER1 and HER4, but not of HER2 or HER3, in interleukin-3 (IL-3)-dependent lymphoid cell lines transfected individually with the various EGF receptors (Riese *et al.*, 1996a). Interestingly, HB-EGF does not activate HER4 in lymphoid cells expressing this receptor alone for reasons that are not clear (Riese *et al.*, 1996b). One possibility is that the lymphoid cells lack cell surface heparan sulfate proteoglycans which are known to enhance markedly HB-EGF binding and chemotactic activity (Higashiyama *et al.*, 1993).

HB-EGF induces differential cellular responses when activating HER1 or HER4. In particular, while HB-EGF is chemotactic for cells expressing either receptor, it is mitogenic for cells expressing HER1 but not for cells expressing HER4. Lack of HB-EGF mitogenic activity in cells expressing HER4 is not due to a lack of any growth potential in these cells since they are capable of a mitogenic response, for example in response to FGF-2. The mechanisms by which HB-EGF is able to stimulate proliferation of cells expressing HER1 but not HER4 are not understood as of yet. We have found that HB-EGF stimulates relatively less tyrosine phosphorylation of HER4, ~2- to 3-fold, as compared with HER1. In addition, while the K_ds for both the high and the low affinity components of HB-EGF binding to HER1 as compared with HER4 are similar, there are about twice the number of available binding sites for HB-EGF on the surface of cells expressing HER1 compared with HER4. Thus, we speculate that HB-EGF, due to lowered binding and tyrosine phosphorylating ability, may not be able to mobilize the necessary signal transduction pathway molecules needed to stimulate proliferation via HER4. An alternative explanation for the lack of HB-EGF-induced proliferation of cells expressing HER4 is that signal transduction pathways that down-regulate proliferation are activated when HER4 is stimulated by HB-EGF. For example, phosphorylation of EGF-activated HER1 at Thr654 by protein kinase C (PKC) results in a reduced proliferative response without affecting the migratory response (Bowen *et al.*, 1991; Chen *et al.*, 1996).

Besides HB-EGF, we have found that the other known ligands for HER4, HRG-β1 (Figure 8B) and βC (our unpublished data), stimulate the migration of cells expressing HER4. On the other hand, EGF (Figure 8A), TGF-α and AR do not stimulate the migration of these cells (our unpublished data) as expected, given that they do not bind to HER4. Unlike HB-EGF, HRG-β1 stimulates proliferation in cells expressing HER4. One possible explanation for this differential effect is that HRG-β1 interacts with HER4 to a greater extent than does HB-EGF. Compared with HB-EGF, HRG-β1 has a 2-fold higher affinity, has twice the number of high affinity binding sites and shows a 2-fold enhanced level of HER4 tyrosine phosphorylation. Thus HRG-β1 activation of HER4 may result in signal transduction that is quantitatively or qualitatively different from that induced by HB-EGF resulting in proliferation. The ability of members of a receptor family, such as HER1 and HER4, to transduce different biological responses indicates that multiple homologous EGF receptor subtypes have evolved to provide additional specificity in response to ligand binding.

Our results suggest that activation and recruitment of PI3-K might be involved in HER4-mediated chemotaxis. First of all, HB-EGF (Figure 9), HRG-β1 (our unpublished data) and HRG-β2 (Cohen *et al.*, 1996) stimulate association of PI3-K with HER4. Secondly, wortmannin, an inhibitor of PI3-K activity (Yano *et al.*, 1993), inhibits HB-EGF-stimulated migration of cells expressing HER4 in a dose-dependent manner. However, a definitive role for PI3-K in HB-EGF-stimulated chemotaxis would require inactivation of this receptor by site-directed mutagenesis of the HER4 PI3-K binding domain (Tyr1056; Cohen *et al.*, 1996) or a dominant-negative strategy that would inactivate PI3-K (Wennström *et al.*, 1994a). Interestingly, HB-EGF does not stimulate direct association of PI3-K with HER1, yet wortmannin inhibits chemotaxis of cells expressing HER1 (our unpublished data). A plausible explanation is that HER1 associates with PI3-K indirectly in these cells, for example via Grb2-associated binder-1 (Gab1) that can link Grb2 to PI3-K (Holgado-Madruga *et al.*, 1996). HB-EGF might also stimulate indirect PI3-K interaction with HER4, resulting in an increased level of total cellular PI3-K above and beyond the increase in direct HER4-associated PI3-K, but this possibility has not been tested yet. In any case, the differential ability of HER4 and HER1 to bind PI3-K in response to HB-EGF demonstrates clearly that there are differences in the signaling cascades generated by activating HER4 as compared with HER1. These differences could affect the local concentrations of PI3-K and/or its activity as well as the availability of binding sites for other signal transduction molecules. Differential recruitment of PI3-K activity in response to HER4 or HER1 stimulation is supported further by our finding that wortmannin inhibits HER4-mediated chemotaxis at significantly lower concentrations compared with HER1-mediated chemotaxis (our unpublished data).

A role for PI3-K in HER4-mediated chemotaxis, but not necessarily mitogenesis, is consistent with previous

reports for other receptors. For example, both PI3-K and phospholipase C-γ (PLC-γ) are required for PDGFR-β-mediated chemotaxis in response to PDGF-BB (Kundra *et al.*, 1994; Wennström *et al.*, 1994b). Mutating the binding sites of PI3-K for PDGFR-β blocks PDGF-stimulated chemotaxis and membrane ruffling without affecting mitogenesis (Wennström *et al.*, 1994b). Wortmannin also inhibits hepatocyte growth factor (HGF)-stimulated chemotaxis and tubulogenesis of renal tubular cells with a small effect on mitogenesis (Derman *et al.*, 1995). Although PLC-γ has also been implicated in mediating cell movement, for example in response to PDGF via PDGFR-β (Kundra *et al.*, 1994) and EGF via HER1 (Chen *et al.*, 1994), it is not clear yet whether this enzyme plays a role in the HB-EGF-stimulated chemotaxis of cells expressing HER4.

Whether HB-EGF might be a ligand for HER4 *in vivo* is not known since no extensive immunolocalization studies have yet been reported for either molecule. Northern blot analyses, however, indicate that both HB-EGF and HER4 transcripts are strongly expressed in heart, skeletal muscle, brain and kidney (Abraham *et al.*, 1993; Plowman *et al.*, 1993a). Targeted disruption of the HER4 gene generates clearly defined defects in the developing central nervous system and heart (Gassmann *et al.*, 1995). Based on analysis of these mice, it has been suggested that putative HER4 ligands other than HRG might be involved in hindbrain development (Gassmann *et al.*, 1995; Meyer and Birchmeier, 1995). Targeted disruption of the HB-EGF gene has yet to be reported.

In summary, we have shown for the first time that HB-EGF is a ligand for HER4 as well as for HER1, and that interaction of HB-EGF with these two receptors results in different biological responses. In particular, HB-EGF activation of HER4 stimulates chemotaxis but not proliferation, while activation of HER1 stimulates both processes. Our future goals include determining the biological significance of HB-EGF–HER4 interactions and delineating the mechanisms by which chemotaxis and proliferation are dissociated in cells expressing HER4.

Materials and methods

Growth factors and antibodies

Recombinant human HB-EGF and FGF-2 were provided by Dr J.Abraham (Scios Nova, Mountainview, CA). Recombinant human HRG-β1 (residues 177–241) was provided by Dr M.Sliwkowski (Genetech, Inc., South San Francisco, CA). [^{125}I]ARIA-1 (the extracellular domain of recombinant chicken proARIA-1; 1.4×10^4 c.p.m./ng) (Falls *et al.*, 1993) was provided by Drs A.Goodearl and G.Fischbach (Harvard Medical School, Boston, MA). Mouse monoclonal anti-phosphotyrosine antibody (4G10) was provided by Dr B.Drucker (Dana Farber Cancer Institute, Boston, MA), and mouse monoclonal antibody directed against the p85 subunit of PI3-K by Dr L.Cantley (Harvard Medical School, Boston, MA). Recombinant human EGF was purchased from Intergen. Sheep polyclonal (#06-129) and mouse monoclonal (clone LA1) antibodies directed against human HER1 were purchased from UBI. Mouse monoclonal antibody (clone H4.72.8) that detects the extracellular domain of human HER4 was purchased from Neomarkers. Rabbit polyclonal antibodies raised against peptides corresponding to sequences from the cytoplasmic domains of human HER2 (C-18), HER3 (C-17) and HER4 (C-18) were purchased from Santa Cruz Biotechnology Inc. Peroxidase-conjugated rabbit anti-mouse IgG antibody was purchased from Sigma. Polyclonal rabbit anti-cow glial fibrillary acidic protein (GFAP) was purchased from DAKO.

Cell culture

NIH 3T3, clone 7 cell lines expressing HER1, HER2, HER3 or HER4 in a stable manner were a generous gift of Dr K.Zhang (Amgen, Thousand Oaks, CA). These cells had been transfected with full-length human cDNAs, cloned and characterized. Western blot analysis indicated that the four cell lines have similar HER expression levels (Zhang *et al.*, 1996). The transfectants were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 1% glutamine/penicillin/streptomycin supplement (GPS; Irvine Scientific), 4.5 g/l glucose and 250 μg/ml G418 (Geneticin; Gibco BRL). No changes in receptor expression levels were observed upon passage. The MDA-MB-453 human breast cancer cell line (ATCC; HB 8506) was grown in DMEM containing 10% FBS and 1% GPS. Primary cultures of rat astrocytes were produced as described (McCarthy and de Vellis, 1980). The astrocytes were passaged once before use. The purity of the astrocyte cultures obtained (~99%) was confirmed by staining with GFAP-specific antibodies.

Radioiodination and cross-linking

HB-EGF, EGF and HRG-β1 were iodinated using IODO-BEADS (Pierce) according to the manufacturer's instructions. Na^{125}I (NEN) was used at 100 μCi/μg of protein. Free iodine was separated from radioiodinated proteins using Sephadex G-25 size exclusion columns (NAP-5; Pharmacia). Specific activities of 2×10^4–1.2×10^5 c.p.m./ng were obtained. For cross-linking of growth factors to cell surface receptors, cells were plated on 28 cm^2 dishes and grown to confluency. Cell layers were washed once with PBS and 5–20 ng/ml of radioiodinated growth factor was added in 1.5 ml of binding buffer (DMEM, 20 mM HEPES, pH 7.4, 0.1 mg/ml gelatin). After a 2 h incubation in a horizontal shaker, cells were washed with PBS and treated for 14 min with 200 μM disuccinimidyl suberate (DSS; Pierce) in PBS. To stop the cross-linking reaction, 200 μl of 10× concentrated stop buffer (10 mM Tris–HCl, pH 7.4, 250 mM glycine, 2 mM EDTA) was added for 1 min. Cells were washed twice with PBS and scraped into 1.4 ml of scrape buffer [PBS containing 2.5 mM EDTA and 1 mM aminoethylbenzenesulfonyl fluoride (AEBSF)]. The harvested cells were pelleted and lysed for 20 min with 40 μl of lysis buffer (10 mM Tris–HCl, pH 7.0, 1% NP-40, 1 mM EDTA, 1 mM AEBSF). All steps were performed at 4°C except for the 14 min DSS treatment which was done at room temperature. Aliquots of the lysates were centrifuged and the supernatants were combined with SDS–PAGE sample buffer, boiled for 5 min and analyzed by 6% SDS–PAGE (Laemmli *et al.*, 1970). Cross-linked complexes were visualized by exposing dried gels to X-ray films. For neutralization of growth factor–receptor interactions, 5 μg/ml of neutralizing anti-HER1 antibody (LA1) or 5 μg/ml of neutralizing anti-HER4 antibody (H4.72.8) were added along with 10 ng/ml of [^{125}I]HB-EGF in the cross-linking reactions.

Ligand binding assay and Scatchard blot

Transfected NIH 3T3 cells were grown to confluency in 96-well plates, washed once with 2 M NaCl, 10 mM Tris–HCl, pH 7.4, and twice with ice-cold PBS. Increasing concentrations of radioiodinated ligands were added to the wells in 100 μl of PBS containing 1 mg/ml bovine serum albumin. After a 90 min incubation on ice, the cell layers were washed three times with ice-cold 2 M NaCl, 10 mM Tris–HCl, pH 7.4, to remove any ligand bound with low affinity to cell surface heparan sulfate (Moscatelli, 1987). The cells were lysed with 0.2 M NaOH and bound radioactivity was measured with a γ-counter. Non-specific binding was defined as the amount of binding obtained after adding a 200-fold excess of unlabeled ligand to cells together with the labeled ligand. The non-specific binding, typically 10%, was subtracted from the total binding. Ligand binding data were used to generate saturation curves and Scatchard blots, and apparent K_d values were determined by linear regression analysis using the LIGAND computer program (Munson and Rodbard, 1980).

Immunoprecipitation

Immunoprecipitation of growth factor–receptor complexes was carried out following growth factor stimulation or [^{125}I]growth factor cross-linking to cells. For immunoprecipitation following growth factor stimulation, cells were grown to confluency in 176.6 cm^2 dishes and starved for 24 h in a serum-free DMEM, 1% GPS. Cells were treated with HB-EGF, EGF or HRG-β1 (100 ng/ml) suspended in DMEM, 1% GPS for 10 min on ice followed by 5 min at 37°C. The cells were scraped into a lysis buffer containing 1% NP-40, 10 mM Tris–HCl, pH 7.0, 1 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate. Lysates were immunoprecipi-

tated with polyclonal anti-HER1, anti-HER2, anti-HER3, anti-HER4 or monoclonal anti-p85 antibodies (all in 1:100 dilutions). All steps were performed at 4°C. The immunoprecipitated samples were analyzed for phosphotyrosine content by Western blotting or for PI3-K activity as described below. For immunoprecipitation following cross-linking, cells were lysed after the final PBS wash following the cross-linking reaction with DSS, with lysis buffer supplemented with 10 μg/ml aprotinin and 10 μg/ml leupeptin, and immunoprecipitation with receptor-specific antibodies was performed as described above. After immunoprecipitation, samples were analyzed by 6% SDS–PAGE and autoradiography.

Western blot analysis for tyrosine-phosphorylated proteins

For analysis of receptor tyrosine phosphorylation, immunoprecipitated proteins were separated on 6% SDS–PAGE gels and transferred onto nitrocellulose filters (0.1 μm; Schleicher and Schuell) as previously described (Higashiyama *et al.*, 1994). Non-specific binding to filters was blocked with a 2 h incubation in 5% non-fat dry milk, TBS-T (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.05% Tween-20), followed by successive incubations with the anti-phosphotyrosine antibody (4G10; 1:10 000) and peroxidase-conjugated anti-mouse antibody (1:40 000), both in 5% milk, TBS-T. The immobilized peroxidase activity was detected by enhanced chemiluminescence (ECL; Amersham).

Receptor-associated PI3-K activity

Lysates from growth factor-treated or untreated NIH 3T3 transfectants were prepared and immunoprecipitated with polyclonal anti-HER4 and monoclonal anti-p85 antibodies as described above. An *in vitro* kinase assay for PI3-K was used to measure PI3-K activity in the immunoprecipitates as previously described (Whitman *et al.*, 1985; Auger *et al.*, 1989). Briefly, the phosphorylation of PI (Avanti Polar Lipids) with [γ-^{32}P]ATP (DuPont) to form [^{32}P]PI was analyzed using TLC, and [^{32}P]PI was visualized by autoradiography and quantitated with a PhosphorImager (Molecular Dynamics).

Cell proliferation and chemotaxis

For proliferation assays, cells were plated at 5×10^4 cells/well in 24-well cell culture plates in DMEM, 10% FBS, 1% GPS, 4.5 g/l glucose and 250 μg/ml G418. Next day the medium was replaced with DMEM, 1% GPS. Six hours later, the medium was aspirated and growth factors were added in DMEM, 1% GPS. Seventy two hours after growth factor addition, the cells were trypsinized and cell numbers were estimated using a Coulter counter (Coulter Electronics). Chemotaxis measurements using a modified Boyden chamber assay and checkerboard analysis were carried out by methods previously described (Boyden, 1962; Yoshida *et al.*, 1996). In inhibition studies, 5 μg/ml of neutralizing anti-HER1 (LA1) antibodies, 5 μg/ml of neutralizing anti-HER4 (H4.72.8) antibodies or various concentrations of wortmannin (Sigma) were added to cell suspensions prior to addition of the cells to the Boyden chamber wells.

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