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Epidermal growth factor and betacellulin mediate signal transduction through co-expressed ErbB2 and ErbB3 receptors

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Interleukin-3 (IL-3)-dependent murine 32D cells do not detectably express epidermal growth factor receptors (EGFRs) and do not proliferate in response to EGF, heregulin (HRG) or other known EGF-like ligands. Here, we report that EGF specifically binds to and can be crosslinked to 32D transfectants co-expressing ErbB2 and ErbB3 (32D.E2/E3), but not to transfectants expressing either ErbB2 or ErbB3 individually. [^{125}I]EGF-crosslinked species detected in 32D.E2/E3 cells were displaced by HRG and betacellulin (BTC) but not by other EGF-like ligands that were analyzed. EGF, BTC and HRG also induced receptor tyrosine phosphorylation, activation of downstream signaling molecules and proliferation of 32D.E2/E3 cells. 32D transfectants were also generated which expressed an ErbB3–EGFR chimera alone (32D.E3-E1) or in combination with ErbB2 (32D.E2/E3-E1). While HRG stimulation of 32D.E3-E1 cells resulted in DNA synthesis and receptor phosphorylation, EGF and BTC were inactive. However, EGF and BTC were as effective as HRG in mediating signaling when ErbB2 was co-expressed with the chimera in the 32D.E2/E3-E1 transfectant. These results provide evidence that ErbB2/ErbB3 binding sites for EGF and BTC are formed by a previously undescribed mechanism that requires co-expression of two distinct receptors. Additional data utilizing MDA MB134 human breast carcinoma cells, which naturally express ErbB2 and ErbB3 in the absence of EGFRs, supported the results obtained employing 32D cells and suggest that EGF and BTC may contribute to the progression of carcinomas that co-express ErbB2 and ErbB3.

Keywords: betacellulin/epidermal growth factor/ErbB2 receptor/ErbB3 receptor/signal transduction

Introduction

The ErbB family of receptor tyrosine kinases includes the epidermal growth factor receptor (EGFR), ErbB2, ErbB3 and ErbB4. Members of the ErbB receptor family have been implicated in the development of a variety of human carcinomas (reviewed in Gullick, 1991; Hynes and Stern, 1994; Salomon *et al.*, 1995; Pinkas-Kramarski *et al.*, 1997). These receptors are thought to contribute to tumorigenesis as a result of their overexpression due to gene amplification or enhanced transcription. The EGFR has been found to be amplified and/or overexpressed in human glioblastomas, epidermoid carcinomas, breast carcinomas and other tumor types (reviewed in Gullick, 1991; Saloman *et al.*, 1995; Pinkas-Kramarski *et al.*, 1997) and overexpression of ErbB2 has been documented in mammary, ovarian, endometrial and non-small cell lung carcinomas (reviewed in Hynes and Stern, 1994; Saloman *et al.*, 1995; Pinkas-Kramarski *et al.*, 1997). Although ErbB3 overexpression in human tumors has not been as frequently observed, it has been found to be overexpressed and/or constitutively activated in a subset of mammary tumor cell lines (Alimandi *et al.*, 1995). While analysis of ErbB4 is at a very early stage, overexpression of this receptor has not yet been reported in human tumors (Plowman *et al.*, 1993a,b). However, simultaneous overexpression of at least two of the other three ErbB receptors has often been observed in human carcinomas (reviewed in Salomon *et al.*, 1995; Pinkas-Kramarshi *et al.*, 1997).

Regulation of ErbB receptor activation is very complex. A large number of ErbB ligands have been described (reviewed in Peles and Yarden, 1993; Groenen *et al.*, 1994; Salomon *et al.*, 1995; Pinkas-Kramarshi *et al.*, 1997). They can be divided into two classes according to their binding specificities. The first group of ligands binds to EGFRs and includes epidermal growth factor (EGF), transforming growth factor-α (TGF-α), amphiregulin (AR), heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC) and epiregulin (Savage *et al.*, 1972; Marquart *et al.*, 1984; Shoyab *et al.*, 1989; Higashiyama *et al.*, 1991; Shing *et al.*, 1993; Toyoda *et al.*, 1995). Unlike the rest of the members of this group, BTC and HB-EGF have been demonstrated to bind to one other ErbB family member, ErbB4 (Riese *et al.*, 1996a; Elenius *et al.*, 1997). The second group is the heregulins (HRGs) which are known to bind to ErbB3 and ErbB4 (Plowman *et al.*, 1993a,b; Carraway *et al.*, 1994; Kita *et al.*, 1994; Sliwkowski *et al.*, 1994; Tazhar *et al.*, 1994). These polypeptides are also referred to as neuregulin, Neu differentiation factor, acetylcholine receptor-inducing activity and glial growth factor (Holmes *et al.*, 1992; Peles *et al.*, 1992; Wen *et al.*, 1992; Falls *et al.*, 1993; Marchionni *et al.*, 1993). HRGs are glycoproteins containing both immunoglobulin- and EGF-like domains. There are at least 12 different isoforms of HRG that arise from a single gene by alternative splicing, and they are classified either as α or β isoforms depending on minor differences within the coding sequences of the EGF-like domain (Holmes

et al., 1992; Peles *et al.*, 1992; Wen *et al.*, 1992; Falls *et al.*, 1993; Marchioni *et al.*, 1993). Despite the presence of a 65 amino acid region of homology within HRG and EGF (Wen *et al.*, 1992), these ligands are thought to bind to their respective receptors with strict specificity.

The recognition that ligand binding induces the formation of heterodimeric as well as homodimeric ErbB receptors has introduced an additional level of complexity to ErbB receptor-mediated signaling (reviewed in Carraway and Cantley, 1994; Hynes and Stern, 1994; Earp *et al.*, 1995; Pinkas-Kramarshi *et al.*, 1997). Recent work has suggested that ErbB2 is the principal receptor that co-operates with other ErbB receptor family members in the formation of heterodimers (Peles *et al.*, 1993; Karunagaran *et al.*, 1996; Pinkas-Kramarski *et al.*, 1996a; Graus-Porta *et al.*, 1997). HRG has been shown to bind to ErbB3 with low affinity (Carraway *et al.*, 1994; Sliwkowski *et al.*, 1994; Tazhar *et al.*, 1994). However, it is thought to bind with high affinity when ErbB2 and ErbB3 are co-expressed (Sliwkowski *et al.*, 1994). Although the significance of these events in tumor formation has not been established, the ability of ErbB2 and ErbB3 to co-operate in cellular transformation of NIH 3T3 cells has been demonstrated (Alimandi *et al.*, 1995; Wallasch *et al.*, 1995).

Natural co-expression of different ErbB receptors is frequently observed and has complicated attempts to characterize specific ligand–receptor interactions. The development of naive cell systems in which specific ErbB receptors have been exogeneously expressed has helped to elucidate which EGF or HRG ligands signal through which ErbB receptor family members (Pierce *et al.*, 1988; Riese *et al.*, 1995, 1996a,b; Pinkas-Kramarski *et al.*, 1996a,b). The IL-3-dependent murine 32D cell line is thought to be devoid of all endogenous ErbB receptors (Pinkas-Kramarski *et al.*, 1996a,b). We previously demonstrated that EGF was capable of mediating proliferation in 32D transfectants expressing exogenous human EGFRs (Pierce *et al.*, 1988), and recent studies revealed that HRG can elicit mitogenesis in 32D transfectants co-expressing ErbB2 and ErbB3 (Pinkas-Kramarski *et al.*, 1996a,b). Thus, these cells appear to possess the intracellular components required for mediating signal transduction through different ErbB receptors. In the present study, we provide evidence that EGF and BTC bind to cells co-expressing ErbB2 and ErbB3 by a novel mechanism and demonstrate that these two ligands stimulate cell proliferation in the absence of EGFR expression.

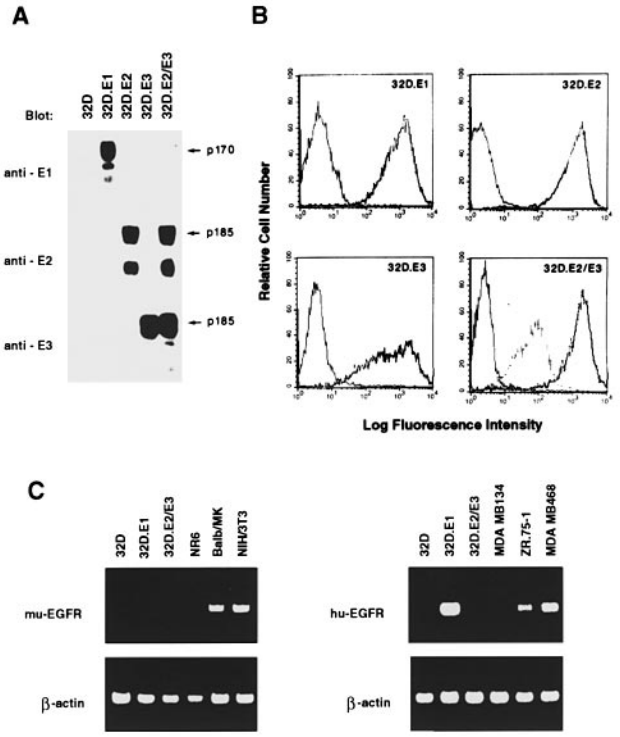

Fig. 1. Analysis of exogenous and endogenous ErbB receptor expression in 32D and MDA MB134 cells. (**A**) Immunoblot analysis to determine the pattern of ErbB receptor expression in 32D transfectants. Anti-EGFR (anti-E1), anti-ErbB2 (anti-E2) or anti-ErbB3 (anti-E3) serum was utilized for immunoblot (Blot) analysis of proteins from lysates of 32D cells or the various transfectants as designated. The 170 kDa mature form of the EGFR and the 185 kDa mature forms of ErbB2 and ErbB3 are marked by arrows. (**B**) Flow cytometric analysis to determine the relative levels of expression of ErbB family receptors on the cell surface of the 32D transfectants. The histograms for untransfected 32D cells or 32D transfectants are represented by thin solid or thick solid lines respectively, in each panel. The histograms for 32D.E2/E3 cells incubated with anti-ErbB2 or anti-ErbB3 are represented by thick solid or dotted lines respectively, in the lower-right panel. (**C**) Analysis of endogenous murine or human EGFR RNA transcript expression in 32D and MDA MB134 cells. The amplified gene products generated by RT–PCR analysis of total RNA from designated cell lines utilizing oligonucleotide primers specific for murine (mu) EGFR, human (hu) EGFR or β-actin are shown.

Results

Analysis of endogenous and exogenous ErbB receptor expression in 32D and MDA MB134 cells

To determine whether EGF or EGF-like ligands possess the ability to bind and signal through co-expressed ErbB2 and ErbB3 receptors, murine IL-3-dependent 32D myeloid progenitor cells were electroporated with expression vectors containing human *EGFR*, *erbB2* or *erbB3* cDNAs individually, or *erbB2* and *erbB3* together (Pierce *et al.*, 1988; Di Fiore *et al.*, 1990), and transfectants were designated 32D.El, 32D.E2, 32D.E3 and 32D.E2/E3 respectively. Immunoblot analysis revealed that lysates from each transfectant expressed readily detectable levels of the transfected ErbB receptor(s), while no ErbB receptor-related proteins were observed in lysates from the untransfected line (Figure 1A). Flow cytometric analysis verified that each transfectant expressed the appropriate receptors on the cell surface (Figure 1B). Similarities in the mean fluorescence intensities also demonstrated that the levels of ErbB2 and ErbB3 expressed on 32D.E2 and 32.E3 cells were comparable with those observed on 32D.E2/E3 cotransfectants (Figure 1B).

Reverse transcriptase–polymerase chain reaction (RT–PCR) analysis was performed utilizing oligonucleotide primers specific for the murine EGFR to verify that EGFR transcripts were not endogenously expressed in these cells. While amplified cDNA products of 760 bp were readily detected when total RNA preparations isolated from murine EGF-dependent Balb/MK epithelial or NIH 3T3 fibroblast cells were analyzed, none were observed when RNA samples from 32D, 32D.E1, 32D.E2/E3 or NR6 fibroblasts were examined (Figure 1C). NR6 fibroblasts are known to be devoid of murine EGFRs (Kokai *et al.*,

1989). We were also interested in analyzing the effects of EGF and EGF-like ligands on MDA MB134 human mammary carcinoma cells, since these cells had been previously reported to express ErbB2 and ErbB3 in the absence of EGFR expression (Alimandi *et al.*, 1995). Therefore, RT–PCR was also performed utilizing oligonucleotide primers specific for the human EGFR. The expected 391 bp gene products were observed when RNA preparations isolated from MDA MB468 and ZR.75-1 human mammary carcinoma cells or 32D.E1 transfectants were analyzed, but not when MDA MB134, 32D or 32D.E2/E3 RNA preparations were examined (Figure 1C). Since BTC and HB-EGF have been demonstrated to bind to ErbB4, we analyzed MB134 and 32D cells for ErbB4 expression by RT–PCR and immunoblot analysis (data not shown). While we did detect a faint ErbB4-specific cDNA product amplified from MB134 RNA, no ErbB4 protein was detected in MB134 cell lysates. In contrast, endogenous ErbB4 protein was observed in lysates from human T47D and OVCAR carcinoma cells. RT–PCR analysis and immunoblot analysis of 32D cells revealed that neither ErbB4 RNA nor protein is expressed in these cells. Murine brain extracts were positive in both analyses. The above results demonstrate that the 32D and MDA MB134 cell lines are appropriate choices to study ErbB ligand interactions with ErbB2 and ErbB3, since they do not express EGFR or ErbB4 cell surface receptors.

EGF binds to cells co-expressing ErbB2 and ErbB3 but not to cells expressing ErbB2 or ErbB3 individually

The capacity of the various 32D transfectants to bind [^{125}I]EGF under saturating conditions was investigated. Both 32D.E1 and 32D.E2/E3 cells were able to bind EGF specifically, while 32D, 32D.E2 and 32D.E3 cells displayed no EGF binding (Figure 2A). These data revealed that ~4.5×10^4 and 1.8×10^5 EGF binding sites per cell were expressed on the surface of 32D.E2/E3 and 32D.E1 transfectants respectively. We next assessed whether [^{125}I]EGF could specifically interact with cell surface receptors on 32D.E2/E3 and MDA MB134 cells by covalent affinity crosslinking analysis. [^{125}I]EGF-crosslinked species were identified with approximate molecular sizes of 190 and 350–500 kDa in lysates from 32D.E2/E3 cells or MDA MB134 cells (Figure 2B). The crosslinked species detected in both lines were effectively displaced by addition of unlabeled HRG, EGF or BTC, but not by transforming growth factor-α (TGF-α), amphiregulin (AR) or heparin-binding-EGF (HB-EGF) (Figure 2B).

The binding affinity of [^{125}I]EGF for 32D.E2/E3 cells was estimated to be 20 nM, as determined by displacement with unlabeled ligand (Figure 2C). HRG was more efficient than EGF at displacing [^{125}I]EGF (Figure 2C). Scatchard analysis of [^{125}I]EGF binding to 32D.E2/E3 cells revealed a single affinity constant (K_d) of ~13 nM (data not shown). To further ascertain whether EGF could specifically interact with ErbB2 or ErbB3 when these receptors were co-expressed or expressed individually in 32D transfectants, lysates from cells crosslinked with [^{125}I]EGF were immunoprecipitated with anti-ErbB2 or anti-ErbB3 serum, and immunoprecipitated proteins were resolved by SDS–PAGE. As shown in Figure 2D, no [^{125}I]EGF-crosslinked proteins were observed in anti-ErbB2 or anti-ErbB3 immunoprecipitates from 32D.E2 or 32D.E3 cells. In contrast, similar levels of crosslinked species were readily detected in anti-ErbB2 or anti-ErbB3 immunoprecipitates from 32D.E2/E3 cells. Addition of a 100-fold excess of unlabeled EGF prior to crosslinking abolished the detection of the crosslinked species (Figure 2D).

EGF, BTC and HRG, but not other EGF-like ligands, induce DNA synthesis and long-term growth of 32D.E2/E3 cells

We next investigated whether the different ligands would mediate DNA synthesis in the various 32D transfectants (Figure 3A). EGF, TGF-α, AR, BTC and HB-EGF stimulated readily detectable mitogenic responses in 32D.E1 cells. While AR was less effective than the other ligands, HRG displaced no activity on 32D.E1 cells (Figure 3A). None of these factors had detectable effects on DNA synthesis in 32D.E2 or 32D.E3 cells. In contrast, EGF, BTC and HRG induced striking DNA synthesis in 32D.E2/E3 cells, while TGF-α, AR and HB-EGF were inactive. Dose–response analysis revealed that EGF, BTC and HRG induced half-maximal DNA synthesis in 32D.E2/E3 cells at ~10 nM, 3 nM and 4 nM respectively (Figure 3B). Maximal [^{3}H]thymidine ([^{3}H]TdR) incorporation achieved in response to EGF or BTC stimulation of 32D.E2/E3 cells was similar to that attained with HRG. Although we attempted to determine whether treatment of MDA MB134 cells with the various ligands would induce DNA synthesis, the basal level of [^{3}H]TdR incorporated in these cells was observed to be extremely high, and we were unable to observe increased DNA synthesis in response to ligand stimulation (data not shown).

We then assessed the ability of 32D.E2/E3 cells to proliferate continuously in growth medium containing EGF or HRG (Figure 3C). Initially, growth of 32D.E2/E3 cells in EGF-containing medium was slower than that achieved in IL-3. After an adaptation period of ~2 weeks, these cells could be cultured continuously in EGF-containing medium, remained EGF dependent and possessed a similar doubling time compared with the same transfectant propagated in IL-3. Similar results were obtained when HRG was utilized for propagation of 32D.E2/E3 cells (Figure 3C). RT–PCR analysis demonstrated that no amplified gene products corresponding to murine or human EGFR RNA transcripts were detected when RNA preparations isolated from 32D.E2/E3 cells propagated in EGF or HRG were utilized as templates (Figure 3D).

EGF or HRG stimulation of 32D.E2/E3 or MDA MB134 cells activates phosphatidylinositol 3′-kinase and mitogen-activated protein kinase

It has been reported that tyrosine phosphorylation of ErbB3 results in its association with the 85 kDa subunit (p85) of phosphatidylinositol 3′-kinase (PI 3′-K) and that HRG activates mitogen-activated protein kinase (MAPK) in human breast carcinoma lines (Fedi *et al.*, 1994; Soltoff *et al.*, 1994; Carraway *et al.*, 1995; Marte *et al.*, 1995). As shown in Figure 4A, co-precipitation of p85 with tyrosine-phosphorylated proteins in the size range of ErbB2 and ErbB3 was greatly enhanced in response to EGF or HRG treatment of 32D.E2/E3 and MDA MB134 cells. We have determined that the phosphotyrosine-

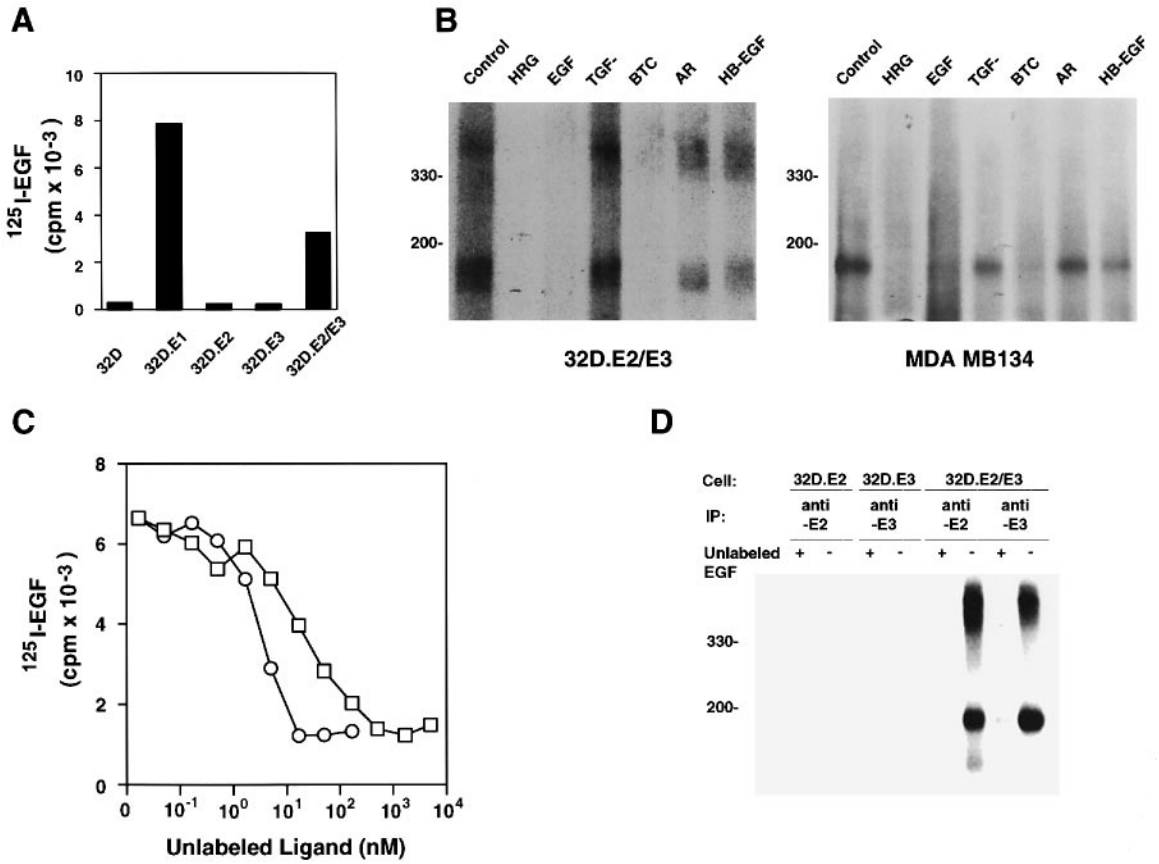

Fig. 2. Characterization of EGF binding to 32D.E2/E3 and MDA MB134 cells. (**A**) Saturation binding of [^{125}I]EGF to 32D and various 32D transfectants. Results are expressed as specific c.p.m. bound and are the mean of duplicate samples. (**B**) Covalent affinity crosslinking of [^{125}I]EGF to cell surface receptors on 32D.E2/E3 and MDA MB134 cells. Cells were exposed to [^{125}I]EGF in the absence of unlabeled ligand (Control) or in the presence of unlabeled HRG, EGF, TGF-α, BTC, AR or HB-EGF prior to crosslinking as designated. (**C**) Displacement of [^{125}I]EGF binding to 32D.E2/E3 cells by various concentrations of unlabeled EGF (□) or HRG (○). (**D**) Analysis of covalent affinity crosslinking of [^{125}I]EGF to ErbB2 and ErbB3 when expressed individually or co-expressed in 32D cells. [^{125}I]EGF was crosslinked to 32D.E2, 32D.E3 or 32D.E2/E3 transfectants in the absence (–) or presence (+) of a 100-fold excess of unlabeled EGF. Cell lysates from the designated transfectants were immunoprecipitated (IP) with anti-ErbB2 (anti-E2) or anti-ErbB3 (anti-E3) as designated under Materials and methods. Molecular mass markers are shown in kDa.

containing protein which co-immunoprecipitates with p85 in response to EGF or HRG is ErbB3 by immunoprecipitating cell lysates with anti-p85 and immunoblotting with either ErbB2 or ErbB3 antiserum (data not shown). An *in vitro* kinase assay also revealed that the amounts of PI 3′-K present in anti-phosphotyrosine (anti-pTyr) immunoprecipitates were increased in response to EGF or HRG stimulation of the co-transfectant and MDA MB134 line (Figure 4A).

To determine whether EGF treatment affected MAPK activity in 32D.E2/E3 cells, the ability of EGF or HRG to induce tyrosine phosphorylation of p42^{ERK1} and p44^{ERK2} was investigated. *De novo* tyrosine phosphorylation of p44^{ERK2} and enhanced phosphorylation of p42^{ERK1} was observed after treatment with factors (Figure 4B). To ascertain whether there were differences in the ability of EGF or HRG to sustain MAPK activation, an *in vitro* kinase assay was performed after EGF or HRG treatment of 32D.E2/E3 cells for time periods ranging from 5 to 60 min. Increases in the levels of myelin basic protein (MBP) phosphorylation peaked at 5 min and were equally reduced by 60 min in response to treatment with either factor (Figure 4B).

EGF and HRG induce different patterns of ErbB receptor tyrosine phosphorylation in 32D.E2/E3 and MDA MB134 cells

The ability of the various ligands to mediate tyrosine phosphorylation of proteins in the ErbB receptor size range was evaluated in 32D.E1 and 32D.E2/E3 cells (Figure 5A). TGF-α, EGF, BTC, HB-EGF or AR treatment of 32D.E1 cells induced pronounced increases in the phosphotyrosine content of proteins in the molecular size range of the EGFR. As expected, HRG did not induce tyrosine phosphorylation in these cells. EGF, HRG and BTC stimulation of 32D.E2/E3 cells resulted in readily detectable increases in the phosphotyrosine content of protein species in the size range of ErbB2 and ErbB3, while TGF-α, HB-EGF and AR did not affect protein tyrosine phosphorylation in this transfectant (Figure 5A).

The pattern of tyrosine phosphorylation of specific ErbB receptors in response to EGF or HRG stimulation of 32D.E1, 32D.E2/E3 and MDA MB134 cells was analyzed utilizing different anti-ErbB receptor sera for immunoprecipitation followed by immunoblot analysis with anti-pTyr (Figure 5B). EGF, but not HRG, treatment of 32D.E1 cells induced a pronounced increase in the phosphotyrosine

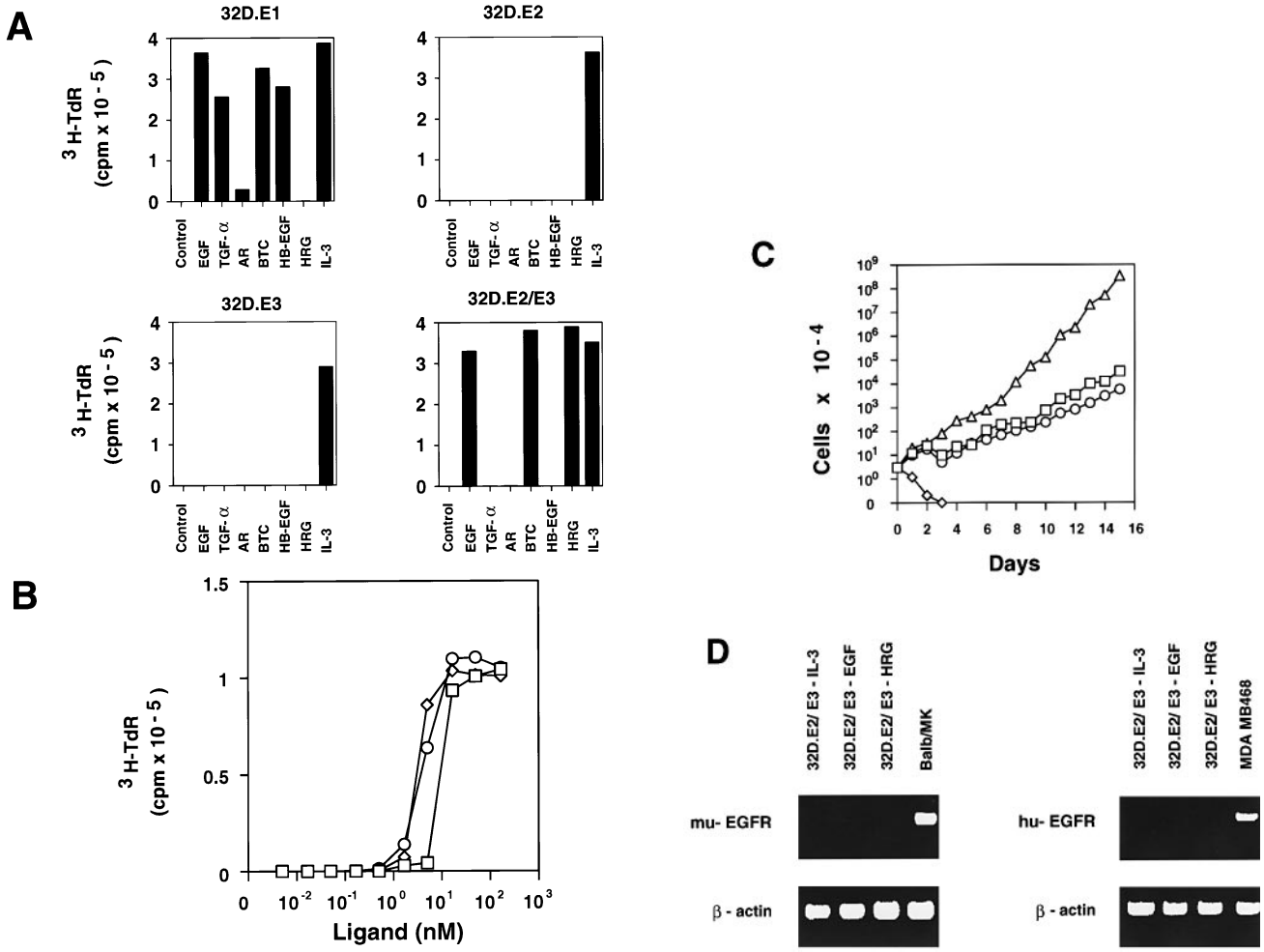

Fig. 3. EGF, HRG and BTC induce DNA synthesis and long-term growth of 32D.E2/E3 cells. (**A**) Analysis of the ability of HRG, EGF and EGF-like ligands to induce DNA synthesis in the various 32D transfectants. 32D.E1, 32D.E2, 32D.E3 or 32D.E2/E3 cells were untreated (Control) or treated with EGF, TGF-α, AR, BTC, HB-EGF, HRG or IL-3. Data points are the mean of triplicate samples and are expressed as amount of [^{3}H]TdR incorporation in c.p.m. (**B**) Dose–response analysis of [^{3}H]TdR incorporation induced by EGF, BTC or HRG in 32D.E2/E3 cells. The 32D.E2/E3 cells treated with various concentrations of EGF (□), BTC (◇) or HRG (○) are shown. (**C**) Comparison of the growth of 32D.E2/E3 cells in medium with no added factors (◇), EGF (□), HRG (○) or IL-3 (△). Results are shown for one representative experiment out of three performed. (**D**) Analysis of EGFR RNA transcript expression in 32D.E2/E3 cells propagated in IL-3, EGF or HRG. RT–PCR analysis utilizing oligonucleotide primers specific for murine (mu) or human (hu) EGFR or β-actin was performed on total RNA preparations from 32D.E2/E3 cells grown in IL-3 (32D.E2/E3-IL-3), EGF (32D.E2/E3-EGF) or HRG (32D.E2/E3-HRG), or on murine Balb/MK or human MDA MB468 RNA, as designated.

content of EGFRs. No detectable EGF-mediated phosphorylation of EGFRs was observed in MDA MB134 cells, consistent with our present and previously published findings regarding their lack of EGFR expression (see Figure 1C; Alimandi *et al.*, 1995). HRG induced an increase in the phosphotyrosine content of both receptors in 32D.E2/E3 and MDA MB134 cells. Although EGF stimulation of 32D.E2/E3 cells resulted in a slight decrease in the mobility of ErbB2, enhanced tyrosine phosphorylation of this receptor was not detected, and EGF-mediated phosphorylation of ErbB2 was also much less striking than that induced by HRG in MDA MB134 cells. In contrast, EGF induced a dramatic increase in the phosphotyrosine content of ErbB3 in both lines.

While HRG mediates signaling through a chimeric ErbB3-EGF receptor expressed in 32D, EGF and BTC require the additional expression of ErbB2

To ascertain further whether EGF and BTC bind to ErbB2/ErbB3 complexes by a distinct mechanism from HRG, an expression vector containing a chimeric receptor cDNA comprising the extracellular domain of human *erbB3* and the transmembrane and intracellular domains of the human *EGFR* was generated. 32D cells were transfected with this vector alone or co-transfected with the *erbB2*-containing vector, and transfectants were designated 32D.E3-E1 and 32D.E2/E3-E1 respectively. FACS analysis revealed that the extracellular domain of ErbB3 was expressed at similar levels on both transfectants and that ErbB2 was expressed only on 32D.E2/E3-E1 cells (Figure 6A). The ability of EGF, HRG and BTC to stimulate DNA synthesis in these transfectants revealed that HRG, but not EGF or BTC, was able to induce [^{3}H]TdR incorporation in 32D.E3-E1 cells (Figure 6B). However, EGF- and BTC-induced DNA synthesis was restored in the 32D.E2/E3-E1 line (Figure 6B). The ability of EGF, HRG and BTC to mediate tyrosine phosphorylation of molecules in the ErbB receptor size range was also investigated. Only HRG mediated tyrosine phosphorylation events in 32D.E3-E1 cells, while EGF, HRG and BTC all induced readily detectable

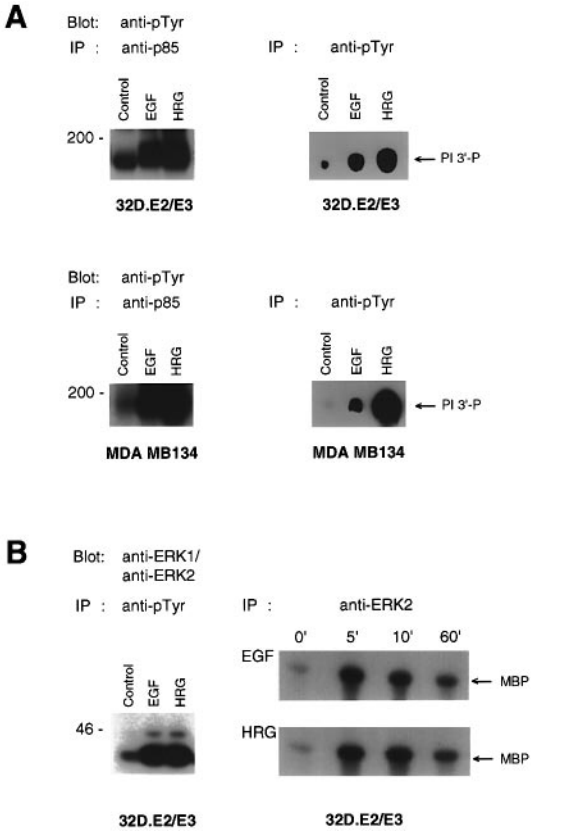

Fig. 4. EGF and HRG stimulation of 32D.E2/E3 or MDA MB134 cells activates PI 3′-K and MAPK. (**A**) Detection of PI 3′-K activity associated with tyrosine-phosphorylated proteins after EGF or HRG stimulation of 32D.E2/E3 or MDA MB134 cells. Lysates from untreated or factor-treated cells were immunoprecipitated (IP) with anti-p85 serum and subsequently immunoblotted (Blot) with anti-pTyr or immunoprecipitated with anti-pTyr and subjected to an *in vitro* PI 3′-K assay. The final PI 3′-P product is marked by an arrow. (**B**) The effects of EGF or HRG stimulation on MAPK tyrosine phosphorylation and activity in 32D.E2/E3 cells. Lysates from untreated or factor-treated cells were immunoprecipitated (IP) with anti-pTyr and immunoblotted (Blot) with a combination of anti-ERK1 and anti-ERK2 sera. The *in vitro* MAPK assay was performed on anti-ERK2 immunoprecipitates from cells treated for 0, 5, 10 or 60 min with EGF or HRG. Phosphorylated MBP is marked by an arrow.

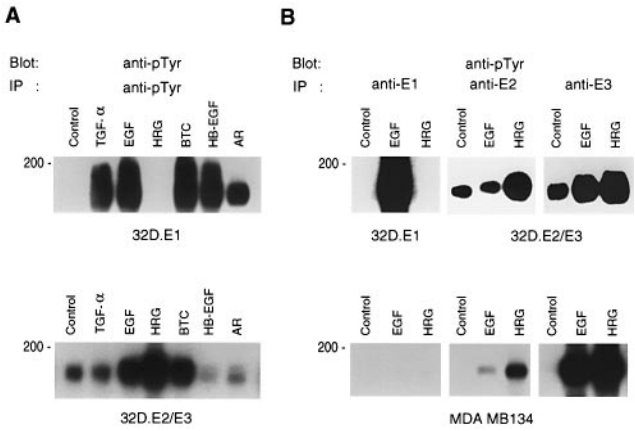

Fig. 5. EGF and HRG induce different patterns of ErbB receptor tyrosine phosphorylation in cells co-expressing ErbB2 and ErbB3. (**A**) Detection of ligand-induced tyrosine phosphorylation of proteins migrating with the mobility of ErbB receptors in 32D.E1 and 32D.E2/E3 cells. Lysates from untreated cells (Control) or cells treated with TGF-α, EGF, HRG, BTC HB-EGF or AR were immunoprecipitated (IP) and subsequently immunoblotted (Blot) with anti-pTyr as designated. (**B**) Detection of ErbB receptor tyrosine phosphorylation in response to EGF or HRG treatment of 32D.E1, 32D.E2/E3 or MDA MB134 cells. Cells were untreated (Control) or stimulated with EGF or HRG, lysates were immunoprecipitated (IP) with anti-EGFR (anti-E1), anti-ErbB2 (anti-E2) or anti-ErbB3 (anti-E3) and subsequently immunoblotted (Blot) with anti-pTyr. Molecular mass markers are given in kDa.

increases in the phosphotyrosine content of proteins migrating within the size range of ErbB receptor proteins in lysates from 32D.E2/E3-E1 cells (Figure 6C).

Discussion

While previous studies have demonstrated that HRG binds to ErbB3 with low to moderate affinity and to ErbB2–ErbB3 heterodimers with high affinity (Carraway *et al.*, 1994; Sliwkowski *et al.*, 1994; Tazhar *et al.*, 1994), our present study provides evidence that EGF can only bind to ErbB2 and ErbB3 when they are expressed simultaneously. Binding and crosslinking analyses revealed that HRG and BTC, but not TGF-α, AR or HB-EGF, could competitively displace [^{125}I]EGF binding to receptors on 32D.E2/E3 or MDA MB134 cells. Moreover, only EGF, BTC and HRG were able to induce proliferative effects in 32D.E2/D3 cells. In contrast, EGF, BTC, TGF-α, HB-EGF and AR (to a lesser extent), but not HRG, were all active on 32D.E1 cells. The distinct patterns of ligand binding, biological activity and receptor tyrosine phosphorylation displayed by these two receptor systems provide evidence that the interactions of EGF with ErbB2/ErbB3 complexes are qualitatively distinct from those which occur with EGFRs. While our data do not rule out the possibility that a third receptor component present in both 32D and MDA MB134 cells could be involved in mediating EGF and BTC signaling through ErbB2 and ErbB3, it is clear that co-expression of these two receptors is required for EGF and BTC binding and signaling.

The effects of EGF stimulation on ErbB receptor tyrosine phosphorylation were found to differ from those induced by HRG in cells co-expressing ErbB2 and ErbB3. HRG stimulation led to a pronounced increase in the phosphotyrosine content of ErbB2 in cell lines co-expressing both receptors, while EGF treatment did not appreciably enhance tyrosine phosphorylation of this receptor in the 32D co-transfectant and did so only weakly in the MDA MB134 line. In contrast, both EGF and HRG stimulation induced pronounced tyrosine phosphorylation of ErbB3. These results suggest that binding of EGF to ErbB2 and ErbB3 may induce a spatial relationship between the intracellular domains of these two receptors that hinders ErbB2 autophosphorylation without inhibiting its ability to transphosphorylate ErbB3 and to phosphorylate downstream effector molecules. Alternatively, it is possible that EGF binding may result in the preferential activation of a phosphatase that rapidly and specifically dephosphorylates ErbB2.

To evaluate further whether EGF and BTC interact with Erb2–ErbB3 complexes by a different mechanism from HRG, we analyzed 32D cells expressing an ErbB3–EGFR chimera alone or in combination with ErbB2. It is known that ErbB3 is kinase defective (Guy *et al.*, 1994). Therefore, the chimeric receptor was generated, since it con-

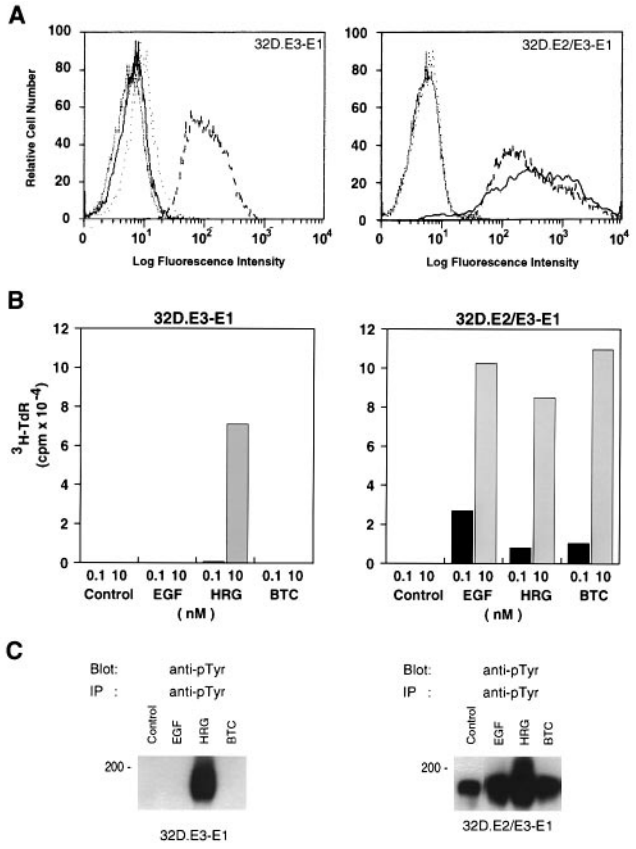

Fig. 6. EGF and BTC, unlike HRG, induce DNA synthesis and ErbB receptor tyrosine phosphorylation in 32D.E1-E3 cells only when ErbB2 is co-expressed. (**A**) Flow cytometric analysis to assess the levels of expression of the ErbB3–EGFR chimeric receptor and ErbB2 on the cell surface of 32D.E3-E1 and 32D.E2/E3-E1 transfectants. The histograms for 32D.E1-E3 or 32D.E2/E1-E3 cells incubated with anti-ErbB2 or anti-ErbB3 are represented by solid or broken lines respectively, in each panel. The histograms for untransfected 32D cells incubated with anti-ErbB2 or anti-ErbB3 are represented by pinpoint or dotted lines respectively, and are shown in both panels. (**B**) Analysis of the ability of EGF, HRG or BTC to induce DNA synthesis in 32D.E3-E1 or 32D.E2/E3-E1 cells. 32D transfectants were untreated (Control) or treated with 0.1 nM (gray bars) or 10 nM (solid bars) of EGF, HRG or BTC as described under Materials and methods. Results are the mean of triplicate samples and are expressed as the amount of [^{3}H]TdR incorporation in c.p.m. (**C**) Analysis of the ability of EGF, HRG or BTC to induce tyrosine phosphorylation of proteins migrating in the ErbB receptor size range in 32D.E3-E1 or 32D.E2/E3-E1 cells. Lysates from untreated (Control) or factor-treated transfectants were immunoprecipitated (IP) and immunoblotted (Blot) with anti-pTyr. Molecular mass markers are shown in kDa.

tained the active tyrosine kinase domain of the EGFR and could potentially mediate signaling upon ligand binding. Indeed, HRG was able to induce DNA synthesis and receptor phosphorylation in 32D.E3-E1 cells, while EGF and BTC had no biological or biochemical effects on this transfectant. However, co-expression of ErbB2 with the ErbB3–EGFR chimera in 32D cells restored the ability of EGF and BTC to induce signal transduction. The above results provide evidence that the binding affinity of HRG for ErbB3 is sufficient to mediate signaling with the provision that the intracellular region of the receptor possesses an unimpaired tyrosine kinase domain. Moreover, these data substantiate that EGF and BTC binding, unlike HRG, require the simultaneous expression of both ErbB2 and ErbB3 extracellular domains.

Although ErbB2 and ErbB3 were co-expressed in 32D cells in two recent studies, the ability of EGF and BTC to induce proliferation or receptor tyrosine phosphorylation in these co-transfectants was not analyzed (Pinkas-Kramarski *et al.*, 1996a,b). ErbB2 and ErbB3 were also co-expressed in IL-3-dependent Ba/F3 cells in separate studies. BTC was reported to lack mitogenic potential in these cells, while EGF was not analyzed (Riese *et al.*, 1996a,b). EGF and BTC were also reported to be unable to induce tyrosine phosphorylation of ErbB2 or ErbB3 in the Ba/F3 co-transfectants (Riese *et al.*, 1995, 1996b). Although we did not detect tyrosine phosphorylation of ErbB2 in 32D.E2/E3 cells, we did observe significant tyrosine phosphorylation of ErbB3 and, more importantly, cellular proliferation in response to EGF or BTC treatment of this co-transfectant. Since the levels of ErbB2 and ErbB3 receptors expressed in the Ba/F3 cells were not reported (Riese *et al.*, 1995, 1996a,b), it is possible that the lack of responsiveness of these transfectants may have resulted from low-level ErbB receptor expression. Alternatively, a third component present in 32D and MDA MB134 cells may be lacking in Ba/F3 cells.

Several models of growth factor–receptor interaction and subsequent receptor activation have been described for receptors containing a single transmembrane domain (reviewed in Ullrich and Schlessinger, 1990; Heldin, 1995). One model postulates that a monomeric ligand binds to one receptor subunit and that formation of dimeric receptor complexes occurs without additional ligand binding. This model has been proposed for the interaction of HRG with ErbB3 and its subsequent dimerization with ErbB2 (reviewed by Carraway and Cantley, 1994). In an alternative model, a monomeric ligand is thought be bivalent and to bind different sites on two identical receptor subunits, leading to homodimerization. Evidence supporting this model has been provided for the interaction of growth hormone or erythropoietin with their respective receptors (Cunningham *et al.*, 1991; Philo *et al.*, 1996). In contrast, results from a recent study utilizing titration calorimetry and small-angle X-ray scattering analyses provide evidence that one EGF molecule binds to one EGF receptor and that two monomeric ligand–receptor complexes subsequently dimerize (Lemmon *et al.*, 1997). Dimeric ligands, such as the platelet-derived growth factor, are thought to form a bridge between two receptors and consequently induce dimerization (reviewed in Heldin, 1995). We propose that EGF and BTC bind to ErbB2 and ErbB3 by a novel mechanism requiring the simultaneous expression of two distinct receptors and that EGF and BTC binding sites are created by the non-covalent association of these two receptors in the absence of ligand, sites that do not exist when either receptor is expressed alone. Thus, our results suggest that EGF or BTC binding evokes additional allosteric effects beyond those which occur through dimerization that result in full activation of the ErbB2 kinase domain.

It has previously been reported that 32D.EGFR cells exhibit high- and low-affinity constants of ~0.1 nM and 3 nM respectively, for EGF binding (Fazioli *et al.*, 1993). 32D.E2/E3 cells were demonstrated to possess a single affinity constant of ~15 nM for EGF binding. Thus, we would envisage that high-affinity EGFR sites would preferentially bind EGF if EGFR, ErbB2 and ErbB3 were

all present on the same cell. However, ErbB2–ErbB3 complexes would probably compete with low-affinity EGFR binding sites for EGF binding. Therefore, the relative levels of EGFR compared with ErbB2 and ErbB3 and the amounts of EGF available would likely influence EGF binding to these respective receptors. Recent evidence has suggested that ErbB2 is the preferred heterodimerization partner for all ErbB receptors (Karunagaran *et al.*, 1996; Graus-Porta *et al.*, 1997). In agreement with our present findings, Graus-Porta *et al.* (1997) detected an EGF-induced direct ErbB2–ErbB3 interaction in SKBR3 cells. They also suggest that ErbB2 may first dimerize with the EGFR in response to EGF and then, in an activated state, be released and dimerize with and activate ErbB3. Our results do not preclude this latter possibility and, indeed, if EGFRs were expressed in conjuction with ErbB2 and ErbB3, we would expect both direct EGF interaction with ErbB2 and ErbB3 and interplay between the three receptors.

Several human carcinoma lines have been shown to synthesize stimulatory growth factors (reviewed in Goustin *et al.*, 1986). For example, various human breast tissues and cell lines have been demonstrated to express EGF, TGF-α, AR or HRG (Perroteau *et al.*, 1986; Derynck *et al.*, 1987; Bates *et al.*, 1988; Travers *et al.*, 1988; Ciardiello *et al.*, 1989). Since betacellulin was only recently isolated, analysis of its expression pattern in carcinoma cells has not been reported. In model systems, expression of TGF-α or EGF as the result of transfection has been demonstrated to mediate transformation of human mammary epithelial cells or NIH 3T3 cells that overexpressed human EGFRs (Stern *et al.*, 1987; DiMarco *et al.*, 1989; Ciardiello *et al.*, 1990). These data suggest that EGF and related peptides might perform a role in the autocrine growth regulation of breast carcinoma cells. Alternatively, paracrine or juxtacrine roles for these ligands could also be involved in mediating the progression of carcinomas overexpressing ErbB receptor family members. Therefore, the recognition of EGF and BTC as ligands for ErbB2–ErbB3 complexes implicates these growth factors in the development of human carcinomas that express both receptors.

Materials and methods

Growth factors, antibodies and cell lines

Recombinant HRGβ3 was generated by bacterial expression and purified essentially as described (Sliwkowski *et al.*, 1994) with the exception that the HRGβ3 preparation utilized in this study encompassed amino acid residues 178–241 of HRGβ3 rather than residues 177–244 of HRGβ1. Recombinant human EGF and TGF-α were purchased from Peprotech, Inc. and BTC, HB-EGF and AR were from R&D Systems. Rabbit antisera utilized for detection of human ErbB receptors by immunoblot analysis were generated against synthetic peptides derived from the human EGFR [amino acids (aa) 1172–1186), ErbB2 (aa 1218–1232) or ErbB3 (aa 1307–1323; Santa Cruz Biotechnology, Inc.)]. The monoclonal antibodies (mAbs) utilized for flow cytometry include anti-EGFR (Ab-1; Oncogene Science), anti-ErbB2 (Ab-2; Oncogene Science) and anti-ErbB3 (Ab-4; Neomarkers). Antisera utilized for immunoprecipitation include anti-EGFR mAb (Ab-1), anti-ErbB2 mAb (Ab-2) and rabbit anti-ErbB3 peptide serum (aa 1307–1323). The 4G10 anti-pTyr mAb was utilized in immunoprecipitation and immunoblot analysis (Upstate Biotechnology, Inc.). The polyclonal rabbit antiserum utilized for detection of the PI 3′-K p85 subunit was obtained from Upstate Biotechnology, Inc. and for detection of MAPK we utililzed rabbit antisera directed against synthetic peptides encompassing rat aa 352–367 of ERK1 and 345–358 of ERK2 (Santa Cruz Biotechnology, Inc.). The murine IL-3-dependent 32D myeloid progenitor cell line, the EGF-dependent Balb/MK epithelial cell line, NIH 3T3 and NR6 fibroblast lines and the human MDA MB134, MDA MB468 and ZR.75-1 mammary carcinoma cell lines have been described previously(Weissman *et al.*, 1983; Pierce *et al.*, 1988; Alimandi *et al.*, 1995). 32D cells were transfected with LTR-*EGFRgpt*, LTR-*erbB2neo* or LTR-*erbB3gpt* expression vectors by electroporation (Pierce *et al.*, 1988; Di Fiore *et al.*, 1990; Alimandi *et al.*, 1995). The chimeric *erbB3–EGFR* cDNA was engineered by joining the extracellular domain of ErbB3 (aa 1–643; Kraus *et al.*, 1989) with the transmembrane and intracellular portions of the EGFR (aa 647–1210; Ullrich *et al.*, 1984). An *EGFR* cDNA containing a unique *Cla*I site in the extracellular domain close to the transmembrane region of the coding sequence was used (Lonardo *et al.*, 1990), and an identical restriction site was generated in the homologous region of the *erbB3* coding sequence (nucleotide 2025) by site-directed mutagenesis of a unique *Dra*I–*Bam*HI *erbB3* segment (nucleotides 1856–2284; Kraus *et al.*, 1989). After gel purification and ligation, the chimeric cDNA was cloned in the unique *Sal*I cloning site of pZip*neo*. To obtain dual transfectants, the pZip-*erbB3–EGFRneo* vector was co-transfected with LTR-*erbB2gpt* (Di Fiore *et al.*, 1990). Stable transfectants were generated by their ability to grow in medium containing 80 μM mycophenolic acid and the addition of hypoxanthine, aminopterin and thymidine (HAT) to confer resistance to *gpt* and/or 750 μg/ml geneticin to confer resistance to *neo*. Clonal populations were generated by single-cell dilution.

Immunoblot, flow-cytometric analysis and RT–PCR

For detection of ErbB receptors by immunoblot analysis, protein (100 μg) from total cell lysates of 32D and transfectants were separated by SDS–PAGE and transferred to polyvinylidene fluoride membranes. Membranes containing identical samples were subjected to immunoblot analysis utilizing the three anti-human ErbB receptor peptide sera (1:500 dilution) and bound antibody was detected by ^{125}I-labeled protein A as previously described (Di Fiore *et al.*, 1990; Alimandi *et al.*, 1995). For detection of cell surface receptors by flow cytometry, cells from the parental 32D line or the transfectants were incubated with anti-ErbB receptor mAbs (1:500 dilution), and phycoerythrin-conjugated goat anti-mouse serum (1:500 dilution) was used as the secondary antibody as previously described (Wang *et al.*, 1993). Amplification of gene products corresponding to murine or human EGFR transcripts was carried out by RT–PCR. Total RNA preparations were isolated using RNA-STAT60 (Tel-Test, Inc.). Total RNA (5 μg) isolated from each cell line was treated with DNase I before hybridization to the oligo(dT) primer. The first strand cDNA was synthesized by reverse transcriptase (Gibco-BRL) at 37°C and was used to examine RNA transcript expression using specific primers. The sense strand and anti-sense strand primers used to detect murine EGFR transcripts were 5′-GAC CCA CGA GAA CTA GAA ATT-3′ and 5′-AGA TGG CCA CAC TTC ACA TC-3′, human EGFR transcripts were 5′-AGG ACA GCA TAG ACG ACA CCT-3′ and 5′-TCC AAT AAA TTC ACT GCT TTG TG-3′, and β-actin transcripts were 5′-CAG GAA GGA AGG CTG GAA GA-3′ and 5′-TTC TAC AAT GAG CTG CGT GTG-3′. The polymerase chain reaction was cycled 35 times with durations of 40 s at 94°C, annealing for 2 min at 55°C and extension for 2 min at 72°C. The amplified gene products were resolved on a 1.5% agarose gel.

Binding and covalent affinity crosslinking analysis

The binding of [^{125}I]EGF was assayed as previously described (Pierce *et al.*, 1988). Cells (5×10^4 per 100 μl) were washed and incubated for 6 h at 16°C in [^{125}I]EGF (50 nM). Free [^{125}I]EGF was removed by washing three times, cells were lysed in 0.5 M NaOH and bound [^{125}I]EGF was measured by γ counting. The extent of non-specific binding was determined by incubating cells in the presence of a 100-fold excess of unlabeled EGF, and these values were subtracted from total bound counts. Displacement of [^{125}I]EGF binding to 32D.E2/E3 cells was performed similarly, using [^{125}I]EGF (50 nM) and various concentrations of unlabeled EGF or HRG. Crosslinking was performed essentially as described previously (Sliwkowski *et al.*, 1994). Approximately 4×10^6 cells were suspended in RPMI 1640 medium containing 0.1% bovine serum albumin and 10 mM HEPES with [^{125}I]EGF (50 nM) in the absence (–) or presence (+) of a 100-fold excess of unlabeled HRG, EGF, TGF-α, BTC, AR or HB-EGF and incubated overnight at 4°C. The chemical crosslinking agent BS^3 was then added at a final concentration of 2.0 mM, and samples were incubated for an additional 30 min. Lysed samples were either immunoprecipitated with anti-ErbB2 or anti-ErbB3 serum or directly resolved by 5.5% SDS–PAGE, and crosslinked complexes were visualized by autoradiography.

DNA synthesis and proliferation assays

The 32D transfectants were washed twice and re-suspended in RPMI 1640 medium with 15% fetal bovine serum (10^5 cells/ml) and exposed to recombinant EGF, TGF-α, AR, BTC, HB-EGF or HRG (50 nM) or to various concentrations of recombinant EGF, BTC or HRG. DNA synthesis was measured after 36 h, and [^{3}H]TdR (0.5 μCi/ml) was added for the last 4 h of the incubation period. The average number of counts per min (c.p.m.) obtained in untreated samples was subtracted from the average number of c.p.m. obtained in factor-treated samples. For long-term proliferation assay, cells were transferred at a 1:10 ratio every 2 days as previously described (Pierce *et al.*, 1988). Cell number was determined every 2 days after testing for cell viability by trypan blue exclusion.

Immunoprecipitation/immunoblot analysis and PI 3′-K and MAPK activity assays

Cells were serum starved for 4 h and either untreated or treated with various ligands (50 nM) for 10 min at 37°C. For detection of PI 3′-K association with ErbB receptors, lysates were prepared in buffer containing 1% Nonidet P-40 (NP-40), immunoprecipitated with anti-p85 serum and subsequently immunoblotted with anti-pTyr as previously described (Fedi *et al.*, 1994; Alimandi *et al.*, 1995). Alternatively, anti-pTyr immunoprecipitates were subjected to an *in vitro* PI 3′-K assay as previously described (Fedi *et al.*, 1994; Alimandi *et al.*, 1995). For detection of MAPK tyrosine phosphorylation, lysates were prepared in buffer containing 0.1% SDS and 0.5% deoxycholate (Fedi *et al.*, 1994; Alimandi *et al.*, 1995), immunoprecipitated with anti-pTyr and subsequently immunoblotted with a combination of anti-ERK1 and anti-ERK2 sera. Alternatively, lysates from untreated cells or cells treated for 5, 10 or 60 min with EGF or HRG were prepared in buffer containing 1% NP-40 and immunoprecipitated with anti-ERK2 serum. MAPK activity was determined on immunoprecipitates in a reaction buffer containing 1 μCi [γ-^{32}P]ATP, 20 μM ATP and 1.5 mg/ml myelin basic protein (MBP) for 20 min at 30°C essentially as described (Marte *et al.*, 1995). For detection of receptor tyrosine phosphorylation, lysates were prepared in buffer containing 0.1% SDS and 0.5% deoxycholate by immunoprecipitation with anti-EGFR, anti-ErbB2, anti-ErbB3 or anti-pTyr, and subsequently underwent immunoblot analysis with anti-pTyr (Alimandi *et al.*, 1995).

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