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Differential endocytic routing of homo- and hetero-dimeric ErbB tyrosine kinases confers signaling superiority to receptor heterodimers

Anne E.G.Lenferink, Ronit Pinkas-Kramarski[1], Monique L.M.van de Poll, Marianne J.H.van Vugt, Leah N.Klapper[2], Eldad Tzahar[1], Hadassa Waterman[1], Michael Sela[2], Everardus J.J.van Zoelen and Yosef Yarden[1,3]

Department of Cell Biology, University of Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands and Departments of [1]Biological Regulation and [2]Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

[3]Corresponding author
e-mail: liyarden@weizmann.weizmann.ac.il

Both homo- and hetero-dimers of ErbB receptor tyrosine kinases mediate signaling by a large group of epidermal growth factor (EGF)-like ligands. However, some ligands are more potent than others, although they bind to the same direct receptor. In addition, signaling by receptor heterodimers is superior to homodimers. We addressed the mechanism underlying these two features of signal tuning by using three ligands: EGF; transforming growth factor α (TGFα); and their chimera, denoted E4T, which act on cells singly expressing ErbB-1 as a weak, a strong, and a very strong agonist, respectively. Co-expression of ErbB-2, a developmentally important co-receptor whose expression is frequently elevated in human cancers, specifically potentiated EGF signaling to the level achieved by TGFα, an effect that was partially mimicked by ErbB-3. Analysis of the mechanism underlying this *trans*-potentiation implied that EGF-driven homodimers of ErbB-1 are destined for intracellular degradation, whereas the corresponding heterodimers with ErbB-2 or with ErbB-3, dissociate in the early endosome. As a consequence, in the presence of either co-receptor, ErbB-1 is recycled to the cell surface and its signaling is enhanced. This latter route is followed by TGFα-driven homodimers of ErbB-1, and also by E4T-bound receptors, whose signaling is further enhanced by repeated cycles of binding and dissociation from the receptors. We conclude that alternative endocytic routes of homo- and hetero-dimeric receptor complexes may contribute to tuning and diversification of signal transduction. In addition, the ability of ErbB-2 to shunt ligand-activated receptors to recycling may explain, in part, its oncogenic potential.

Keywords: endocytosis/ErbB/HER family/oncogene/signal transduction/transforming growth factor α

Introduction

A large group of polypeptide growth factors mediates intercellular signaling by binding to, and activation of, transmembrane allosteric kinases with specificity to tyrosine residues (van der Geer *et al*., 1994). As in other allosteric systems, the monomeric form of the receptor tyrosine kinase (RTK) is inactive, but upon ligand-induced oligomerization (primarily dimerization) it initiates a plethora of intracellular events ranging from stimulation of ion fluxes to cytoskeletal alterations, and culminating in regulation of gene expression. The underlying biochemical mechanism involves autophosphorylation of specific tyrosine residues of the activated receptor. These are turned into docking sites for cytoplasmic signaling proteins containing Src-homology 2 (SH-2) domains (Koch *et al*., 1991), such as the adapter molecules SHC, Sem-5/Grb-2 and the p85 subunit of phosphatidylinositol 3′ kinase (Eagan and Weinberg, 1993). As a consequence thereof, several linear cascades of protein kinases are triggered, including the mitogen-activated protein kinase (MAPK) pathway (Seger and Krebs, 1995) and the S6-kinase pathway (Ming *et al*., 1994).

In addition to this 'vertical' transduction pathway, lateral propagation of growth factor signals is made possible within subgroups of homologous RTKs by means of receptor heterodimerization. The best characterized example of 'lateral' signaling is provided by the type I RTKs (also named ErbB or HER family) (Carraway and Cantley, 1994; Alroy and Yarden, 1997). This subfamily comprises four members whose prototype is ErbB-1, a receptor that binds several ligands, including epidermal growth factor (EGF) and transforming growth factor (TGFα). Likewise, ErbB-3 and ErbB-4 bind three groups of alternatively spliced growth factors, collectively called neuregulins (Burden and Yarden, 1997). The fourth member, ErbB-2, binds no known ligand with high affinity. Nevertheless, impairment of ErbB-2 function by gene targeting resulted in a phenotype shared with that of neuregulin- and ErbB-4-deficient embryos (Lee *et al*., 1995), and a mutant form of this receptor promotes cancer in rodents (Bargmann *et al*., 1986). Overexpression of the wild-type human protein leads to phenotypic transformation of cultured cells (Di Fiore *et al*., 1987; Hudziak *et al*., 1987), and is frequently observed in several types of human carcinomas (Slamon *et al*., 1987, 1989). Moreover, ErbB-2 overexpression predicts poor prognosis and resistance to certain therapeutic modalities, implying that the orphan receptor contributes to tumor virulence (reviewed in Hynes and Stern, 1994; Stancovski *et al*., 1994). Despite the absence of a direct ligand, ErbB-2 plays a central role in a network of inter-receptor interactions; although the four ErbBs can form all 10 possible homo- and hetero-dimeric combinations, ErbB-2-containing heterodimers are preferred over other combinations (Tzahar *et al*., 1996; Graus-Porta *et al*., 1997). Each dimeric receptor complex has a distinct signaling potency, resulting in diversification and fine-tuning of signaling (Riese *et al*., 1995; Pinkas-

Kramarski *et al*., 1996a). In general, signaling by homodimeric complexes is relatively weak, whereas heterodimers, and especially those containing ErbB-2, are more potent transmitters of signals. The collaborative action of two different ErbBs is best exemplified by the potent combination of ErbB-2, the ligandless receptor, with ErbB-3, whose kinase function is defective, and is reflected by the synergistic effect on cell transformation of certain co-expressed pairs of ErbBs (Kokai *et al*., 1989; Alimandi *et al*., 1995; Wallasch *et al*., 1995).

In addition to the receptor level, combinatorial signaling by the ErbB network is further diversified at two additional levels. First, multiple EGF-like ligands exist and they differentially induce certain receptor combinations (Pinkas-Kramarski *et al*., 1996b), probably because each ligand carries not only a high affinity site, but also a 'low affinity/broad specificity' site that recruits the dimer's partner (Tzahar *et al*., 1997). Interestingly, some ligands induce more potent signals than others although they bind to the same receptor. For example, on certain cellular systems, such as keratinocytes (Barrandon and Green, 1987) and endothelial cells (Schreiber *et al*., 1986), TGFα is more potent than EGF, although both ligands bind to ErbB-1 with comparable affinity (Kramer *et al*., 1994). Another level of signal diversification is comprised of the multiple substrates of RTKs; members of this large group of SH-2 domain-containing proteins are differentially recruited to certain ErbBs. Examples include the phosphatidylinositol 3′-kinase and c-Cbl that preferentially engage with ErbB-3 (Soltoff *et al*., 1994) and with ErbB-1 (Levkowitz *et al*., 1996), respectively. Despite differences in second messenger activation, signaling by all ErbBs feeds into the MAPK pathway, raising the question of how signal specificity is maintained intracellularly. One potential answer is provided by results obtained with other growth factors in pheochromocytoma cells, indicating that the kinetics of MAPK activation, and especially its inactivation, may critically determine signal identity (reviewed in Marshall, 1995). Unlike the activation process which has been extensively studied, the inactivation phase of RTK signaling is poorly understood. One obvious candidate is the process that leads to endocytosis, down-regulation and degradation of ligand-activated receptors. Indeed, individual ErbB proteins differ remarkably in their rate of endocytosis and down-regulation (Baulida *et al*., 1996; Pinkas-Kramarski *et al*., 1996a).

Our present study addressed the hypothesis that the multiple ligands of ErbBs differ in their potencies because they differentially recruit certain heterodimeric receptor combinations (Beerli and Hynes, 1996; Pinkas-Kramarski *et al*., 1996b; Gulliford *et al*., 1997). To this end we compared signaling by EGF and TGFα, a pair of ligands that display respectively weak and strong signaling in most tissues, in a well-defined cellular system expressing combinations of ErbB-1 with either ErbB-2 or ErbB-3. In contrast to our working hypothesis, differences in potency were observed even in the absence of either co-receptor, namely ErbB-2 or ErbB-3. However, to our surprise, the co-receptors potentiated the effect of EGF without significantly affecting TGFα signaling. In subsequent experiments we investigated the mechanism of potentiation and found that the co-receptors, by forming heterodimers with ErbB-1, redirected this receptor to an endocytic route that allows receptor recycling and, therefore, enhanced signaling. These results imply that EGF-like ligands whose ErbB specificity is shared are functionally distinct, and suggest that alternative endocytic routing may be critical for controlled inactivation and fine-tuning of signal transduction.

Results

ErbB-2 and ErbB-3 potentiate EGF mitogenicity but not TGFα signaling

To examine possible functional relationships between the multiplicity of EGF-like ligands and the extensive inter-receptor interactions within the ErbB family of receptors we used the two best characterized ligands of the family, namely EGF and TGFα, in combination with a series of cell lines co-expressing ErbB-1 with either ErbB-2 (D12 cells), or with ErbB-3 (D13 cells) (Pinkas-Kramarski *et al*., 1996a). A third cell line that singly expresses ErbB-1 (D1 cells) was used for comparison of ErbB-1 homodimers with heterodimers of this receptor. In addition, a chimeric EGF/TGFα molecule, designated E4T, comprised of the A and B loops of EGF, and the C loop of TGFα, was used because of its superior mitogenic activity to that of other chimeric molecules and the parental ligands (Lenferink *et al*., 1997). Due to their dependence on interleukin-3 (IL-3), the cell lines we employed are extremely sensitive to EGF-like ligands when tested in the absence of IL-3. Thus, TGFα exerted mitogenic stimuli that were at least 10-fold more active than EGF-induced signals when tested on D1 cells (Figure 1A). However, E4T was even more potent in inducing cell proliferation. This pattern of relative potency was also reflected in long-term survival experiments in which IL-3 was replaced by the corresponding ErbB-1 ligand and cell survival monitored daily (Figure 1B). Introduction of ErbB-2 into D1 cells elevated the basal proliferation rate of the resulting cell line, D12, in agreement with previous reports (Kokai *et al*., 1989; Cohen *et al*., 1996; Tzahar *et al*., 1996; Zhang *et al*., 1996). Thus, whereas maximal stimulation of D1 cells by IL-3 was 5.5-fold, only a 2-fold activation was displayed by D12 cells. Interestingly, however, co-expression of ErbB-2 together with ErbB-1 (D12 cells) resulted in remarkable potentiation of the mitogenic action of EGF; whereas half maximal mitogenic effect was induced by 10 ng/ml of this ligand on D1 cells, only 0.7 ng/ml was necessary to stimulate the D12 cells (Figure 1A, compare D1 with D12 panels). In contrast, ErbB-2 co-expression only slightly improved the mitogenic action of TGFα and E4T. In fact, in the presence of ErbB-2, EGF almost approached the high mitogenic activity of TGFα, a phenomenon that was reflected, in part, also in a long-term survival assay (Figure 1B, D12 panel). Interestingly, ErbB-3 only partially potentiated EGF activity in D13 cells (compare the EC_{50} of EGF on D13 cells, which is 2 ng/ml, with that on D1 cells, which is 10 ng/ml). Once again, co-expression exerted no significant effect on the potency of either TGFα, or E4T (D13 panels in Figure 1). In conclusion, ErbB-2, and to some extent also ErbB-3, specifically enhance the EGF-induced mitogenic action of ErbB-1, probably by forming heterodimeric complexes with this receptor.

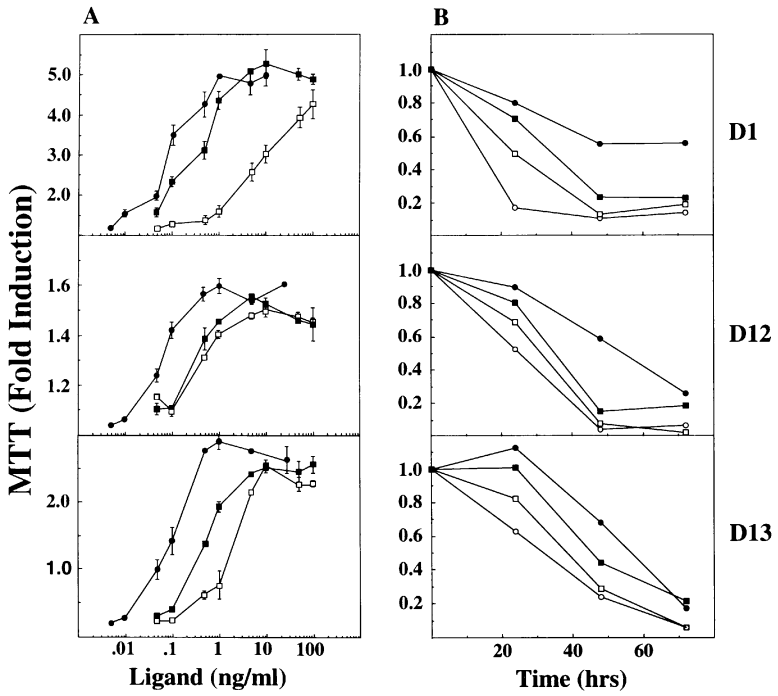

Fig. 1. Ligand-induced proliferation and survival of ErbB-expressing 32D-cells. (**A**) The following derivatives of 32D cells were examined for cell proliferation by using the MTT assay: D1 cells that singly express ErbB-1, D12 cells expressing a combination of ErbB-1 with ErbB-2, and D13 cells expressing a combination of ErbB-1 with ErbB-3. Cells were washed free of serum factors and IL-3, and seeded at a density of 5×10^5 cells/ml in RPMI-1640 medium containing serial dilutions of EGF (□), TGFα (■), or E4T (●). Following 24 h of incubation, the MTT assay was performed as described in Materials and methods. (**B**) The indicated sublines of 32D-cells were plated as described above in the presence of 100 ng/ml EGF, TGFα or E4T [symbols are as in (A)]. Cell proliferation was measured daily using the MTT assay. As a negative control cells were plated in serum- and IL-3-free medium (○). The data from both experiments are given as the means of three determinations. Bars in (A) represent standard deviations. The experiments were repeated three times. The responses to IL-3 (fold induction) of D1, D12 and D13 were 5.54±0.63, 1.96±0.67 and 3.03±0.81, respectively.

Binding parameters may explain superiority of E4T, but not the difference between EGF and TGFα

Perhaps the simplest explanation for the observed differences in mitogenic potencies of EGF, TGFα and E4T might be parallel differences in receptor binding affinities. To examine this possibility we labeled the three ligands with ^{125}I and determined their apparent binding affinities to D1, D12 and D13 cells using ligand displacement analysis. The results of this experiment are shown in Figure 2A. Evidently, the apparent affinities of EGF, TGFα and E4T were not remarkably different when tested on D1 cells, in agreement with a similar analysis that was performed with fibroblasts (Lenferink *et al*., 1997). Co-expression of ErbB-2 (or ErbB-3) only slightly improved the affinity of D12 cells (or D13 cells) to EGF or TGFα (Figure 2A, D12 and D13 panels). Notably, ligand binding assays performed with derivatives of 32D cells usually yield affinities that are consistently lower than those measured with adherent cell types such as fibroblasts or epithelial cells. For example, the K_d values of EGF and TGFα binding to adherent cells are in the range of 0.1–5 nM (Tzahar *et al*., 1994; Lenferink *et al*., 1997), whereas D1 cells bind these ligands with apparent K_d values of 30–50 nM. This may be due to the relatively prolonged washing procedure required in the case of the 32D myeloid cells, which results in an overall reduction in assay sensitivity. We used a ligand dissociation assay as an alternative to partly overcome this limitation. Cells were loaded with the various radiolabeled ligands under saturating conditions, then the unbound ligand was removed and the rates of release of radioactivity were monitored. Clearly, the rates of release of E4T from the surfaces of all three cell lines examined were higher than the dissociation rates of EGF and TGFα (Figure 2B). In addition, the co-expressed co-receptors, namely ErbB-2 and ErbB-3, comparably decelerated the rate of dissociation of EGF and TGFα from ErbB-1, in agreement with previous reports (Kokai *et al*., 1989; Karunagaran *et al*., 1996; Tzahar *et al*., 1996). Taken together, rapid dissociation from the cell surface may be involved in the mitogenic superiority of E4T over EGF and TGFα. However, neither the enhancement of EGF signaling by the co-receptor, nor the superiority of TGFα over EGF may be attributed to binding parameters.

Co-receptors decelerate ligand depletion and internalization, but clearance of the E4T superagonist is defective

Because E4T is released from the cell surface at a much faster rate then EGF or TGFα, we expected that these latter ligands would be depleted from the medium at a much faster rate than E4T. This possibility was tested by incubating D1, D12 and D13 cells with serial dilutions of the ligands for 24 h, thereby allowing their depletion from the medium. Then we determined the relative concentration of each ligand in the conditioned medium by employing a bioassay that uses serum-starved HER-14 fibroblasts overexpressing ErbB-1. As predicted, the rate of ligand depletion inversely correlated with mitogenic potency; the weakest and the strongest mitogens of D1 cells, namely

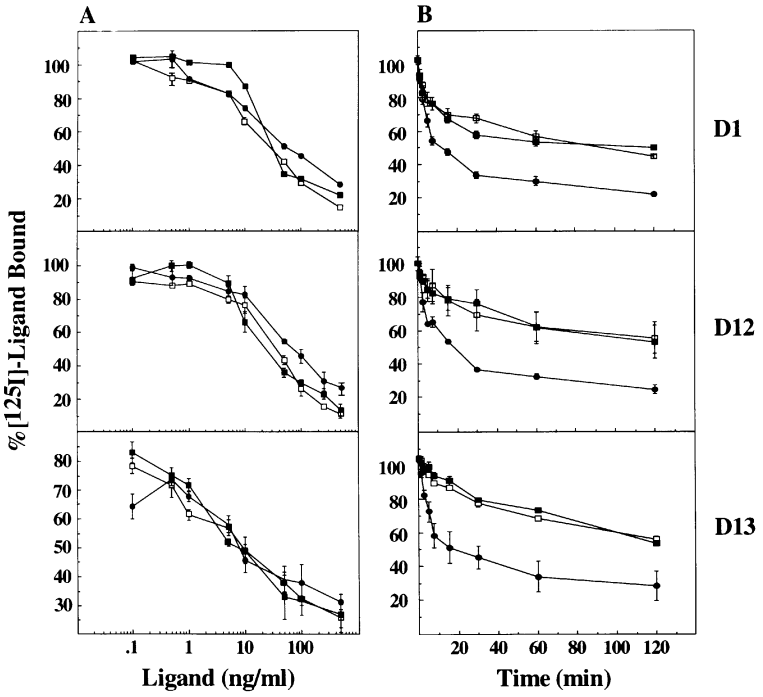

Fig. 2. Ligand displacement and dissociation analyses. (**A**) Displacement analysis was performed with 1.0×10^6 cells of the indicated subclones of the 32D cell line. Cells were washed free of IL-3 and serum factors using binding buffer, and subsequently incubated for 2 h at 4°C with [^{125}I]EGF (1 ng/ml) in the presence of serial dilutions of unlabeled EGF (□), TGFα (■) or E4T (●). Unbound ligand was removed by sedimenting the cells through a cushion of calf serum. The results are presented as the mean ±SD of two determinations. Experiments were repeated three times with similar results. (**B**) The indicated cell lines were incubated for 2 h at 4°C with [^{125}I]EGF (□), [^{125}I]TGFα (■), or [^{125}I]E4T (●), each at 60 ng/ml. Then, the unbound ligand was replaced by an excess of the unlabeled growth factor (3 μg/ml), and cell-bound radioactivity was monitored at the indicated time intervals. Results are expressed as the fractional ligand binding (mean ±SD) relative to the amount of ligand that bound at $t=0$. The experiment was performed in duplicate and repeated twice with similar results.

EGF and E4T, respectively displayed rapid and slow depletion from the medium (Figure 3A). For example, when D1, D12 and D13 cells were incubated for 24 h with a low concentration of EGF (1 ng/ml) and the resulting conditioned media compared with medium similarly incubated in the absence of cells, we observed a 63, 28 and 47% reduction, respectively, in mitogenic activity. The corresponding numbers for TGFα were 28, 36 and 43%, and for E4T, 14, 16 and 24%. Thus, the presence of ErbB-2 significantly decelerated the rate of EGF depletion, but it less efficiently affected removal of E4T or TGFα from the medium. The relative rates of cell-mediated removal of the three ligands correlated with their mitogenic potency, implying that an endocytic mechanism is responsible for the observed differences in signaling potency. Consistent with this model, co-expression of the less potent co-receptor, ErbB-3, together with ErbB-1 only partly extended the half life of EGF (D13 panel in Figure 3A).

To test directly a model involving endocytosis, we comparatively analyzed the internalization rates of the various ligands of ErbB-1, and also determined their dependence on the presence of a co-receptor, either ErbB-2 or ErbB-3. It is notable that our previous experiments, which used a standard ligand internalization assay, detected only minor differences between the rates of ligand internalization through homo- and hetero-dimeric receptors (Pinkas-Kramarski *et al*., 1996a). Therefore, we tested several ligand internalization protocols for their ability to discriminate between the rates of endocytosis of homo- and hetero-dimeric receptors and selected the following assay. Cells were first incubated in the cold with a moderately low concentration of the respective radio-labeled ligand, then the unbound ligand was removed, cells chased at 37°C with a saturating ligand concentration and the ligand distribution between the cell surface and the cytoplasm was determined using an acid wash. This protocol differs from that previously employed (Pinkas-Kramarski *et al*., 1996a) in two aspects. First, a 10-fold lower ligand concentration was used in order to avoid saturation of the coated pit-mediated internalization pathway (reviewed in Sorkin and Waters, 1993). Secondly, other protocols do not include a step that removes unbound ligand prior to initiation of endocytosis. Therefore, continuous uptake of the radiolabeled ligand may mask differences in endocytosis rates. The results of this experiment presented in Figure 3B confirmed that internalization of E4T is significantly slower than that of EGF or TGFα. More importantly, the rate of EGF uptake was remarkably decelerated by a co-expressed ErbB-2, but less so in the presence of ErbB-3 (EGF panel in Figure 3B). The rate of TGFα internalization was similarly affected by the presence of ErbB-2 or ErbB-3 (hTGFα panel in Figure 3B), implying that receptor heterodimers endocytose more slowly than homodimers, irrespective of ligand identity. Because both homodimers and heterodimers of ErbB-1 apparently exist in D12 and in D13 cells, the net kinetics of heterodimer internalization is expected to be even slower than the rates reflected in Figure 3B. Taken together, the data presented in Figure 3 suggest that signaling superiority of E4T is due to the slow rates of internalization and clearance of this ligand from the medium. Possibly,

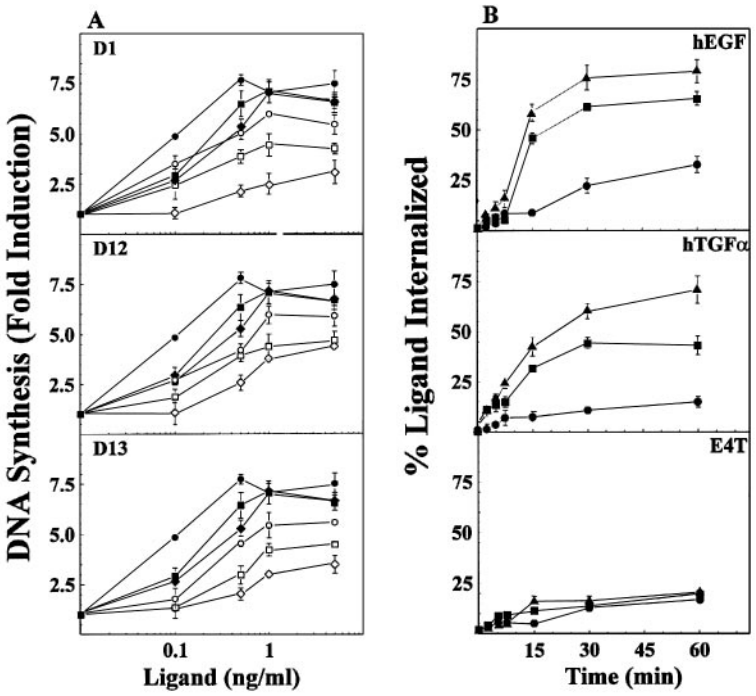

Fig. 3. Receptor-mediated depletion and uptake of ligands. (**A**) Increasing concentrations of the following ligands were incubated for 24 h at 37°C with the indicated derivatives of 32D cells (open symbols): EGF (diamonds), TGFα (squares) or E4T (circles). For control, ligands were similarly incubated in the absence of cells (closed symbols). The capacity of the resulting conditioned media to stimulate DNA synthesis in HER-14 fibroblasts was then determined as described in Materials and methods. Results are given as the mean ± SD of three individual experiments carried out in duplicate. (**B**) For determination of ligand internalization rates, radiolabeled forms of the indicated ligands (each at 1 ng/ml) were incubated for 2 h at 4°C with the following derivatives of 32D cells: D1 (▲), D12 (●) or D13 cells (■). Following incubation on ice, cells were washed free of unbound ligand and incubated at 37°C for various time intervals with excess of the corresponding unlabeled ligand (at 3 μg/ml). Cellular uptake of radioactivity was monitored by removing surface-bound ligand with an acidic ligand-strip buffer. Data are presented as the mean ± SD of duplicate determinations. Each experiment was repeated at least twice.

rapid dissociation of E4T from ErbB-1 (Figure 2) prevents efficient internalization. On the other hand, the relatively weak signaling capacity of EGF through the singly expressed ErbB-1 is attributed by our results to the efficient rate of cellular uptake of this ligand. Moreover, the potentiating effect of ErbB-2 is probably due to its ability to decelerate both the rate of internalization (Figure 3B) and the rate of clearance of EGF from the medium (Figure 3A), in line with the relatively slow down-regulation and endocytosis of ErbB-2 (Sorkin *et al*., 1993; Baulida *et al*., 1996). Despite these consistencies, our results cannot provide a satisfactory explanation for the relatively high potency of TGFα; although this ligand is more potent than EGF on D1 cells, and it is almost equipotent to EGF on D12 cells (Figure 1), its rates of internalization (Figure 3B), depletion from the medium (Figure 3A) and dissociation from the cell surface (Figure 2B), are only slightly different than those of EGF, and they apparently cannot account for the EGF-specific 10–15-fold mitogenic enhancement effect of ErbB-2 (Figure 1A).

EGF and TGFα are comparably degraded, but E4T degradation is limited

According to one possibility, EGF and TGFα are similarly endocytosed, but whereas the former is efficiently degraded in lysosomes, the other escapes intracellular degradation. To test this model we treated cells with each of the radiolabeled ligands under conditions that prevent receptor recycling and retard targeting to the degradative pathway. Upon transfer of chilled cells to 37°C ligand degradation was allowed and monitored using acid precipitation. The results presented in Figure 4 indicate that E4T is degraded at a slower rate than EGF and TGFα, as expected on the basis of its slower rate of uptake (Figure 3B), but intracellular degradation of EGF and TGFα were comparable in kinetics and extent. Remarkably, expression of a co-receptor together with ErbB-1 only slightly affected the rates of ligand degradation. In experiments not shown we confirmed a previous report (Hamel *et al*., 1997) that degradation of both ligands was significantly inhibited by chloroquine, a drug known to inhibit degradation in both endosomal (prelysosomal) and lysosomal compartments, but leupeptin, a tripeptide whose inhibitory action is specific to lysosomes (Cardelli *et al*., 1989), did not affect TGFα degradation. Conceivably, EGF is destined for lysosomal degradation after endocytosis (Renfrew and Hubbard, 1991), whereas TGFα is degraded in a non-lysosomal compartment whose identity is only partly characterized (Hamel *et al*., 1997). Independent of its exact intracellular location, endocytic degradation of EGF and TGFα cannot provide an explanation for the superiority of TGFα and the potentiating effect of ErbB-2.

The presence of a co-receptor specifically increases acid sensitivity of EGF binding

It is well established that binding of EGF and TGFα (Ebner and Derynck, 1991), as well as binding of various chimeras of these two ligands (Lenferink *et al*., 1997), display differential sensitivity to acidic pH. This, in turn, is thought to allow recycling of TGFα-bound receptors to

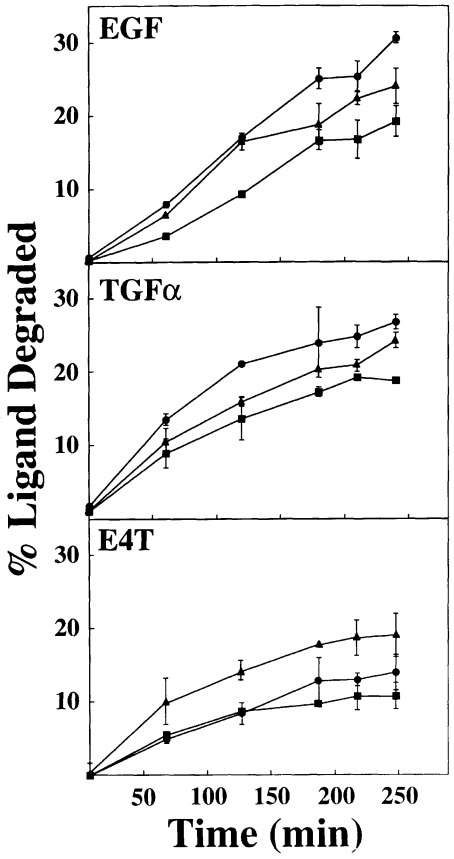

Fig. 4. Kinetics of ErbB-mediated ligand degradation. The indicated radiolabeled ligands (each at 1 nM) were incubated for 1 h at 20°C with the following derivatives of 32D cells: D1 (▲), D12 (●) or D13 cells (■). Thereafter, the cells were spun through a cushion of serum to remove unbound ligand, and then incubated at 37°C for various time intervals. Media were then collected and cells solubilized. The fraction of acid-soluble (degraded) ligand in the medium was determined by counting the acid-soluble radioactivity in the medium and the total cell-associated radioactivity. The results are expressed as the average percentage of acid-soluble radioactivity, relative to the sum of cell-associated and medium-released radioactive counts. Bars represent standard deviations. The experiment was performed in duplicate and repeated twice.

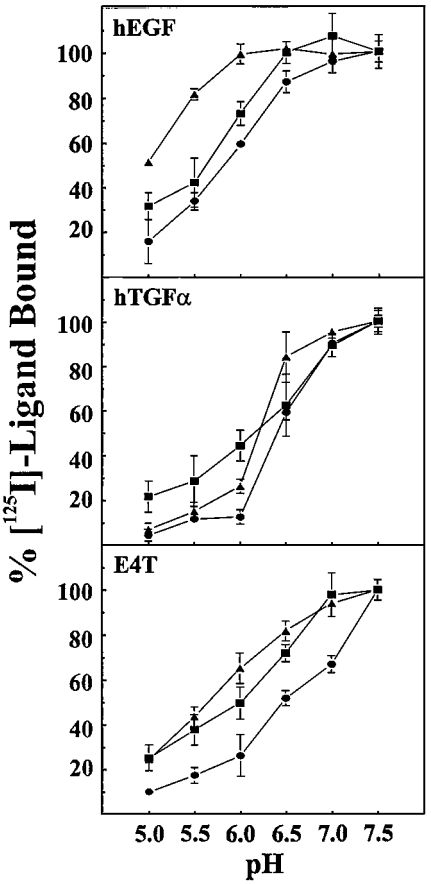

Fig. 5. pH sensitivity of ligand binding to specific combinations of ErbBs. D1 (▲), D12 (●) or D13 cells (■) were incubated for 2 h at 4°C with radiolabeled forms of the indicated ligands (each at 60 ng/ml). The pH of the binding buffer was adjusted to the indicated values. Unbound radioactivity was removed by sedimenting the cells through a cushion of calf serum, prior to γ-counting. Results are shown as the mean ± SD of a triplicate experiment which was repeated twice.

the cell surface, thereby augmenting TGFα biological action (Ebner and Derynck, 1991). On the other hand, because EGF resists the moderately acidic pH of early endosomes, this ligand does not permit receptor recycling, and the ligand–receptor complex is destined for degradation in lysosomes. To examine the possibility that the presence of a co-receptor alters pH sensitivity of ligand binding, we analyzed the interaction between EGF, TGFα and E4T with D1, D12 and D13 cells under various pH conditions. In line with previous observations, EGF binding to ErbB-1 displayed remarkable stability when compared with TGFα and E4T (Figure 5). However, the presence of a co-receptor, either ErbB-2 or ErbB-3, significantly destabilized these interactions. By contrast, the co-receptors only slightly affected the relatively sensitive binding of TGFα (hTGFα panel in Figure 5). In addition, a moderate effect of the co-receptors was observed in the case of E4T (Figure 5). On the basis of these observations we predict that the lysosome-destined EGF-driven ErbB-1 is re-routed to recycling back to the cell surface once a co-receptor is present. On the other hand, co-expression of ErbB-2 or ErbB-3 may not alter routing of a TGFα-driven ErbB-1, because this ligand rapidly dissociates in early endosomes regardless of the dimerization state of its receptor.

EGF-driven homodimers of ErbB-1 are degraded, but heterodimers are recycled to the cell surface

To monitor the fate of ErbB-1 after ligand-induced endocytosis, we induced down-regulation of this receptor using an unlabeled ligand and then determined the status of the remaining surface-associated binding sites by performing a radio-receptor assay. The results of this experiment revealed that ErbB-1 was destined for different fates depending on the activating ligand; upon EGF binding ErbB-1 rapidly disappeared from the surface of D1 cells, but both TGFα and E4T caused re-appearance of binding sites following an initial phase of receptor down-regulation (Figure 6). That re-appearance was due to recycling of endocytosed receptors was indicated by its complete inhibition by monensin (Figure 6, right column), a drug known to inhibit recycling of transmembrane receptors (Basu *et al*., 1981), including the EGF-receptor (Gladhaug

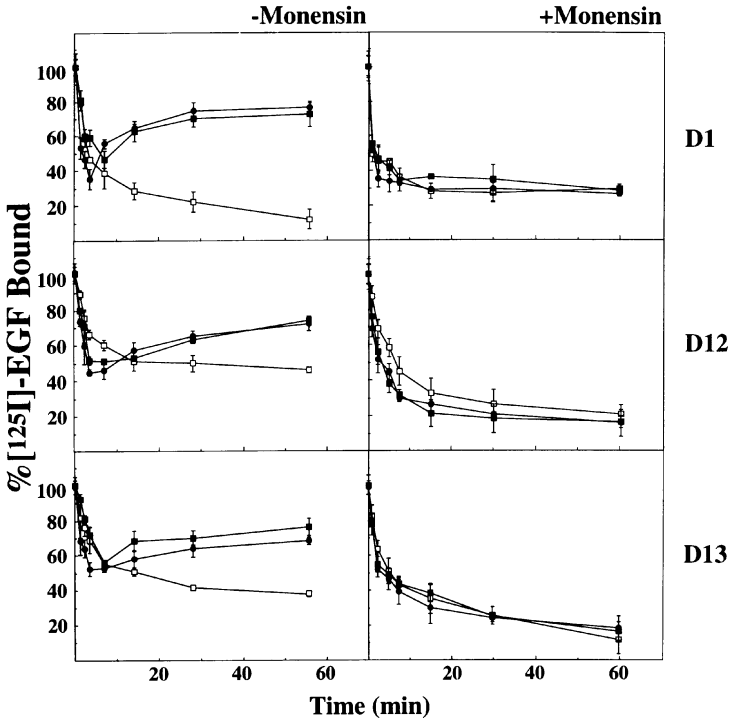

Fig. 6. Dependence of down-regulation and recycling of ErbB-1 on ligand identity and receptor interactions. The indicated derivatives of 32D cells (1.0×10^6 cells per each data point) were incubated for 2 h at 4°C with the following ligands (each at 60 ng/ml): EGF (□), TGFα (■) or E4T (●), in the absence (left panels) or presence (right panels) of monensin (0.3 mM). The cells were then transferred to 37°C and incubated for the indicated time intervals. The residual level of surface receptor that did not undergo down-regulation was determined by performing a direct binding assay with radiolabeled EGF. The results are calculated as the fraction of the initial binding of [^{125}I]EGF at $t=0$, and are presented as the mean ± SD. The experiment was performed in duplicate and repeated twice.

and Christofferson, 1988). It is worthwhile noting, however, that monensin may affect other intracellular processes. For example, it has been reported that treatment with monensin can inhibit the addition of N-linked oligosaccharide chains to ErbB-1 (Mayes and Waterfield, 1984). The patterns of receptor down-regulation exhibited by EGF-treated D12 and D13 cells were different; whereas the behavior of TGFα- or E4T-driven receptors was not significantly altered by either co-receptor, in the presence of either ErbB-2 or ErbB-3 the EGF-induced down-regulation was decelerated and eventually reached a relatively high steady state (D12 and D13 panels in Figure 6). This effect was more pronounced in the case of D12 cells, in correlation with the observation that ErbB-2 potentiates EGF signaling better than does ErbB-3 (Figure 1). The relatively high steady-state of ErbB-1, that was induced by the presence of ErbB-2 or ErbB-3, was completely abolished by monensin (Figure 6). The absence of net re-appearance of binding sites, following an initial drop, in the case of EGF-treated D12 and D13 cells is attributed to the combined contribution of homodimers (that are destined for degradation) and heterodimers (that are destined for recycling). Thereby, heterodimer formation can alter the endocytic fate of an EGF-driven ErbB-1 from degradation to recycling. This scenario is consistent with the observation that the two co-receptors destabilized EGF binding at moderately acidic conditions (Figure 5), and they also attenuated both the rate of EGF uptake (Figure 3B) and the rate of ligand disappearance from the growth medium (Figure 3A).

EGF and TGFα similarly recruit ErbB-2, but engagement of ErbB-3 by heterodimerization is limited

The specificity of the potentiating effect of ErbB-2 to EGF action, but not to the biological effect of TGFα, may be explained by an alternative model which argues that TGFα less efficiently recruits ErbB-2 into heterodimers with ErbB-1 (Gulliford *et al*., 1997), and therefore its action is unaffected by the presence of the co-receptor. Two experimental strategies were employed in order to test the validity of this model. First, the ability of TGFα to induce heterodimers was compared with that of EGF by covalent labeling of ErbB-1 with either ligand and determination of the extent of co-precipitation of the co-receptor (either ErbB-2 or ErbB-3) with ErbB-1. The results of this experiment indicated that EGF- and TGFα-labeled monomers (M) and dimers (D) of ErbB-1 underwent comparable co-immunoprecipitation by antibodies directed to ErbB-2 (Figure 7A), in agreement with recent reports (Beerli and Hynes, 1996; Riese *et al*., 1996). The interaction between ErbB-3 and ErbB-1 was hardly detectable by this assay (D13 lanes in Figure 6B), confirming weak stability of the ErbB-1/ErbB-3 complex (Tzahar *et al*., 1996). Thus, recruitment of a co-receptor cannot explain the differences between EGF and TGFα, because these ligands similarly engage ErbB-2 heterodimerization. This conclusion was independently supported by a second approach using monoclonal antibodies (mAbs) to ErbB-2, denoted L26 and L140, that respectively inhibit or only slightly affect heterodimer formation

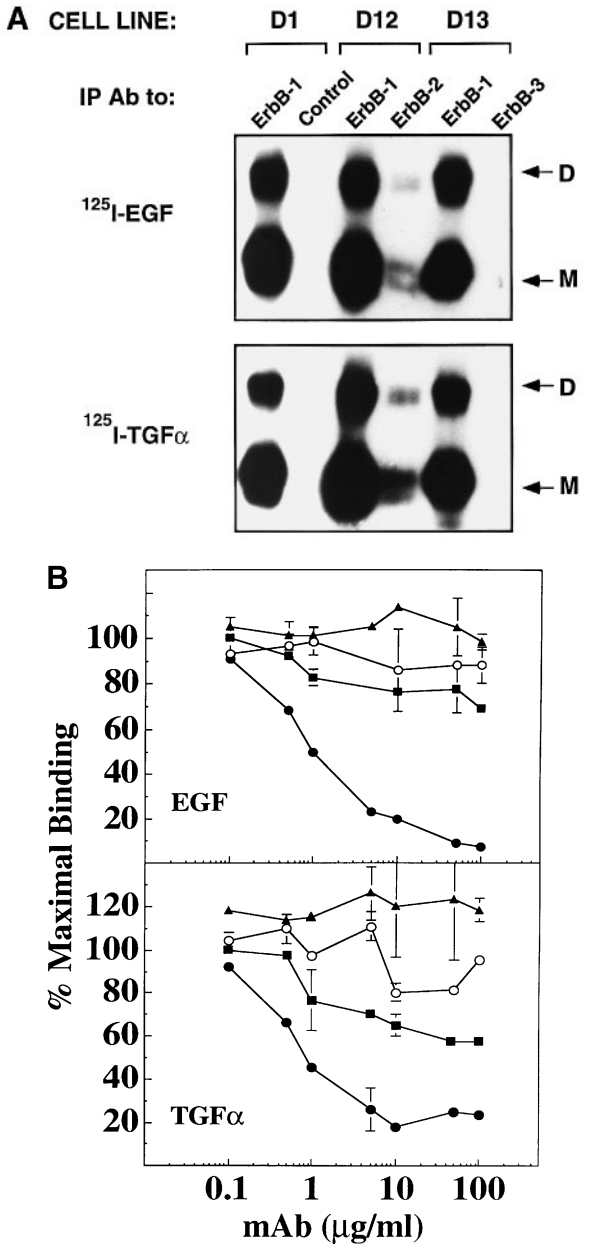

Fig. 7. Ligand-induced formation of ErbB-1-containing heterodimers. (**A**) D1, D12 and D13 cells were incubated with radiolabeled EGF or TGFα (each at 20 ng/ml) for 90 min at 4°C. Covalent crosslinking was performed by further incubation for 1 h with the bivalent crosslinking reagent BS^3. Cell lysis and immunoprecipitation (IP) of the indicated ErbB proteins were then performed and followed by gel electrophoresis. The resulting autoradiograms are shown, along with the locations of monomeric (M_r ~180 kDa) and dimeric (D) ligand–receptor complexes. (**B**) D12 cells were incubated for 2 h at 4°C with either [^{125}I]EGF or [^{125}I]TGFα (each at 10 ng/ml), along with the indicated concentrations of the following anti-ErbB-2 mAbs: L26 (■) and L140 (○). For positive control we used a neutralizing antibody to ErbB-1, mAb 528 (●). As a negative control we used a mAb to a hepatitis B antigen (▲). Binding of the radiolabeled ligands was determined as described under Materials and methods and presented as the mean ±SD of three determinations. The experiment was repeated three times with similar results.

(Klapper *et al.*, 1997). Since by breaking ErbB-2-containing heterodimers these mAbs partly reduce the binding of ligands to their direct receptors (Klapper *et al.*, 1997), ligand binding may be used as a readout of ErbB-2 recruitment into heterodimers. When tested on D12 cells, mAb L26 and to some extent also mAb L140 reduced binding of EGF and TGFα (Figure 7B), implying that both ligands can induce formation of the ErbB-1/ErbB-2 heterodimeric complex. Of note, in these cells TGFα was inhibited more efficiently than EGF. For control, a ligand-competitive mAb to ErbB-1 was used and it reached an almost complete inhibition of both ligands, but an irrelevant mAb was inactive (Figure 7B). Taken together, the results presented in Figure 7 exclude the possibility that differences in heterodimer recruitment account for the EGF-specific potentiating action of a co-receptor, thus strengthening an endocytosis-based mechanism of signal potentiation.

Discussion

Previous analyses concentrating on the relative mitogenic and transforming abilities of ErbB proteins and their ligands established the notion that cells co-expressing ErbB-1 together with ErbB-2 are more effectively transformed than either cells expressing ErbB-1 alone (Kokai *et al.*, 1989), or ErbB-1 in combination with ErbB-3 (Cohen *et al.*, 1996). Likewise, TGFα was shown to be more mitogenic and transforming than EGF in an autocrine or paracrine context (reviewed in Salomon *et al.*, 1995). Our present study links the superiority of receptor heterodimers with ligand specificity and provides a mechanistic basis for this functional linkage. After dealing with the proposed mechanism of signal potentiation, we discuss below the implications of our findings to current open questions, such as the extent of physiological redundancy of the multiple EGF-like ligands and the role of ErbB-2 in cancer.

The observation that ErbB-2 can *trans*-potentiate the proliferative effect of EGF more efficiently than ErbB-3 is best interpreted in terms of heterodimer formation: ErbB-1/ErbB-2 interactions are more prevalent than ErbB-1/ErbB-3 associations (Figure 7A) (Tzahar *et al.*, 1996). Nevertheless, EGF is known to activate ErbB-3 in cells overexpressing ErbB-1 (Kim *et al.*, 1994; Soltoff *et al.*, 1994), and phosphorylation of ErbB-3 apparently takes place within an EGF-driven ErbB-1/ErbB-3 heterodimer (Riese *et al.*, 1995; Pinkas-Kramarski *et al.*, 1996a; Zhang *et al.*, 1996). Thus, the relatively weak interactions between ErbB-1 and ErbB-3 may explain why the potentiating effect of ErbB-3 is weaker than that of ErbB-2 (Figure 1A). Assuming a heterodimerization model, we propose that the three ligands we tested utilize distinct mechanisms for signal potentiation. These mechanisms are described below.

EGF

According to our results, EGF can signal through two alternative pathways that are schematically presented in Figure 8. In the absence of a co-receptor, EGF is rapidly endocytosed, and due to the relatively stable binding to ErbB-1 it resists the low pH of early endosomes (Figure 5). This targets homodimeric complexes of ErbB-1, along with EGF, to degradation in lysosomes (Figure 4), and results in an almost complete disappearance of surface ErbB-1 (Figure 6). On the contrary, in the presence of a co-receptor the ternary complex (EGF, ErbB-1 and the co-receptor), whose internalization rate is relatively slow

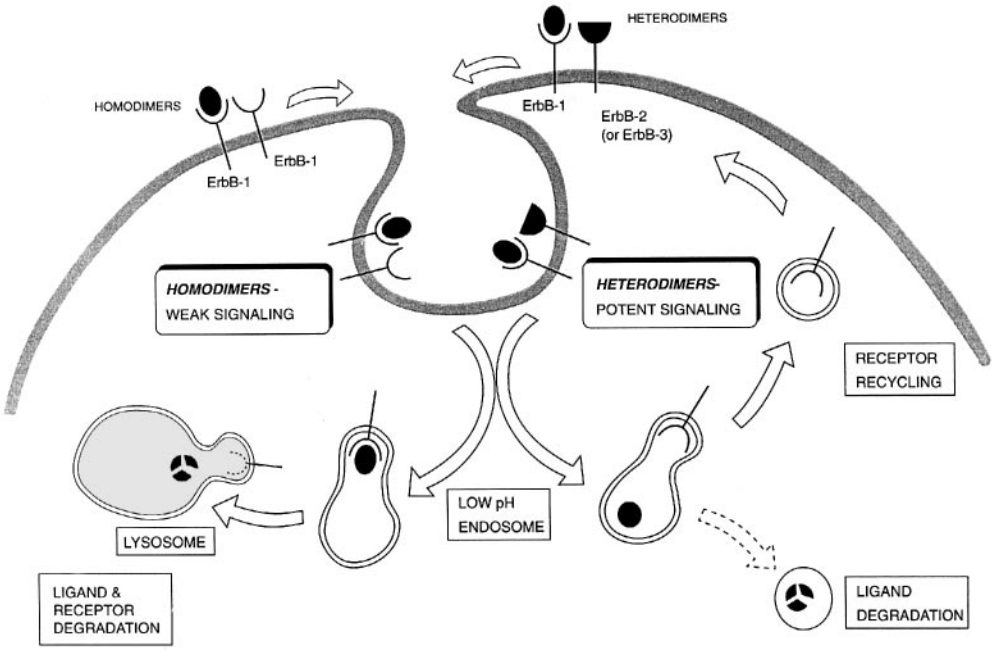

Fig. 8. Proposed endocytic model of heterodimerization-mediated tuning of mitogenic signals. EGF-occupied homodimers of ErbB-1 are destined for rapid endocytosis and lysosomal degradation that efficiently terminate signaling. In the presence of ErbB-2 (or ErbB-3), EGF signals are enhanced because ErbB-1/ErbB-2 heterodimers release EGF when the pH of early endosomes decreases. This allows recycling of the receptor back to the cell surface, thereby augmenting EGF signaling. Not presented are the pathways undertaken by TGFα and E4T. Whereas the former directs ErbB-1 to recycling regardless of the presence of a co-receptor, E4T signaling is further enhanced by its rapid on/off rates of interaction with ErbB-1. Both routes of EGF/ErbB-1 endocytosis result in intracellular degradation of the ligand, either because co-existence of homo- and hetero-dimers allows inter-pathway leakage of ligand molecules, or because the recycling route is coupled to non-lysosomal proteolytic degradation.

(Figure 3B), dissociates under the moderately acidic conditions of early endosomes (Figure 5), and consequently ErbB-1 recycles back to the cell surface (Figure 6). The exact fate of the two other molecular components of the ternary complex is unclear; whereas the co-receptor either escorts ErbB-1 to the plasma membrane, or undergoes enhanced degradation (Worthylake and Wiley, 1997), degradation of EGF takes place in an unknown compartment, probably the same non-lysosomal vesicular compartment that processes TGFα (Hamel *et al*., 1997). Nevertheless, some recycling of undegraded EGF molecules seems to occur, as the rate of depletion of this ligand from the medium is decelerated in the presence of a co-receptor (Figure 3A). Regardless of the exact fate of their molecular components, the altered endocytic routing of ErbB-1-containing complexes may be responsible for signal potentiation, because this pathway constantly delivers unoccupied ErbB-1 molecules to the plasma membrane. By contrast, in the case of a homodimeric ErbB-1, efficient down-regulation of the receptor takes place and, therefore, signaling is short lived. It is relevant that a linkage between defective internalization of ErbB-1 and strong proliferative signals has been previously established by using an endocytosis-impaired mutant of this receptor (Wells *et al*., 1990).

TGFα

Because binding of this ligand to both homo- and heterodimeric complexes of ErbB-1 is pH-sensitive (Figure 5), TGFα directs receptor recycling regardless of the presence of a co-receptor (Figure 6). Consequently, receptor down-regulation (Figure 6) and ligand depletion (Figure 3A) are slower in the case of TGFα than they are with EGF, which may explain the stronger mitogenic signal of TGFα, as compared with EGF (Figure 1). In a parallel set of experiments that examined neuregulin signaling through the extremely potent ErbB-2/ErbB-3 complex we found that the cellular routing of neuregulin-driven ErbB-3 is similar to that of TGFα-driven ErbB-1 complexes (Waterman *et al*., 1998), implying that recycling of ErbBs is a common mechanism of signal potentiation. Interestingly, however, the cellular context may affect intracellular routing of TGFα as human endometrial and other cells display more rapid processing of this ligand relative to EGF, and this correlates with biological potency (Korc and Finman, 1989; Reddy *et al*., 1996b).

E4T

Unlike EGF and TGFα which differ only slightly in binding parameters (Figure 2), examination of the rate of dissociation of the chimeric superagonist E4T revealed a relatively high rate of release from both homo- and hetero-dimeric receptor complexes (Figure 2B). This was confirmed using plasmon resonance to measure in real time the association and dissociation rates of the three ligands from a soluble form of ErbB-1; E4T was found to behave differently to EGF and TGFα, in having both a relatively high association and dissociation rate constant (A.E.G.Lenferink and M.D.O'Connor-McCourt, manuscript in preparation). This kinetic combination may explain why the apparent affinity of E4T is similar to that of EGF or TGFα (Figure 2A). In addition, E4T displayed several significant landmarks, such as relatively slow rates of endocytosis (Figure 3B) and intracellular degradation (Figure 4), combined with pH-sensitive receptor binding (Figure 5), and an ability to induce receptor recycling

(Figure 6). It is relevant that a mutant form of EGF, denoted EGF-Val-47, shares with E4T resistance to intracellular degradation and high biological potency (Walker *et al*., 1990). Collectively, the biochemical features of E4T appear to contribute to high signaling potency in the following way: due to its rapid on/off kinetics, E4T only transiently stimulates its receptor and therefore this ligand causes inefficient endocytosis. Moreover, due to their pH sensitivity, those E4T-bound ErbB-1 molecules that eventually undergo endocytosis rapidly recycle back to the cell surface, probably along with the chimeric ligand. Thus, the relatively strong mitogenic signal of E4T may be entirely due to inefficient signal inactivation processes. An alternative interpretation emerged from a study performed with a chimeric ligand similar to E4T (Puddicombe *et al*., 1996). Like E4T, the other chimera displayed superagonist activity and its rate of depletion from the growth medium was relatively low. However, it has been noted that activation of receptor autophosphorylation by this ligand was more sustained than by EGF, and its mitogenic superiority displayed cell type specificity, suggesting a contextual requirement.

A central issue of the above described models of signal potentiation is the assumption that heterodimer formation by ErbB-1 can affect intracellular routing of this receptor. Most likely heterodimers do not dissociate upon endocytosis, thereby allowing an '*in trans*' effect of the co-receptor on the rate and destination of receptor endocytosis. It has been shown previously that the rates of ligand internalization and receptor down-regulation are high in the case of ErbB-1 and relatively low in the case of ErbB-2, ErbB-3 and ErbB-4 (Baulida *et al*., 1996; Pinkas-Kramarski *et al*., 1996a). Because ErbB-3 is practically devoid of tyrosine kinase activity (Guy *et al*., 1994), and a kinase-defective mutant of ErbB-1 displays altered routing (Glenney *et al*., 1988; Felder *et al*., 1990), it is understandable why ErbB-3-containing heterodimers are less efficiently endocytosed. In fact, our recent results indicate that ErbB-3 undergoes slow endocytosis, which is followed by rapid recycling to the cell surface (Waterman *et al*., 1998), a route that is apparently shared with a kinase-defective mutant of ErbB-1. On the other hand, the slow endocytic rates of ErbB-2 and ErbB-4 are more difficult to reason. One potential explanation may involve their inability to recruit components of the coated pit, such as the adapter protein 2 (Baulida *et al*., 1996), which are necessary for rapid internalization. Alternatively, signals inhibitory for rapid internalization may reside in the structurally distinct cytoplasmic portions of the co-receptors (Sorkin *et al*., 1993).

What is the physiological role of *trans*-potentiation through heterodimer formation? An evolutionary perspective may provide a hint to the answer; while only one EGF-like ligand and one ErbB-like receptor exist in worms (Kornfeld, 1997), several dozen ligands and four receptors are known in mammals. This evolutionary expansion of the number of distinct components was probably aimed at increasing physiological versatility. One such mechanism emerges from the present study: controlled expression of a co-receptor may confer superior signaling properties to others. By inference, the multiple ligands of ErbB-1 may not have redundant functions; within the appropriate context of a receptor and a co-receptor some ligands may be superior to others. An example from mammals may demonstrate the issue: whereas normal hepatocytes respond to TGFα better than to EGF (Guren *et al*., 1996), their embryonic counterparts respond equally well to the two ligands (Lipeski *et al*., 1996), in accordance with the presence of ErbB-2 in fetal cells (W.E.Russell, personal communication) but not in adult hepatocytes (Carver *et al*., 1996).

The biochemical mechanism underlying the prognostic value of ErbB-2 in human cancer is currently unclear (Hynes and Stern, 1994; Stancovski *et al*., 1994). According to an autonomous type of mechanism, ErbB-2 contributes to high proliferation and tissue invasion perhaps because its direct ligand, whose identity is unknown, activates homodimeric ErbB-2 complexes in a manner similar to an oncogenic rat mutation (Weiner *et al*., 1989). Alternatively, an overexpressed ErbB-2 is oncogenic perhaps because the basal tyrosine kinase activity of this receptor is relatively high (Lonardo *et al*., 1990). The non-autonomous type of mechanism (Tzahar and Yarden, 1998) implies that ErbB-2 functions solely as a molecular amplifier of signaling initiated by all stromal EGF-like ligands (Karunagaran *et al*., 1996), because this receptor is the preferred heterodimeric partner of all ErbB proteins (Tzahar *et al*., 1996; Graus-Porta *et al*., 1997), and its coupling to the MAPK pathway is extremely efficient (Ben-Levy *et al*., 1994). The realization that ErbB-2 is a slowly internalizing receptor that can *trans*-potentiate EGF signaling by decelerating the relatively fast rate of ErbB-1 endocytosis (Figure 8) suggests that ErbB-2 supports oncogenesis not only by decelerating the rate of growth factor dissociation from heterodimeric receptor complexes (Karunagaran *et al*., 1996), but also by delaying their inactivation process. One immediate implication is that ErbB-2 overexpression in carcinomas may be related to the type of stromal ligands expressed in the vicinity of each particular tumor. Likewise, this mechanism may be critical in metastasis; successful seeding of ErbB-2-overexpressing tumor cells at selected sites may be determined by the presence of ligands whose action is potentiated by the co-receptor. Establishment of this and other predictions made on the basis of the *trans*-potentiation effect of ErbB-2 will require additional studies.

Materials and methods

Materials, buffers and antibodies

Human recombinant EGF and TGFα were obtained from Boehringer Mannheim. Binding buffer contained RPMI-1640 medium supplemented with 0.5% bovine serum albumin (BSA). mAbs L26 and L140 raised against the extracellular part of the human ErbB-2 receptor were as described (Klapper *et al*., 1997). mAb 528 directed against the extracellular domain of ErbB-1 was a kind gift of John Mendelsohn (MD Anderson Cancer Center, TX). The acidic ligand-strip buffer (pH 2.5) contained 5 mM acetic acid, 2.5 mM KCl, and 135 mM NaCl. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 1 mM $MgCl_2$, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM Na_3VO_4, 5 μg/ml pepstatin A, 5 μg/ml leupeptin and 5 μg/ml aprotinin. HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol.

Mutant growth factor production

The chimeric growth factor E4T, consisting of EGF sequences N-terminal to the fourth cysteine of the EGF-like motif and TGFα sequences C-terminal to this cysteine, was constructed as described (Kramer *et al*.,

1994), cloned into the pEZZ18 expression vector (Pharmacia, Uppsala, Sweden) (van de Poll *et al*., 1995) and harvested as a secreted protein A-containing product from the periplasmic space of *Escherichia coli* KS474, a protease-deficient mutant (Strauch *et al*., 1989). Bacteria were grown overnight in 2YTE medium under continuous agitation (200 r.p.m.). The fusion protein was isolated as described (Nilson and Abrahmsen, 1990) and purified using IgG–Sepharose (Pharmacia). Protein yield was determined by using a binding competition assay with biotin-labeled protein A (van Zoelen *et al*., 1993). E4T was enzymatically cleaved from protein A by factor X digestion and separated by an additional run over an IgG column. Final purification of the sample was done by reverse-phase chromatography as described previously (van de Poll *et al*., 1995). Fractions of 1 ml were collected and tested for binding to HER-14 cells (Lenferink *et al*., 1997). The quantity of E4T was calculated using the peak area representing the binding activity at 229 nm in the chromatography profile. Murine EGF from a natural source was used under the same experimental conditions as a standard (van de Poll *et al*., 1995).

Cell culture

32D murine myeloid cells (Greenberger *et al*., 1983), transfected with the various combinations of erbB-encoding plasmid or viral vectors (Pinkas-Kramarski *et al*., 1996a) were grown in RPMI-1640 medium supplemented with antibiotics, 10% heat-inactivated fetal bovine serum and 0.1% medium conditioned by IL-3-producing X63/0 cells (Karasuyama and Melchers, 1988). Cells were kept under continuous selection using 0.4 mg/ml hygromycin B (Boehringer Mannheim) for D1 cells and additionally 0.6 mg/ml G418 (Boehringer Mannheim) for D12 and D13 cells. NIH 3T3 cells transfected with the wild-type human EGF receptor (HER-14 cells) and expressing 4.0×10^5 ErbB-1 molecules/cell (Honegger *et al*., 1988), were cultured in gelatinized flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% newborn calf serum.

Ligand displacement assays

The rationale of this assay was adopted and modified from a previous protocol (Reddy *et al*., 1996a). Essentially, ligand concentration profiles were determined in media conditioned by preincubation with ErbB-expressing cells. Because a radioimmunoassay and a radio-receptor assay were less satisfactory, we used a bioassay with HER-14 murine fibroblasts overexpressing ErbB-1. Recombinant human EGF, TGFα and the chimera E4T were radiolabeled using the indirect Iodogen method (Pierce, Roxford, IL), as described previously (Peles *et al*., 1993). For ligand displacement analysis, 1.0×10^6 cells were washed once with binding buffer, incubated with a radiolabeled ligand (at 1 ng/ml) for 2 h at 4°C in 0.2 ml of the same buffer, containing serial dilutions of the unlabeled ligand. To terminate ligand binding, cells were sedimented (9000 *g*, 2 min), washed once with 0.5 ml binding buffer and loaded on top of a 0.7 ml cushion of BSA. Tubes were spun again to remove the unbound ligand and radioactivity in the cell pellets was counted directly.

Cellular proliferation assays

To analyze ligand-induced proliferative responses of D1, D12 and D13 cells, 5.0×10^4 cells were washed free of IL-3, resuspended in RPMI-1640 and seeded in 96-wells plates. For dose–response experiments, serial dilutions of a ligand were added in RPMI-1640 medium and cells were incubated for 24 h at 37°C. IL-3 (1:1000 of medium conditioned by a producer cell line) was used as a positive control. Proliferation was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, which determines mitochondrial activity in living cells (Mosman, 1983). During an incubation for 2 h at 37°C with MTT, living cells transform the tetrazolium ring into dark blue formazan crystals which can be quantified by reading the optical density at 540–630 nm after lysis of the cells with acidic 2-propanol. For cell survival experiments, cells were seeded at the same density in 96-well plates and incubated at 37°C with a fixed ligand concentration (100 ng/ml). Cell survival was determined 24, 48 and 72 h after ligand addition using the MTT method.

Ligand depletion assay

The rationale of this assay was adopted and modified from a previous protocol (Reddy *et al*., 1996a). Essentially, ligand concentration profiles were determined in media conditioned by preincubation with ErbB-expressing 32D cells. Because radioimmunoassay and radio-receptor assay was less satisfactory we used a bioassay with HER-14 fibroblasts overexpressing ErbB-1. HER-14 cells were seeded in gelatinized 24-well dishes (1.8 cm^2) at a density of 6.0×10^4 cells/well in 1 ml DMEM/10% serum. After 24 h of incubation the medium was replaced by 0.9 ml of DMEM/Ham's F12 medium (1:1) supplemented with 30 nM Na_2SeO_3, 10 μg/ml human transferrin and 0.5% BSA. After an additional incubation for 48 h, 0.1 ml medium that was conditioned for 24 h by D1, D12 or D13 cells was added. Eight hours later 0.5 μCi [^{3}H]thymidine (TdR) was added in 0.1 ml Ham's F12 medium. Incorporation of the tracer into cellular DNA was determined 24 h after growth factor addition as described previously (van Zoelen *et al*., 1986).

Receptor recycling assays

To quantify receptor recycling, 1.0×10^6 cells were incubated for 2 h at 4°C with various ligands (at 60 ng/ml) in the absence or presence of 0.3 mM monensin (as indicated), and then transferred to 37°C for various time periods. Subsequently, cells were sedimented (9000 *g*, 2 min), resuspended, and incubated in ice-cold ligand-strip buffer for 2 min on ice. Cells were sedimented again, neutralized in binding buffer and incubated in the same buffer for an additional 1 h at 37°C to allow intact internalized receptors to recycle to the cell surface. To quantify the number of ErbB-1 molecules on the cell surface, cells were incubated for 2 h at 4°C with [^{125}I]EGF, sedimented as above, rinsed once in binding buffer and spun through a serum cushion to remove the unbound ligand, prior to γ-counting.

Ligand internalization assays

The fate of various ligands was determined by incubating 32D cells (1.0×10^6 cells) with 1 ng/ml radiolabeled EGF, TGFα or E4T. Following 2 h at 4°C cells were washed in binding buffer, resuspended in the same buffer that contained unlabeled ligand (3 μg/ml) and transferred to 37°C for the indicated time periods. Then, cells were immediately cooled on ice, incubated for 5 min in the acidic ligand-strip buffer (pH 2.5), and sedimented through a serum cushion. The released ligand was considered as cell surface-associated ligand. Cells were lysed in 1% Triton X-100 for 1 h at room temperature prior to γ-counting.

Ligand dissociation assays

Dissociation of radiolabeled human EGF, TGFα and E4T was investigated using 1.0×10^6 D1, D12 or D13 cells. Cells were rinsed once in binding buffer and subsequently incubated (2 h, 4°C) with excess (60 ng/ml) radiolabeled ligand in binding buffer. Then, the tubes were spun and the cell pellet was resuspended and incubated at 4°C in binding buffer supplemented with 3 μg/ml unlabeled ligand for the indicated time spans. Finally, cells were pelleted and lysed in 100 mM NaOH containing 0.1% sodium dodecylsulfate prior to γ-counting.

Ligand degradation assays

Derivatives of 32D cells (1.0×10^6 cells) were washed free of IL-3 and subsequently incubated at 20°C for 60 min with radiolabeled ligand (at 1 nM) in binding buffer. Then, cells were spun through a serum cushion to remove the unbound ligand and incubated, without ligand, for up to 240 min at 37°C. At various time points, trichloroacetic acid-precipitable counts in the medium (degraded ligand) were determined.

Ligand crosslinking analyses

For chemical crosslinking experiments with 32D cells, 5.0×10^6 cells were incubated for 2 h on ice with 20 ng/ml radiolabeled EGF or TGFα. The chemical crosslinker bis(sulfonylsuccinimidyl)-suberate (BS^3, Pierce, Roxford, IL) was added to a final concentration of 1 mM. Cells were then incubated for 45 min at 4°C and subsequently washed with phosphate buffered saline, pelleted by centrifugation, and lysed in solubilization buffer. Lysates were cleared by centrifugation, and immunoprecipitated with antibodies against specific ErbB proteins. Rabbit antibodies were directly coupled to protein A–Sepharose beads while shaking (1 h, 4°C); mouse antibodies were coupled indirectly using rabbit-anti-mouse IgG under the same conditions. ErbB proteins present in the cell lysate were immunoprecipitated with the protein A–Sepharose-antibody complex for 2 h at 4°C. Precipitates were washed three times in HNTG buffer prior to heating for 5 min at 95°C in gel sample buffer under reducing conditions. Samples were analyzed using gel electrophoresis (7.5% acrylamide).

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