The C-terminus of the kinase-defective neuregulin receptor ErbB-3 confers mitogenic superiority and dictates endocytic routing

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Signaling by the epidermal growth factor (EGF) family and the neuregulin group of ligands is mediated by four ErbB receptor tyrosine kinases, that form homo- and heterodimeric complexes. Paradoxically, the neuregulin receptor ErbB-3 is devoid of catalytic activity, but its heterodimerization with other ErbBs, particularly the ligand-less ErbB-2 oncoprotein of carcinomas, reconstitutes superior mitogenic and transforming activities. To understand the underlying mechanism we constructed a chimeric EGF-receptor (ErbB-1) whose autophosphorylation C-terminal domain was replaced by the corresponding portion of ErbB-3. Consistent with the possibility that this domain recruits a relatively potent signaling pathway(s), the mitogenic signals generated by the recombinant fusion protein were superior to those generated by ErbB-1 homodimers and comparable to the proliferative activity of ErbB-2/ErbB-3 heterodimers. Upon ligand binding, the chimeric receptor recruited an ErbB-3-specific repertoire of signaling proteins, including Shc and the phosphatidylinositol 3-kinase, but excluding the ErbB-1-specific substrate, phospholipase C γ 1. Unlike ErbB-1, which is destined to lysosomal degradation through a mechanism that includes recruitment of c-Cbl and receptor poly-ubiquitination, the C-terminal tail of ErbB-3 shunted the chimeric protein to the ErbB-3-characteristic recycling pathway. These observations attribute the mitogenic superiority of ErbB-3 to its C-terminal tail and imply that the flanking kinase domain has lost catalytic activity in order to restrain the relatively potent signaling capability of the C-terminus.

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Introduction

Signal transduction by many polypeptide growth factors is mediated by cell surface receptors with an intrinsic tyrosine kinase activity. Type I receptor tyrosine kinases (also called ErbB or HER) constitute a prototypic family of such receptors, which recognizes a very large group of ligands sharing an epidermal growth factor (EGF) motif of six cysteine residues. Whereas ErbB-1 binds several ligands, represented by EGF and the transforming growth

factor α , ErbB-3 and ErbB-4 bind with different affinities several groups of ligands, collectively termed neuregulins (Burden and Yarden, 1997). Essential to understanding ErbB signaling is the realization that the four ErbB proteins can form all 10 possible homo- and heterodimeric complexes, but ErbB-2, a ligand-less co-receptor (Karunagaran *et al.*, 1996), acts as the preferred heterodimeric partner (Tzahar *et al.*, 1996; Graus-Porta *et al.*, 1997). Thus, signaling by ErbB proteins and EGF/neuregulin-like ligands may be described in terms of a neural-type network (Bray, 1990): ligands and ErbB receptors are at the input layer, a large group of cytoplasmic phosphotyrosine-binding proteins and transcription factors constitute the hidden layers, and cellular responses such as proliferation and differentiation are at the output layer (Alroy and Yarden, 1997).

Evolutionarily, the network evolved from a single nematode ligand called Lin-3, and one receptor tyrosine kinase, Let-23 (Kornfeld, 1997). Along with gene duplication events, at both the ligand and the receptor levels, one receptor, ErbB-3, has lost its kinase activity due to several alterations at conserved enzymatic motifs (Guy *et al.*, 1994). Nevertheless, ErbB-3 retained the ability to form heterodimers with other ErbB proteins, and it also serves as their substrate both *in vitro* and in living cells (Wallasch *et al.*, 1995). Several lines of evidence indicate that ErbB-3 plays an essential role in the ErbB network despite being catalytically impaired. First, targeted inactivation of the rodent gene resulted in severe defects in the sympathetic nervous system, Schwann cells of peripheral axons and cardiac development (Erickson *et al.*, 1997; Riethmacher *et al.*, 1997). Secondly, *in vitro* studies have implicated ErbB-3, in combination with ErbB-2, in the development of keratinocytes (Marikovsky *et al.*, 1995), Schwann cell precursors (Syroid *et al.*, 1996), oligodendrocytes (Vartanian *et al.*, 1997) and the neuromuscular synapse (Zhu *et al.*, 1995). Thirdly, ErbB-3 can transform murine fibroblasts, but only in the presence of ErbB-1 or ErbB-2 (Cohen *et al.*, 1996; Zhang *et al.*, 1996). Particularly potent is the cooperation between ErbB-3 and ErbB-2 in the presence of one of the ErbB-3 ligands, namely neuregulin-1 (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995). These observations are significant to cancer of epithelial cells, which often display overexpression of ErbB-2 (e.g. breast and ovarian tumors; Slamon *et al.*, 1987, 1989) on a background of moderate or high ErbB-3 expression (for review see Klapper *et al.*, 1999).

The mechanism underlying cooperation between the ligand-less receptor (ErbB-2) and the kinase-defective protein (ErbB-3) is well explained by results of *in vitro* studies. First, ErbB-2 can dramatically increase the affinity of neuregulins to ErbB-3 (Peles *et al.*, 1993; Sliwkowski *et al.*, 1994; Tzahar *et al.*, 1996), probably by decelerating the rate of ligand dissociation (Karunagaran *et al.*, 1996). In

fact, the ErbB-2/ErbB-3 combination displays an extended specificity to EGF-like ligands, including EGF itself, betacellulin and a chimeric synthetic molecule termed biregulin (Barbacci *et al.*, 1995; Alimandi *et al.*, 1997; Tzahar *et al.*, 1997; Pinkas-Kramarski *et al.*, 1998). Secondly, within the ErbB-2/ErbB-3 heterodimer the catalytically impaired receptor provides many tyrosine substrate sites for *trans*-phosphorylation by ErbB-2 (Wallasch *et al.*, 1995; Kim *et al.*, 1998). These sites are located on the relatively large C-terminal domain of ErbB-3. Upon phosphorylation they serve as docking sites for multiple signaling proteins carrying one of a few types of phosphotyrosine-binding motifs (Carraway and Cantley, 1994). Thirdly, ErbB-3 is characterized by a defective mechanism of clearance from the cell surface (Baulida *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996): instead of ligand-induced internalization and degradation in lysosomes, a mechanism shared by many growth factor receptors (Sorkin and Waters, 1993), ErbB-3 is destined primarily to enter the recycling pathway (Waterman *et al.*, 1998). Presumably, through repeated engagement into heterodimers with an overexpressed ErbB-2, the kinase-defective receptor can prolong signaling and thereby contribute to cell transformation.

The present study addressed the molecular enigma presented by ErbB-3, namely a receptor whose kinase activity is impaired but it nevertheless exerts extremely potent mitogenic and oncogenic signals. By combining the C-terminal domain of ErbB-3 with a catalytically intact tyrosine kinase (of ErbB-1) we hoped to learn why a similar combination has been denied throughout evolution of the ErbB network. Our results indicate that the chimeric receptor, by coupling to an ErbB-3-specific signaling pathway and by avoiding downregulation, acquired a dramatically potent mitogenic activity. Thus, inactivation of the kinase domain placed the very effective C-tail of ErbB-3 under a two-layer control: both a ligand (neuregulin) and a co-receptor with an active kinase (primarily ErbB-2) are essential to initiate signaling from ErbB-3. Apparently, this mechanism allows more stringent regulation of the most active combinations of the ErbB network.

Results

Construction, expression and ligand-induced activation of a chimeric ErbB-1/ErbB-3 receptor in 32D cells

In order to examine the possibility that the C-terminus of ErbB-3 confers to this receptor signaling superiority relative to kinase-intact receptors like ErbB-1, we replaced by molecular cloning the C-terminus (C-tail) of the latter receptor with the corresponding domain of ErbB-3. The resulting chimeric receptor, denoted ErbB-1T3 (Figure 1), consisted of the extracellular, transmembrane and kinase domains of ErbB-1 fused to the relatively long C-terminus of ErbB-3. This tail harbors multiple tyrosine phosphorylation sites that recruit cytoplasmic-signaling proteins (Prigent and Gullick, 1994). To allow sensitive determination of the relative ability of the constructed chimera to generate proliferative signals we expressed it in the interleukin-3 (IL-3)-dependent 32D myeloid cell line, which expresses no

Fig. 1. Structural representations of ErbB-1, ErbB-3 and the derivative ErbB-1T3 chimeric protein. The domain structure of ErbB-1 (EGF-receptor; open rectangles) and ErbB-3 (closed rectangles) is shown by boxes and segments corresponding to the double cysteine-rich domain of the extracellular (EC) region, the transmembrane domain (TM), the juxtamembrane domain (JM), the tyrosine kinase domain (TK), and the C-terminal tail (CT). Tyrosine phosphorylation sites located at the C-terminal tails are schematically presented by bars. P represents autophosphorylation. The catalytic domain of ErbB-3 is crossed to indicate its enzymatic defect. Note that the chimeric protein ErbB-1T3 includes the extracellular, transmembrane, juxtamembrane and kinase domains of ErbB-1 (open boxes) fused to the C-terminal tail of ErbB-3.

endogenous ErbB protein (Pinkas-Kramarski *et al.*, 1996). Cell clones that express ErbB-1T3 were drug-selected and their levels of receptor expression tested by immunoblotting. Several positive clones that expressed the chimeric receptor were selected and designated D1T3. Specific recognition of EGF by the recombinant receptor was confirmed by saturable binding and covalent crosslinking of radiolabeled EGF molecules (Figure 2A; data not shown). An *in vitro* kinase assay performed on receptor immunoprecipitates indicated that the chimeric receptor was able to undergo phosphorylation *in vitro* (Figure 2B). In experiments that are not presented we found that only very low phosphorylation activity could be recovered from cells singly expressing ErbB-3, in agreement with a previous report (Guy *et al.*, 1994). Consistent with the results of the *in vitro* assays, a 190 kDa protein corresponding to ErbB-1T3 underwent EGF-induced tyrosine phosphorylation in transfected 32D cells (Figure 2C). As expected, the chimeric receptor displayed no response to an ErbB-3 ligand, namely neu differentiation factor (NDF). However, unlike cells singly expressing ErbB-1 (D1 cells), in which a 170 kDa protein, corresponding to the EGF-receptor, along with a 120 kDa protein (probably c-Cbl, see below) and other proteins were modified in response to EGF, only the receptor was prominently phosphorylated in D1T3 cells. Taken together, the results shown in Figure 2 indicate proper construction and

expression at the cell surface of the chimeric ErbB-1/ErbB-3 protein, which retained ligand-dependent tyrosine phosphorylation.

Fig. 2. Expression of the ErbB-1T3 chimeric receptor and its phosphorylation *in vitro* and *in vivo*. (A) 32D myeloid cells that ectopically express the chimeric receptor ErbB-1T3, denoted D1T3 cells (an equivalent of 10^7 cells per lane), were incubated for 1.5 h at 4°C with a radiolabeled EGF (100 ng/ml). Covalent cross-linking analysis of the radioactive ligand to its receptor was then performed by using the bivalent reagent BS³ (1 mM). This was followed by cell lysis and immunoprecipitation (I.P.) with antibodies directed to either the extracellular (EC) domain of ErbB-1 (left lane), the C-terminal domain (CT) of ErbB-1 (middle lane), or the C-terminal domain of ErbB-3 (right panel). The extensively washed immunocomplexes were resolved by SDS-PAGE. Arrows mark the locations of the monomeric (M) and dimeric (D) receptor species. The corresponding two species of ErbB-1T3 are resolvable in a shorter exposure autoradiogram. (B) Whole-cell lysates were prepared from 10^7 cells of the indicated sublines of 32D cells. A monoclonal antibody directed to the extracellular domain of ErbB-1 was used for immunoprecipitation. Immunocomplexes were subjected to an *in vitro* kinase reaction by adding radioactive ATP. They were then washed and resolved by SDS-PAGE. The resulting autoradiogram is shown, along with the locations of molecular weight marker proteins. Note the doublet appearance of ErbB-1T3 in both panels. (C) The indicated lines of 32D cells were incubated for 10 min at 37°C with or without the indicated ligands (each at 100 ng/ml). Whole-cell lysates were prepared, resolved by SDS-PAGE and subjected to an immunoblot (I.B.) analysis with an anti-phosphotyrosine (P-TYR) antibody.

The chimeric ErbB-1T3 receptor can mediate potent cell growth and survival signals

We have reported previously that both ErbB-2 and ErbB-1 can reconstitute *in trans* relatively potent mitogenic signals of an NDF-bound ErbB-3 (Pinkas-Kramarski *et al.*, 1996). However, when singly expressed in 32D cells, ErbB-1 and ErbB-3 can mediate only a weak or a null signal, respectively. To determine the relative mitogenic potential of the ErbB-1T3 receptor we replaced IL-3 in the medium of D1T3 cells with increasing concentrations of EGF, and quantified cell proliferation by using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay. For reference, we used two derivatives of 32D cells: D1 cells, which singly express ErbB-1; and D23 cells co-expressing ErbB-2 and ErbB-3. As expected, EGF-driven homodimers of ErbB-1 generated significantly weaker signals compared with the NDF-driven heterodimers between ErbB-2 and ErbB-3 (Figure 3A). It was surprising that the response of D1T3 cells to EGF exceeded the effect of the most mitogenic receptor combination, ErbB-2/ErbB-3 (Figure 3A, and data not shown). To differentiate between the strong proliferative effect of ErbB-1T3 and its ability to extend cell survival, we analyzed the cell cycle distribution of EGF-treated D1 and D1T3 cells (Figure 3B). Cell sorting revealed that most (>90%) EGF-treated D1 cells underwent apoptosis after 72 h of incubation in the absence of IL-3. In contrast, <35% of D1T3 cells were found in the sub-G₁ phase of the cell cycle, confirming a potent cell survival effect of the C-terminus of ErbB-3. A long-term cell growth assay further supported the potent proliferative and survival activities of the C-tail. This assay involves IL-3 withdrawal and incubation of cells in the absence or presence of an ErbB ligand for up to 3 days. Whereas EGF only slightly extended the survival of D1 cells relative to the control untreated cells (Figure 3C, D1 panel), NDF exerted extensive cell proliferation (Figure 3C, D1T3 panel). This was comparable to the effects of IL-3, the ultimate mitogen of 32D cells. In contrast, stimulation of the chimeric receptor with EGF yielded a proliferation signal that exceeded that of IL-3 (panel D1T3 in Figure 3C). Taken together, the results of short- and long-term cell growth assays clearly indicate that the C-terminal portion of ErbB-3 can confer to ErbB-1 an extremely potent mitogenic potential, which appears to exhaust the proliferative cellular machinery.

The chimeric ErbB-1T3 receptor recruits Shc and the regulatory subunit of the phosphoinositide 3-kinase, but not PLC-γ1

To provide an initial mechanistic basis for the mitogenic superiority of the C-terminal tail of ErbB-3, we analyzed its ability to confer recruitment of several signaling proteins. First, we tested association of the chimeric receptor with the Shc adaptor protein. It is noteworthy that in the myeloid 32D cells only p46^{Shc} and p52^{Shc} are expressed, but the larger isoform, p66^{Shc}, is absent (Figure 4A, lower panel). Immunoprecipitation with either anti-receptor or anti-phosphotyrosine antibodies followed by immunoblotting detected the p52 isoform, which underwent increased physical association with the stimulating receptor following ligand binding (Figure 4A). The other isoform, p46^{Shc}, was found primarily in constitutive complexes with the

Fig. 3. Ligand-induced proliferative responses of ErbB-expressing 32D cells. (A) Sublines of 32D cells that express either ErbB-1 alone (D1 cells; squares), a combination of ErbB-2 and ErbB-3 (D23 cells; circles), or the chimeric ErbB-1T3 receptor (D1T3 cells; triangles) were tested for ligand-induced cell proliferation by using the MTT assay. Cells were deprived of IL-3 and plated at a density of 5×10^5 cells/ml in media containing serial dilutions of either EGF (D1 and D1T3 cells) or NDF (D23 cells). The MTT assay was performed 24 h later. The results obtained are presented as fold induction over a control culture maintained for 24 h in the absence of added growth factors. (B) D1 (black line) and D1T3 (gray line) cells (10^6 /ml) were cultured at 37°C for 72 h in the absence (Cont.) or presence of EGF (100 ng/ml). No IL-3 was included in the culture medium. Cells were harvested 72 h later and analyzed for their DNA content by flow cytometry. The sub-G₁ phase of the cell cycle, which represents primarily cells undergoing apoptosis, is indicated. (C) The indicated sublines of 32D cells (5×10^5 cells/ml) were incubated for various time intervals with the following ligands (each at 100 ng/ml): EGF (squares; D1 and D1T3 cells) or NDF (squares; D23 cells). Control cultures were incubated in the absence (open triangles) or presence of IL-3 (closed triangles). Cell growth was determined daily by using the colorimetric MTT assay, and compared with the MTT signal obtained with a fresh culture at time zero. The data presented are the mean $\pm$ SD of four determinations. Each experiment was repeated at least twice.

receptors. The ability to elicit p52 recruitment was shared by ErbB-1 homodimers (in D1 cells), the ErbB-2/ErbB-3 heterodimer (after stimulation with NDF, D23 cells), as well as by homodimers of the ErbB-1T3 chimera (after EGF stimulation). In contrast with Shc, ErbB-1 but not ErbB-3 can interact with the phosphatidylinositol-specific phospholipase, PLC- γ 1 (Fedi *et al.*, 1994). This differential coupling was confirmed, by co-immunoprecipitation analysis, in the transfected 32D cells we used (compare D1 and D23 lanes in Figure 4B). However, examination of ErbB-1T3-expressing cells revealed that the chimeric receptor lost significant interaction with PLC- γ 1; this enzyme was hardly detectable in immunoprecipitates of an EGF-stimulated ErbB-1T3 (Figure 4B). From the results of other experiments not presented here, we learned that ErbB-1T3 lost the ability to interact with another ErbB-1-specific effector, the Ras-GTPase activating protein (Ras-GAP) (Fedi *et al.*, 1994), although this effector underwent physical association with the ligand-activated ErbB-1 in D1 cells. Unlike Ras-GAP and PLC- γ 1, the phosphoinositide 3-kinase can interact efficiently with ErbB-3, but in most cell types the interaction between the lipid kinase and ErbB-1 is almost undetectable (Soltoff *et al.*, 1994). In accordance with previous reports, we detected only weak precipitation of the regulatory subunit, p85, by anti-phosphotyrosine antibodies in response to

ErbB-1 activation (Figure 4C, D1 lanes). In contrast, relatively large quantities of p85 could be co-immunoprecipitated from extracts of ligand-stimulated D23 or D1T3 cells by using anti-phosphotyrosine or anti-receptor antibodies (Figure 4C). Thus, p85 phosphorylation on tyrosine residues, or its association with a tyrosine-phosphorylated complex, displayed an increase following NDF binding to an ErbB-2/ErbB-3 heterodimer or EGF binding to ErbB-1T3 homodimers. However, ErbB-1 homodimers were unable to recruit the lipid kinase with high efficiency. A similar conclusion was reached on the basis of determination of the lipid kinase activity recovered by anti-phosphotyrosine antibodies (data not shown). When combined with the observed ability of ErbB-1T3 to recruit Shc but not PLC- γ 1, these results indicate that the C-terminal portion of ErbB-3 can confer to ErbB-1 an ability to recruit, at least in part, an ErbB-3-specific repertoire of signaling proteins.

Because receptor coupling to various phosphotyrosine-binding proteins is ultimately followed by activation of linear cascades of protein kinases (for review see Seger and Krebs, 1995), we analyzed the capacity of ErbB-1T3 to funnel signals into two such cascades, the mitogen-activated protein kinase (MAPK or Erk) and the Akt/protein kinase-B pathway. Antibodies specific to the active phosphorylated forms of the two kinases enabled sensitive

Fig. 4. Downstream signaling by the chimeric ErbB-1T3 receptor and its parental ErbB-1 and ErbB-3 molecules. The indicated sublines of 32D cells were incubated for 10 min at 37°C in the absence (–) or presence of EGF or NDF (each at 100 ng/ml). Whole-cell lysates were then prepared and subjected to immunoprecipitation (I.P.) with either a mAb to phosphotyrosine (P-TYR) or anti-receptor antibodies; either anti-ErbB-1 (D1 and D1T3 cells) or anti-ErbB-3 (D23 cells). The anti-receptor immunocomplexes were resolved by SDS–PAGE and subjected to immunoblot analysis (I.B.) with antibodies specific to one of the following signaling molecules: (A) Shc, (B) phospholipase C γ 1 (PLC- γ 1) or (C) the α subunit of the phosphoinositide 3-kinase (p85). Aliquots of whole-cell lysates were directly resolved by SDS–PAGE, followed by immunoblotting to test for equal loading. Arrowheads indicate the locations of the corresponding signaling proteins.

determination of ligand-induced activation. Although ErbB-1T3, like ErbB-1 and the ErbB-2/ErbB-3 combination, rapidly activated the Erk pathway, signaling by this receptor was more sustained than that initiated by ErbB-1 (Figure 5A); whereas the ErbB-1-initiated signal almost disappeared after 10 min, Erk activation through ErbB-1T3 was still detectable after 60 min, and the kinetics exhibited by D23 cells was slightly shorter. Control experiments excluded the possibility that Erk underwent degradation in ligand-stimulated cells (data not shown). It is worthwhile noting that differences in kinetics were not reflected in the time course of receptor phosphorylation (Figure 5A),

Fig. 5. Comparative kinetic analyses of receptor phosphorylation and activation of the linear kinase cascades Erk and Akt. (A) The indicated ErbB-expressing derivatives of 32D cells were incubated at 37°C for various time intervals (indicated in minutes) with the appropriate ligand, either EGF (D1 and D1T3 cells) or NDF (D23 cells), each at 100 ng/ml. At the end of the incubation period, whole-cell lysates were prepared, resolved by gel electrophoresis and subjected to immunoblotting (I.B.) with either an antibody to phosphotyrosine, or with an antibody specific to the active doubly phosphorylated form of Erk (P-Erk). A separate immunoblot assay indicated that Erk expression level was similar in D1 and in D1T3 cells and it underwent no significant change following ligand stimulation. (B) Whole-cell lysates were prepared as described above and resolved by SDS–PAGE followed by immunoblotting (I.B.) with an antibody directed to the active Ser-473 phosphorylated form of Akt (P-Akt). Signal detection was performed by using a chemiluminescence kit.

implying a post-receptor mechanism. Similar to Erk activation, all three receptors we examined were able to stimulate Akt phosphorylation (Figure 5B). However, the differences in kinetics of Akt activation were moderate compared with those exhibited by Erk. Although Akt is considered a down-stream effector of the phosphoinositide 3-kinase, its activation by ErbB-1 may be explained by recent studies, which uncovered wortmannin-independent pathways of Akt activation, including a route involving the calcium/calmodulin-dependent protein kinase kinase (Yano *et al.*, 1998). We conclude that despite differences in coupling to the phosphoinositide 3-kinase and PLC- γ 1, both ErbB-1 and ErbB-3 can recruit the MAPK and the Akt pathways, albeit with disparate kinetics.

The C-terminal domain of ErbB-3 impairs endocytic sorting of the EGF receptor

The superior mitogenic activity mediated by ErbB-1T3, as well as its prolonged signaling, raised the possibility that this receptor is less susceptible than ErbB-1 to inactivation mechanisms. To examine this scenario we followed the major mechanism of signal inactivation,

Fig. 6. Ligand-dependent receptor downregulation and degradation. (A) D1 cells (squares) or D1T3 cells (circles) were incubated with EGF (100 ng/ml) at 37°C for the time intervals indicated. Cells were then placed on ice and their surface-bound ligand removed using an acidic buffer. Radiolabeled EGF was then added and its binding to the cell surface at neutral pH used to quantify the extent of receptor disappearance. The results are expressed as the average fraction of original binding sites that remained on the cell surface after exposure to the unlabeled ligand at 37°C. (B) To follow receptor degradation directly, cells were treated with EGF as described above, except that whole-cell lysates were prepared at the end of each time interval. Following separation by SDS-PAGE and transfer to nitrocellulose membrane filters, membranes were immunoblotted (I.B.) with antibodies directed to the respective receptors, as indicated.

namely the decrease in the number of cell-surface receptors following stimulation with a growth factor, a process termed 'downregulation'. Evidently, and in agreement with previous reports, EGF induced rapid and extensive downregulation of ErbB-1 in 32D cells (Figure 6A). In contrast, almost no parallel decrease in the number of EGF-binding sites was observed with D1T3 cells following ligand binding. This observation implied that the C-terminus of ErbB-3, when replacing the corresponding domain of ErbB-1, can impair downregulation of the latter receptor. Immunoblotting of whole-cell lysates with anti-receptor antibodies confirmed this conclusion since rapid disappearance of ErbB-1, due to intracellular degradation, was observed upon exposure of D1 cells, but not D1T3 cells, to EGF (Figure 6B).

It is important to note that the parental molecules of ErbB-1T3 are differentially destined to intracellular degradation; whereas ErbB-3 is destined primarily to the recycling pathway (Waterman *et al.*, 1998), endocytic targeting of ErbB-1 to degradation in lysosomes involves recruitment of the c-Cbl adaptor protein and concomitant conjugation of ubiquitin molecules to ErbB-1 (Levkowitz *et al.*, 1998). Therefore, our subsequent experiments analyzed ligand-induced ubiquitination of the ErbB-1T3

Fig. 7. Ligand-induced ubiquitination of ErbB proteins and their coupling to the c-Cbl protein. (A) The indicated ErbB-expressing sublines of 32D cells (10^7 cells/lane) were treated for the time intervals indicated at 37°C with or without ligands, either EGF (D1 and D1T3 cells) or NDF (D23 cells), each at 100 ng/ml. Whole-cell lysates were then prepared and analyzed by immunoprecipitation (I.P.) using antibodies directed to the respective receptor [anti-ErbB-1 antibodies (D1 and D1T3 cells) or anti-ErbB-3 antibodies (D23 cells)]. Immunocomplexes were washed extensively, resolved by SDS-PAGE and analyzed by immunoblotting (I.B.) with either anti-ubiquitin or anti-c-Cbl antibodies. To detect receptor phosphorylation, whole-cell lysates were directly resolved by SDS-PAGE (panel marked as NONE), transferred to membrane filters and immunoblotted with anti-phosphotyrosine (P-TYR) antibodies. The locations of molecular weight marker proteins are indicated in kilodaltons. The filled arrowhead in the anti-ubiquitin panel indicates the location of the major ErbB-1 protein band, whereas the open arrowhead marks the ubiquitinated species. (B) 32D sublines were treated for 10 min with the indicated ligands as described above and whole-cell lysates were either directly resolved by SDS-PAGE (NONE) or first subjected to immunoprecipitation (I.P.) with antibodies to phosphotyrosine (P-TYR). Following transfer to nitrocellulose membranes, filters were immunoblotted with anti-c-Cbl antibodies. Antibody detection was performed using a chemiluminescence kit.

chimeric receptor. Although rapid EGF-induced modification of ErbB-1 was readily detectable in D1 cells, in agreement with observations made with other cell types (Galcheva-Gargova *et al.*, 1995), no ubiquitination of either ErbB-1T3 or ErbB-3 was detectable following stimulation with EGF or NDF, respectively (Figure 7A). Consistent with a requirement for c-Cbl recruitment, this adaptor became physically associated only with ErbB-1; no c-Cbl protein could be detected in immunoprecipitates of an activated ErbB-1T3 or the parental ErbB-3 molecule (Figure 7A, middle panel). Control experiments verified that both receptors, as well as ErbB-2, underwent extensive tyrosine phosphorylation upon ligand binding (Figure 7A, lower panel; data not shown). Because recruitment of c-Cbl by ErbB-1, as well as by other receptors, is followed by extensive tyrosine phosphorylation of the adaptor molecule (reviewed in Thien and Langdon, 1998), we examined the phosphorylation state of c-Cbl in ligand-

stimulated D1, D1T3 and D23 cells. In line with differential coupling, extensive tyrosine phosphorylation of the adaptor (or its recruitment into a tyrosine-phosphorylated complex) was observed only in EGF-stimulated D1 cells (Figure 7B). Taken together, the results shown in Figures 6 and 7 suggest that the defective downregulation behavior of the ErbB-1T3 molecule is due to its inability to recruit c-Cbl and undergo ubiquitination. By inference, this inability to undergo ubiquitination and downregulation may explain the relatively potent and sustained signaling by ErbB-3-containing receptor combinations, such as the ErbB-2/ErbB-3 heterodimer.

c-Cbl can inhibit signal transduction downstream to ErbB-1, but ErbB-1T3 signaling is refractory to c-Cbl

Sli-1, the *Caenorhabditis elegans* homologue of c-Cbl, was identified by a genetic screen that searched for inhibitors of Ras signaling downstream to Let-23, the worm homologue of the EGF-receptor (Yoon *et al.*, 1995). To examine the possibility that ErbB-1T3 is endowed with relatively potent signaling because it escaped inhibition by c-Cbl, we examined the effect of the latter adaptor protein on transcription from the serum response element (SRE). This DNA sequence of the Fos promoter integrates several signaling pathways, including the Ras-MAPK route (Whitmarsh *et al.*, 1995). Due to the relatively inefficient transient expression of genes introduced into 32D cells we performed these experiments in adherent Chinese hamster ovary (CHO) cells. Preliminary experiments that tested the suitability of this cell system confirmed that ErbB-1, but not ErbB-1T3, undergoes ligand-induced ubiquitination in CHO cells, and showed that this modification can be enhanced by an overexpressed c-Cbl (data not shown). ErbB-1 and ErbB-1T3 were transiently expressed in CHO cells, along with an SRE reporter plasmid, and the response to EGF quantified. Incubation of ErbB-1-expressing cells with EGF enhanced by several-fold transcription from the SRE (Figure 8A). However, overexpression of c-Cbl almost abolished EGF-induced transcription from the SRE; background transcription from this element was significantly reduced and EGF only weakly stimulated expression of the reporter under these conditions (Figure 8A, left panel). Overexpression of ErbB-1T3 increased basal SRE activity in CHO cells by 2- to 3-fold. Despite this high background activity, cells expressing ErbB-1T3 retained their ability to upregulate SRE activity in response to EGF. However, ectopic overexpression of c-Cbl in these cells exerted only very small effects on both basal and EGF-induced SRE activity (Figure 8A, right panel). Analyses of receptor phosphorylation and MAPK activation yielded results that were in line with the effects on transcription from the SRE (Figure 8B). First, despite relatively high basal phosphorylation of ErbB-1T3, this receptor, like ErbB-1, exhibited an increase in autophosphorylation upon ligand binding. However, Erk activation by ErbB-1T3 was more sustained than the stimulation observed following ErbB-1 activation. Moreover, overexpression of c-Cbl almost abolished Erk activation via ErbB-1, but it exerted no significant effect on the ErbB-1T3-mediated activation of the MAPK pathway. Taken together with the results of receptor downregulation and degradation, these results imply that the potent signal-

ing capacity of ErbB-3 is due to its C-terminus; by avoiding recruitment of c-Cbl, this region enables ErbB-3 to evade negative regulation and, thereby, it can transmit prolonged signals through the MAPK-SRE pathway.

Discussion

The family of growth factor receptors with intrinsic tyrosine kinase activity includes several members whose catalytic activity is defective. This relatively small group includes, in addition to ErbB-3, also CCK-4, Vik/Ryk, Klg and two Ror isoforms (Mossie *et al.*, 1995 and references therein). Because all of the kinase-defective receptors except ErbB-3 remained orphan of ligands, their study is extremely difficult and lessons learned with ErbB-3 are considered relevant to the function of other kinase-defective receptors. The enzymatic domains of these receptors differ from the active kinases within a few highly conserved motifs essential for catalysis (van der Geer *et al.*, 1994). For example, the highly conserved kinase motif VIb, HRDLA, is present as HRNLA in ErbB-3, but the canonical DFG sequence (kinase motif VII), which is modified in most kinase-defective receptors, is unmodified in ErbB-3. Another ErbB-3 structural landmark that is unique among ErbBs, but is not shared with other kinase-defective receptors, is exhibited by the flanking C-terminus. This relatively long and hydrophilic region carries more potential sites for tyrosine phosphorylation than any other ErbB protein (Carraway and Cantley, 1994). However, conversion of these sites to active docking points for cytoplasmic-signaling proteins appears strictly regulated; only upon *trans*-phosphorylation by other ErbBs does ErbB-3 recruit its own signaling proteins. Attempts to relieve the activity of ErbB-3 by replacing certain amino acids of the catalytic domain have failed (Prigent and Gullick, 1994), indicating that catalysis, at least in the case of ErbB-3, is defected at several regions of the enzyme. This observation, together with the fact that ErbB-3 proteins from different mammalian species are all kinase-dead, suggests that inactivation of the enzyme is not accidental; it probably fulfills a functional role. The present study addressed this role by using an experimental strategy that may be applicable to other defective receptor tyrosine kinases; instead of attempting to relieve the kinase by mutagenesis we constructed a chimeric active kinase that appears to recruit ErbB-3-specific partners.

Several lines of evidence indicate that the chimeric ErbB-1/ErbB-3 molecule we constructed reliably represents signaling through ErbB-3, rather than via its other parent, ErbB-1. First, the recombinant molecule underwent efficient phosphorylation on tyrosine residues (Figure 2C) in response to ligand binding and homodimerization (Figure 2A) in cells lacking endogenous ErbBs. Secondly, as predicted for ErbB-3, the proliferative signals generated by the chimeric receptor were significantly stronger than ErbB-1-driven mitogenesis (Figure 3). Thirdly, although several pathways common to ErbB-1 and ErbB-3 were utilized by the chimeric receptor (e.g. Shc, Erk and Akt), it nevertheless exhibited ErbB-3 specificity in that the phosphoinositide 3-kinase, but not PLC γ 1, Ras-GAP or c-Cbl, was recruited (Figures 4 and 7). Lastly, like ErbB-3, but unlike ErbB-1, the chimeric receptor underwent neither

Fig. 8. c-Cbl negatively regulates signaling downstream of ErbB-1, but the ErbB-1T3 chimeric receptor is refractory to c-Cbl. **(A)** Monolayers of CHO cells (in triplicate) were co-transfected with a plasmid vector directing expression of the indicated receptor (1 μ g of DNA, either pcDNA-3/erbB-1 or pcDNA-3/erbB-1T3) together with a c-Cbl plasmid (1 μ g or an empty control vector). To follow activation of transcription from the SRE, all cell monolayers were also transfected with an SRE-luciferase reporter plasmid. At 36 h post transfection cells were untreated (open bars) or treated (closed bars) with EGF (20 ng/ml), and 16 h later we performed a luciferase assay on cell extracts. Signals obtained were normalized to the concentration of proteins in each cell extract and are presented as the relative light units [average $\pm$ SD (bars)] of triplicate determinations. **(B)** Monolayers of CHO cells were co-transfected with a plasmid encoding c-Cbl, along with ErbB-1- or ErbB-1T3-encoding expression vectors (as indicated). Following stimulation with EGF (100 ng/ml) for the indicated time intervals, whole-cell lysates were prepared and analyzed by immunoblotting with antibodies to phosphotyrosine (P-TYR), the activated form of Erk (P-Erk), or an anti-HA antibody that detects expression of a recombinant c-Cbl. The arrowheads mark the locations of the c-Cbl protein band.

downregulation nor ligand-dependent ubiquitination (Figures 6 and 7). These biochemical characteristics of the chimeric ErbB-1T3 receptor are not necessarily mutually independent. For example, recruitment of c-Cbl, an ErbB-1-specific substrate (Levkowitz *et al.*, 1996) promotes receptor ubiquitination and downregulation, two landmarks of signaling through the EGF receptor, rather than through the neuregulin receptor (Levkowitz *et al.*, 1998). Likewise, efficient recruitment of the lipid kinase, an ErbB-3 landmark (Soltoff *et al.*, 1994), is known to be essential for receptor recycling (Joly *et al.*, 1994), another intrinsic property of ErbB-3 (Waterman *et al.*, 1998). Despite these consistent observations, we cannot exclude the possibility that ErbB-1T3 has lost interactions with

some of the original ErbB-3 effectors. Moreover, because the identity of tyrosine autophosphorylated sites within ligand-activated ErbB dimers may depend on the specific partner (Olayioye *et al.*, 1998), different sets of signaling proteins may be recruited to ErbB-3 when it heterodimerizes with each of the three other ErbBs.

It is important to consider potential mechanisms that may explain the mitogenic superiority of ErbB-3 relative to ErbB-1. According to one scenario, superiority is determined by the specific repertoire of signaling proteins the neuregulin receptor can recruit. For example, the phosphoinositide 3-kinase, which is specifically recruited by ErbB-3 and the ErbB-1T3 chimeric receptor, is a well-known regulator of cell growth and survival (reviewed in

Carpenter and Cantley, 1996). Another candidate is Shc, whose relatively strong interaction with ErbB-3 appears crucial for neuregulin-induced MAPK activation and mitogenic effect (Vijapurkar *et al.*, 1998). However, superiority of ErbB-3 is not limited to positive regulators; it is possible that the specific partners of ErbB-1 are in fact negative regulators. This possibility may imply that PLC γ can diminish the mitogenic potential of the EGF receptor. Consistently, accelerated generation of inositol trisphosphate and a rise in cytoplasmic calcium concentrations, two effectors of PLC γ , can attenuate mitogenic signals when the interaction of this enzyme with ErbB-1 is artificially enhanced (Obermeier *et al.*, 1996). Besides PLC γ , Ras-GAP (Fedi *et al.*, 1994) and Grb-2 (Prigent and Gullick, 1994; Fiddes *et al.*, 1998) interact with ErbB-1, but not with ErbB-3. We are currently investigating whether or not these targets can restrict ErbB-1 mitogenesis. However, unlike the unclear role of specific phosphotyrosine-binding proteins in determining signaling potency, causative relationships between receptor downregulation and weaker signaling are well established. Thus, an endocytosis-defective mutant of ErbB-1 is endowed with enhanced mitogenic ability and oncogenic potential (Wells *et al.*, 1990). Similarly, a dominant-negative dynamin mutant, which inhibits internalization of the EGF-receptor via clathrin-coated areas of the plasma membrane augments signaling by EGF (Vieira *et al.*, 1996). On the ligand side, TGF α , a ligand that directs ErbB-1 to recycling (Ebner and Derynck, 1991) is more potent than EGF on many types of cells. Likewise, growth factors encoded by poxviruses are significantly more potent than their mammalian counterparts because they escape the normal downregulation process (Tzahar *et al.*, 1998). It is therefore conceivable that the ErbB-1T3 receptor we studied acquired high signaling potency by avoiding the c-Cbl-mediated process of receptor downregulation (Figure 6). This possibility is strongly supported by our analysis of SRE-mediated transcription, which is sensitive to c-Cbl only when stimulated via ErbB-1 (Figure 8A). At present, however, we cannot exclude alternative mechanisms or their combinations. Nevertheless, it is clear that the C-terminal region of ErbB-3 is necessary and sufficient for full expression of the extremely potent mitogenic power of ErbB-3.

What is the biological rationale behind the evolution and perseverance of kinase-impaired receptors like ErbB-3? In the case the neuregulin receptor this question may be approached from an evolutionary perspective of network signaling. The gradual increase in the complexity of the ErbB network throughout evolution, an outcome of adding ligands, receptors and effectors (Tzahar and Yarden, 1998), remarkably increased the diversification potential of the network by means of combinatorial assemblies. Inevitably, added complexity necessitated more stringent control, especially over the more potent combinations. ErbB-3, by means of its uniquely active C-terminal domain, is probably the most potent signal generator of the network. Therefore, its separation from an active kinase created a two-layer regulatory mechanism; unlike homodimers of active kinases, which require only a ligand for full activation, stimulation of the more potent heterodimers with ErbB-3 (Pinkas-Kramarski *et al.*, 1996) strictly depends on a ligand and a co-receptor. Thus, ErbB-3 may

have lost enzymatic activity in order to restrain its potent collection of C-terminally-located tyrosine docking sites. The preferred partner of ErbB-3, namely ErbB-2, raises another interesting question: what is the significance of the likely possibility that ErbB-2 has no direct ligand of its own? Besides providing an intact kinase that *trans*-phosphorylates ErbB-3, ErbB-2 itself carries a C-terminal region with unique signaling properties. For example, this region strongly couples to the MAPK pathway (Ben-Levy *et al.*, 1994) and it exerts an inhibitory effect on ErbB-2 internalization (Sorkin *et al.*, 1993). More mechanistic studies of signaling from the ErbB-2/ErbB-3 heterodimer, as well as from other ErbB combinations, appear crucial for understanding the normal physiological role of ErbB-3 and its contribution to human cancers.

Materials and methods

Materials, buffers and antibodies

A recombinant form of NDF- β 1₁₇₇₋₂₄₆ was prepared by Amgen (Thousand Oaks, CA). Human recombinant EGF was purchased from Sigma (St Louis, MO). Radioactive materials were from Amersham (Aylesbury, UK). IODOGEN and BS³ were from Pierce. Rabbit anti-c-Cbl (C-15) antibodies, anti-ErbB-1, anti-ErbB-3 and a monoclonal antibody (mAb) to phosphotyrosine were from Santa-Cruz Biotechnology (Santa-Cruz, CA). Rabbit anti-Shc antibodies, and murine anti-PLC- γ 1 antibodies were purchased from Transduction Laboratories (Lexington, KY). An anti-phosphoinositide 3-kinase (p85) was from Upstate Biotechnology (Lake Placid, NY). A mAb to the active doubly phosphorylated form of Erk (Thr-183 and Tyr-185) was from Sigma, and an antibody to the active phosphorylated form of Akt (Ser-473) was from New England Biolabs. An anti-ubiquitin antibody was kindly provided by Dr S. Yokota (Yamanashi Medical University, Japan). mAbs to the extracellular domain of ErbB-1 and ErbB-3 used for immunoprecipitation were antibodies SG199 and X252, respectively (Chen *et al.*, 1996). Binding buffer contained RPMI 1640 supplemented with 0.5% bovine serum albumin and 20 mM HEPES. Solubilization buffer contained 50 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 10 μ g/ml pepstatin A, 10 μ g/ml aprotinin and 10 μ g/ml leupeptin. The HNTG buffer contained 20 mM HEPES pH 7.5, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol.

Establishment of transfected cell lines

To construct the ErbB-1T3 chimeric receptor we synthesized by PCR the portion of the human erbB-3 cDNA corresponding to the C-terminus of the protein, using the following primers: 5'-CCAATGCATAGAGA-GAGTGGGCTGG-3' (this primer was fused to an *Nsi*I site at the 5' end) and 3'-GCTCTAGAGGGAATAGGGAGAAGACGG-5'.

The DNA fragment was cloned into an *Eco*RV-restricted pBluescript plasmid. The resulting shuttle plasmid was cut with *Nsi*I and *Ksp*I to match a pBluescript/erbB-1 from which the C-terminus-encoding portion was removed using the two enzymes. Proper insertion of the fragment was verified by nucleotide sequence analysis. The resulting chimeric DNA was cut out of pBluescript using *Xba*I and cloned into the same site in the poly-linker of the pCDNA3-mammalian expression vector (Invitrogen). Right orientation of the insert was tested by restriction analysis. The establishment of a series of IL-3-dependent 32D myeloid cells expressing ErbB-1 (D1 cells), ErbB-3 (D3 cells) or the combination of ErbB-2 with ErbB-3 (D23 cells) has been described elsewhere (Pinkas-Kramarski *et al.*, 1996). Stable expression of ErbB-1T3 in 32D cells was achieved using essentially the same procedures as those described above.

Cell proliferation and survival assays

Cells were washed free of IL-3, resuspended in RPMI 1640 medium at 5×10^5 cells/ml, and treated without or with growth factors or IL-3 (1:1000 dilution of medium conditioned by IL-3-producing cells). Cell proliferation was determined by using the MTT assay (Mosman, 1983). MTT (0.1 mg/ml) was incubated for 2 h at 37°C with the cells analyzed. Living cells can 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 isopropyl alcohol. For cell cycle analysis, cells were seeded at 10^6 cells/ml with or without growth factors. After 72 h, the treated cells were washed once with phosphate-buffered saline and fixed in cold methanol for 1 h at 4°C. RNase A (5 µg) and propidium iodide (10 µg) were added and incubated with the cells for 10 min. The stained cells were analyzed in a fluorescence-activated cell sorter (FACScan, Becton Dickinson) within 2 h. The percentage of cells in the different phases of the cell cycle was determined by using the Cellquest program.

Lysate preparation, immunoprecipitation and Western blotting

Cells were exposed to the indicated stimuli in RPMI 1640 medium. After treatment, cells were pelleted by centrifugation and extracted in solubilization buffer, mixed harshly and lysates cleared by centrifugation. For direct electrophoretic analysis, boiling gel sample buffer was added to cell lysates. For other experiments, lysates were first subjected to immunoprecipitation with immobilized antibodies. Rabbit antibodies were coupled directly to protein A-Sepharose while shaking for 1 h at 4°C. Mouse antibodies were first coupled to anti-mouse IgG-agarose by the same procedure. The proteins in the lysate supernatants were immunoprecipitated for 2 h at 4°C. The immunoprecipitates were washed three times with HNTG, resolved by SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in TBST buffer containing 1% milk, blotted for 2 h with a primary antibody (1 µg/ml), followed by a secondary antibody (0.5 µg/ml) linked to horseradish peroxidase. Immunoreactive protein bands were detected with the enhanced chemiluminescence reagent (Pharmacia-Amersham Corporation).

Ligand radiolabeling and covalent cross-linking

EGF was labeled by using IODOGEN as described (Karunagaran *et al.*, 1996). The specific activity was $\sim 5 \times 10^5$ c.p.m./ng. For covalent cross-linking analysis, cells (1×10^6) were incubated on ice for 1.5 h with 125 I-radiolabeled EGF at 100 ng/ml. The chemical cross-linking reagent BS³ was then added (1 mM), and after 90 min on ice, cells were pelleted and extracted in solubilization buffer.

Receptor downregulation assay

To quantify receptor downregulation, 1×10^6 cells were washed with binding buffer and incubated with 100 ng/ml EGF at 37°C for various time intervals. Subsequently, cells were precipitated by centrifugation (9000 g, 1 min), resuspended, and incubated with ice-cold ligand stripping buffer (100 mM acetic acid, 150 mM NaCl, pH 2.7) for 2 min on ice. Cells were then pelleted and their medium neutralized by two sequential washes with binding buffer. To determine the number of receptor molecules on the cell surface, cells were incubated for 2 h at 4°C with a radioactive EGF, precipitated as above, rinsed once in binding buffer and spun through a serum cushion to remove the unbound ligand, prior to determination of radioactivity.

In vitro kinase assay

To phosphorylate the EGF receptor *in vitro*, cell lysates were subjected to immunoprecipitation with the indicated antibodies and the washed immunoprecipitates incubated for 15 min on ice in phosphorylation solution containing 5 mM MnCl₂ and 0.01 µCi [γ - 32 P]ATP in HNTG. The immobilized immunocomplexes were washed three times with HNTG, eluted with gel sample buffer and resolved by SDS-PAGE.

SRE transcription assay

CHO cells were transiently transfected by using the Lipofectamine reagent (Gibco-BRL). Cells were seeded 24 h prior to transfection at 2×10^5 cells/60 mm dish and then transfected in triplicates with ErbB- and Cbl-expression vectors as specified. All transfection experiments were carried out with a reporter pSRE-Fluc plasmid (4 µg), containing one copy of the serum response element (SRE) cloned upstream to the Fos minimal promoter (nucleotides -53/+45) and the luciferase gene (Johansen and Prywes, 1994). Cells were left in transfection medium for 14–16 h and then washed and refed with growth medium. Ten hours later, cells were treated with EGF (20 ng/ml) in growth medium, or left untreated for 12 h, and then harvested. Whole-cell extracts for luciferase assays were prepared by resuspending the cells in 30 µl $1 \times$ lysis buffer (Promega). Following a 10 min incubation at room temperature, the cellular debris were removed and a 10 µl aliquot of the resulting supernatant was mixed with 100 µl luciferin buffer (0.1 M Tris-acetic acid, 10 mM Mg-acetate, 1 mM EDTA, pH 8.0, 74 mM luciferin and 2.2 µM ATP). Light intensity was measured by using a luminometer,

and the results were normalized to protein concentrations, which were determined by using a kit from Bio-Rad Laboratories (Hercules, CA).

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