The Mitogenic Action of Insulin-like Growth Factor I in Normal Human Mammary Epithelial Cells Requires the Epidermal Growth Factor Receptor Tyrosine Kinase*

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The signals used by insulin-like growth factor I (IGF-I) to stimulate proliferation in human mammary epithelial cells have been investigated. IGF-I caused the activation of both ERKs and Akt. Activation of ERKs was slower and more transient than that of Akt. ZD1839, a specific epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor, prevented activation of ERKs but not Akt by IGF-I. Inhibition of the EGFR with function-blocking monoclonal antibodies also specifically blocked IGF-I-induced ERK activation. These effects occurred in primary mammary epithelial cells and in two cell lines derived from normal mammary epithelium but not in mammary fibroblasts or IGF-I-responsive breast carcinoma cell lines. Although IGF-I stimulated the proliferation of both normal and carcinoma cell lines, ZD1839 blocked this only in the normal line. ZD1839 had no effect on IGF-I receptor (IGF-IR) autophosphorylation in intact cells. IGF-I-induced ERK activation was insensitive to a broad spectrum matrix-metalloproteinase inhibitor and to CRM-197, an inhibitor of the EGFR ligand heparin-bound epidermal growth factor. EGFR was detectable within IGF-IR immunoprecipitates from normal mammary epithelial cells. Treatment of cells with IGF-I led to an increase in the amount of tyrosine-phosphorylated EGFR within these complexes. ZD1839 had no effect on complex formation but completely abolished their associated EGFR tyrosine phosphorylation. These findings indicate that IGF-I utilizes a novel EGFR-dependent signaling pathway involving the formation of a complex between the IGF-IR and the EGFR to activate the ERK pathway and to stimulate proliferation in normal human mammary epithelial cells. This form of regulation may be lost during malignant progression.

The growth, development, and normal functioning of the mammary gland are regulated by a variety of hormones, growth factors, and cytokines. Numerous studies indicate that the IGF-1 system is important not only for normal mammary gland function but also during tumorigenesis (1, 2). The precise role that IGF-I plays remains to be elucidated, but studies with epithelial cells isolated from both normal and malignant breast tissue indicate that IGF-I can promote cell survival, proliferation, and motility (3–7). Increased expression of multiple components of the IGF-I signaling system has also been noted, particularly in estrogen-dependent breast cancer cells (8, 9).

The signaling pathways through which IGF-I promotes mitogenesis and other responses have been extensively studied in several experimental systems (10). IGF-I binds to the IGF-IR, which is a heterotetrameric transmembrane glycoprotein comprising two α subunits and two β subunits. The β subunits express intrinsic tyrosine kinase activity, which is activated upon ligand binding to the α subunits. Tyrosine kinase activation results in autophosphorylation of the β subunits on specific tyrosine residues, which then act as docking sites for a range of signaling molecules, which then transmit downstream signals. In addition, the activated IGF-IR phosphorylates adaptor proteins, such as SHC (Src homology/3-collagen) and IRS-1, which themselves are coupled to major signaling pathways such as the Ras-Raf-mitogen-activated protein kinase/ERK and phosphatidylinositol 3-kinase-Akt pathways. The roles played by SHC- and IRS-dependent pathways in mediating biological responses to IGF-I appear to be dependent upon the cell context (11, 12).

Recent studies have provided evidence for an involvement of heterologous type I tyrosine kinase receptors such as the EGFR and ErbB2 in IGF-I action (13–15). For example, in COS-7 cells transfected to overexpress the IGF-IR, an EGFR tyrosine kinase inhibitor was shown to attenuate IGF-I-induced activation of ERK mitogen-activated protein kinases. The mechanisms involved have not been fully elucidated but are believed to involve matrix metalloproteinase-mediated cleavage of tethered EGFR ligands from the cell surface (14).

Here we have investigated the involvement of the EGFR in the mitogenic actions of IGF-I in epithelial cells isolated from the normal human mammary gland. Although we find that the EGFR is required for IGF-I-signaling in some cellular contexts, this is not a global requirement. Furthermore, we identify a novel mechanism responsible for the activation of ERKs, involving direct interaction between the IGF-IR and the EGFR.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco’s modified Eagle’s medium, RPMI, and FCS were obtained from Invitrogen. EGF was purchased from R&D Systems (Abingdon, UK). Commercial sources of the antibodies used were as follows: TyrP(H9251)-EGFR, ThrP(H9252)-EGFR, TyrP(H9251)-Akt, SerP(H9252)-Akt, and Akt-α (Cell Signaling, Hitchin, UK); EGFR and ERK1 (Santa Cruz, Calif., signal regulated kinase; EGF, epidermal growth factor; EGFR, EGF receptor; FCS, fetal calf serum; HB-EGF, heparin-binding EGF.

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§ The abbreviations used are: IGF-I, insulin-like growth factor I; IGF-IR, IGF-I receptor; IRS-1, insulin receptor substrate-1; ERK, extracellular

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UK. Secondary horseradish peroxidase-conjugated anti-mouse and anti-rabbit antibodies were obtained from Amersham Biosciences. SuperSignal chemiluminescent substrate and protein A-agarose beads were obtained from Perbio (Warrington, UK). IGF-I and all other reagents were purchased from Sigma. ZD1839 was a kind gift from Dr. Alan Wakeling (Astrazeneca Pharmaceuticals, Macclesfield, UK).

Cell Culture—MCF-7, CAL-51, ZR75-1, MDA-MB-231, MDA-MB-468, Hs578T, and SKBr3 breast carcinoma and murine NIH3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium containing FCS (10%) and l-glutamine (2 mM). The immortalized human mammary epithelial cell lines, HB4A (16) (a kind gift from Professor Michael O’Hare, University College London) and MCF-10F, were cultured in RPMI 1640 supplemented with l-glutamine (2 mM), FCS (10%), hydrocortisone (5 μg/ml), insulin (5 μg/ml), and cholesterin (25 ng/ml). Isolation and Culture of Human Mammary Cells—Tissue obtained from patients undergoing reduction mammoplasty surgery was diced into pieces of <1 cm³ before being digested overnight with gentle agitation at 37 °C in an equal volume of L-15 medium (Leibovitz) containing FCS (5%), type I collagenase (Sigma, 0.05%), penicillin/streptomycin (100 units/ml), gentamicin (50 μg/ml), kanamycin (100 μg/ml), and fungizone (2.5 μg/ml). The digested material was centrifuged at 1600 × g for 5 min, washed three times with L-15 medium, resuspended in 30 ml L-15 medium, and allowed to sediment at 4 °C for 2 h. The sedimented organoids were then further digested in medium containing 0.25% collagenase at 37 °C for 1 h to remove stromal connective tissue. Following three further washes, the suspensions were passed through a 120 μm filter followed by a 53-μm nylon filter sheet. Retained epithelial organoids were combined and plated in RPMI 1640 medium supplemented with l-glutamine (2 mM), FCS (10%), penicillin/streptomycin (100 units/ml), hydrocortisone (5 μg/ml), insulin (5 μg/ml), cholesterin (25 ng/ml), EGF (5 ng/ml), and bovine serum albumin (0.07%). The cells that passed through the nylon filters, comprising mainly stromal fibroblasts, were collected by centrifugation, washed as above, and the plated in Dulbecco’s modified Eagle’s medium containing FCS (10%), l-glutamine (2 mM), and penicillin/streptomycin (100 units/ml). The purity of the epithelial and fibroblast populations was checked by immunostaining primary cultures with antibodies to cytokeratin-18 (an epithelial marker), smooth muscle actin (mesenchymal), and vimentin (fibroblast). Only cultures exhibiting at least 85% purity for the respective enriched cell type were used in the experiments reported here.

Cell Treatment and Lysis—In each case the cells were plated in their respective serum-free medium overnight. The serum-free medium was then replaced for 1 h prior to treatment with growth factors and other agents. Following treatment, the cells were rinsed twice in ice-cold phosphate-buffered saline and then scraped into lysis buffer (25 mM HEPES, pH 7.4, 5 mM EDTA, 50 mM sodium chloride, 30 mM tetr-sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 1% sodium pyrophosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1% Triton X-100, 1% sodium orthovanadate) before being resuspended in reduced medium containing no sodium orthovanadate. Immunoprecipitates were assessed by relating absorbances to those obtained from cells treated with appropriate drug vehicle and evaluated statistically using analysis of variance.

RESULTS

To study the mitogenic actions of IGF-I in normal breast epithelial cells, we selected HB4A cells, a conditionally immortalized human breast epithelial line, with phenotypic characteristics very similar to normal breast epithelial cells (16). We first examined the ability of IGF-I to activate two pathways strongly linked with transducing mitogenic signals in mammary and other epithelial cells, the ERK and Akt pathways. Treatment of HB4A cells with IGF-I led to a time-dependent increase in the phosphorylation of ERK2 and, to a lesser extent, ERK1, with a peak of phosphorylation occurring at 5–10 min before returning to basal levels by 30 min (Fig. 1). By contrast IGF-I induced a more rapid (detectable by 30 s) and sustained (remaining high at 60 min) phosphorylation of Akt. The apparently slower kinetics of ERK activation was not due to relatively poorer detection by the phospho-ERK antibodies because phosphorylation of ERKs in response to EGF was detectable within 2 min (Fig. 1).

The relatively slow induction of ERK phosphorylation by IGF-I suggested that activation of this pathway occurred via an indirect mechanism. Previous studies, using COS-7 cells transfected with the IGF-IR, showed that IGF-I signaling can involve transactivation of the EGFR (14). We therefore tested whether the EGFR was involved in IGF-I-induced ERK activation in normal breast epithelial cells. Pretreatment of HB4A cells with ZD1839 (Iressa™), a highly specific inhibitor of the EGFR tyrosine kinase, which blocks EGF-stimulated tyrosine phosphorylation of the EGFR and subsequent downstream signals and cellular responses (17–19), completely blocked the addition of 200 μl of ice-cold EDTA (10 mM). The complexes were pelleted, rinsed, and boiled in 2× concentrated Laemmli buffer before immunoblotting samples with anti-phosphotyrosine antibodies.

Measurement of Proliferation—The cells (5,000) were seeded in serum-free medium overnight prior to treatment with IGF-I (10 nm, upper panels) or EGF (10 nm, lower panels) for the times shown. The cells were then lysed, and the samples containing equivalent amounts of protein were processed for immunoblotting with antibodies to phospho-ERK (P-ERK 1/2), total ERK (ERK 1/2), phospho-Ser473 Akt (P-AKT), or total Akt (AKT) as described fully under “Experimental Procedures.” The blots shown are representative of three independent experiments.
ability of EGF to promote both ERK and Akt phosphorylation (Fig. 2A). Such treatment also prevented ERK phosphorylation in response to IGF-I, whereas IGF-I-induced Akt phosphorylation was unaffected. These results indicate a divergence in IGF-I signaling, with an EGFR-dependent pathway leading to ERK phosphorylation and an EGFR-independent pathway leading to Akt phosphorylation. We next examined the relative potency of ZD1839 in preventing ERK activation by EGF compared with IGF-I. Pretreatment of cells with a range of ZD1839 concentrations caused a dose-dependent decrease in subsequent ERK activation induced by both growth factors (Fig. 2B). ZD1839 appeared more potent in blocking IGF-I-induced ERK phosphorylation, indicating an absolute requirement for the EGFR tyrosine kinase in this pathway and arguing against nonselective effects of the drug at higher concentrations.

Although ZD1839 is a highly specific inhibitor of the EGFR tyrosine kinase, displaying little inhibitory activity when tested against other several other type I family tyrosine kinases (19), we wanted to exclude the possibility that its ability to block IGF-I-induced ERK phosphorylation in normal breast epithelial cells was due to any direct effect on the IGF-IR tyrosine kinase. We tested firstly whether ZD1839 affected IGF-I-induced tyrosine phosphorylation of its receptor in intact cells. As shown in Fig. 3A, pretreatment of HB4A cells with ZD1839 had little or no effect on the level of IGF-IR tyrosine phosphorylation induced by IGF-I. ZD1839 was also without effect on IGF-IR tyrosine phosphorylation in MCF-7 cells. We also tested whether ZD1839 had any effect on IGF-IR tyrosine kinase activity in vitro. The addition of IGF-I to IGF-IR immunoprecipitates in the presence of ATP/Mg\(^{2+}\) led to the expected increase in IGF-IR tyrosine phosphorylation (Fig. 3B). However, pretreatment of parallel samples with ZD1839 had no inhibitory effect on the extent of IGF-IR tyrosine phosphorylation induced by IGF-I. These results indicate that ZD1839 does not inhibit IGF-IR autophosphorylation.

To further exclude the possibility that the inhibitory actions of ZD1839 were mediated by an EGFR-independent mechanism, we used an alternative strategy to block EGFR function. The cells were treated with function-blocking EGFR monoclonal antibodies prior to growth factor treatment. These experiments revealed that ERK phosphorylation induced by both EGF and IGF-I was severely abrogated and, in the case of IGF-I, completely blocked, by monoclonal antibody treatment (Fig. 4). As with ZD1839, monoclonal antibodies failed to block the activation of Akt by IGF-I. These data provide compelling support for the idea that the actions of ZD1839 are mediated through a direct effect on EGFR tyrosine kinase activity.

The potential in vitro relevance of these results was explored further using primary cultures of human breast luminal epithelial cells. As was found in HB4A cells, both EGF and IGF-I promoted phosphorylation of ERK2 and to a lesser extent ERK1, with EGF being the more potent activator (Fig. 5). Both growth factors also caused phosphorylation of Akt to an equivalent degree. Pretreatment of cells with ZD1839 prevented EGF-induced activation of both ERK and Akt and selectively inhibited phosphorylation of Akt in response to IGF-I. These results are consistent with the observations made in intact cells and in primary cultures and indicate a distinct pathway for Akt activation in response to IGF-I.
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Fig. 5. Requirement of EGFR in IGF-I-stimulated ERK phosphorylation in primary breast cells. Primary cultures of cells isolated from human breast tissue and enriched into epithelial and fibroblast populations, as described under “Experimental Procedures,” were incubated in serum-free medium overnight prior to the addition of 1 μM ZD1839 for 10 min. The cells were then left with no further treatment (ns) or incubated with EGF (10 nM) or IGF-I (10 nM) for a further 5 min. The cells were then lysed, and the samples containing equivalent amounts of protein were processed for immunoblotting with antibodies to phospho-ERK, total ERK, phospho-Akt, and total Akt. The blots shown are representative of at least three independent experiments.

Fig. 6. Lack of requirement of EGFR in IGF-I-stimulated ERK phosphorylation in breast cancer cell lines. MCF-7 (upper panel) or CAL-51 (lower panel) cells were incubated in serum-free medium overnight prior to the addition of 1 μM ZD1839 for 10 min. The cells were then left with no further treatment (ns) or incubated with EGF (10 nM) or IGF-I (10 nM) for a further 5 min. The cells were then lysed, and the samples containing equivalent amounts of protein were processed for immunoblotting with antibodies to phospho-ERK, total ERK, phospho-Akt, and total Akt. The blots shown are representative of at least three independent experiments.

Phosphorylation of ERK, Akt, and their upstream kinases, as well as changes in cytoskeleton associated protein levels, was observed following 10 min of concurrent IGF-I and EGF stimulation in MCF-7 and CAL-51 cells. The extent of ERK and Akt phosphorylation was similar between the upper panel (MCF-7 cells) and lower panel (CAL-51 cells). The presence of ZD1839 in the medium partially blocked ERK phosphorylation in MCF-7 cells, although Akt phosphorylation was unaffected. In CAL-51 cells, both ERK and Akt phosphorylation were partially blocked by ZD1839.

The preceding data suggest that IGF-I-induced ERK signaling involves the EGFR only within epithelial cells of the breast. Because of the potential implications of using EGFR tyrosine kinase inhibitors therapeutically, we next considered whether the same mechanism might occur in breast cancer cells. Using MCF-7 cells, which express high levels of the IGF-IR and respond mitogenically to IGF-I (8), we found that, although IGF-I induced robust ERK phosphorylation, this response was not mediated via the EGFR (Fig. 6). Similar experiments were then conducted on a range of other breast carcinoma cell lines. Of the lines tested (ZR-75–1, CAL-51, SKBr3, MDA-MB231, MDA-MB468, and Hs578T), only one (CAL-51) exhibited reproducible activation of ERK in response to IGF-I treatment. Prior treatment of these cells with ZD1839 failed to affect the subsequent degree of ERK phosphorylation induced by IGF-I (Fig. 6). Therefore, based on this survey of available breast epithelial cell lines, the EGFR involvement in IGF-I signaling seems to be restricted to normal breast epithelial cells.

We next examined whether EGFR blockade affected the mitogenic response of cells to IGF-I. Treatment of HB4A or MCF-7 cells with IGF-I in serum-free medium increased viable cell number by ~3- and ~2-fold, respectively (Fig. 7). CAL-51 cells failed to respond to IGF-I, a result that may be partly explained by their lack of phosphatase and tensin homolog deleted from chromosome 10 expression leading to a constitutively active phosphatidylinositol 3-kinase/Akt pathway (20). When ZD1839 was included in the incubation medium, IGF-I-induced mitogenesis was completely blocked in HB4A but unaffected in MCF-7 cells. These results indicate that the EGFR is required for IGF-I-induced mitogenesis in normal but not malignant breast epithelial cells.

To investigate the mechanism by which EGFR participates in IGF-I-induced ERK phosphorylation and mitogenesis, we first sought direct evidence for EGFR activation in response to IGF-I. Following treatment of cells with IGF-I, EGFR tyrosine phosphorylation was measured either by probing immunoprecipitates of the receptor with anti-phosphotyrosine antibodies or by direct immunoblotting of cell lysates with an antibody that detects phosphorylation of the EGFR at residue Tyr^{1068}, one of its major autophosphorylation sites. As shown in Fig. 8A, these experiments failed to detect any increase in EGFR phosphorylation in response to IGF-I, despite clear induction of phosphorylation in response to EGF. Because it was possible that the above approaches lacked sufficient sensitivity to detect small increases in EGFR phosphorylation, we used a third approach, based on recent studies indicating that EGFR transactivation can occur as a result of matrix metalloproteinase-dependent release of the membrane-tethered EGFR ligand, HB-EGF (21). The cells were therefore pretreated with GM6001 (Iomastat^{TM}), a broad spectrum inhibitor of matrix metalloproteinases (22) or with CRM-197, an analogue of diphtheria toxin, which blocks the action of HB-EGF (23). Neither GM6001 nor CRM-197 had any discernible effect on either basal or IGF-I-induced ERK phosphorylation (Fig. 8B), indicating that IGF-I-induced ERK phosphorylation in HB4A cells is not mediated by metalloproteinase-mediated release of HB-EGF.

An alternative mechanism to explain the participation of the EGFR in IGF-I signaling could involve a direct and functional interaction between the EGFR and IGF-IR. To explore this
possibility the IGF-IR was quantitatively immunoprecipitated from HB4A cells, and the resulting immunoprecipitates were probed with EGFR-specific antibodies. These experiments revealed the presence of a ~175-kDa immunoreactive band in IGF-IR immunoprecipitates from both untreated and IGF-I-treated cells (Fig. 8A). This band co-migrated with the EGFR immunoreactive band from cell lysates subjected to the immunoprecipitation procedure in the absence of IGF-IR antibody. Control immunoprecipitations using either unbound protein A-agarose or control rabbit IgG failed to precipitate EGFR, and probing of IGF-IR immunoprecipitates with secondary antibody alone did not result in the appearance of the 175-kDa band. Overall these experiments support the conclusion that the IGF-IR co-precipitates with the EGFR and that the two receptors interact in HB4A cells.

Inspection of the amounts of EGFR precipitated under different conditions indicated that more EGFR co-precipitated when cells had been treated with IGF-I or, interestingly, EGF. Reprobing of these immunoblots with anti-phosphotyrosine antibodies revealed that the amount of phosphotyrosine associated with the EGFR within IGF-IR immunoprecipitates increased in parallel (Fig. 8B). Under conditions where cells had been preincubated with ZD1839 prior to IGF-I treatment, EGFR tyrosine phosphorylation within IGF-IR immunoprecipitates was completely abolished. Conversely, ZD1839 had little or no effect on the total amount of EGFR co-precipitating with the IGF-IR under the same conditions. Overall these experiments indicate that the IGF-IR forms a complex with the EGFR in intact HB4A cells and suggest that the absolute amounts and tyrosine phosphorylation of both proteins within these complexes are regulated by IGF-I, EGF, and ZD1839. Activation of the ERK pathway by IGF-I may therefore occur through the formation of EGFR-IGF-IR hetero-oligomers.

FIG. 8. IGF-I-stimulated ERK phosphorylation in HB4A cells is not mediated via pro-HB-EGF mediated transactivation of the EGFR. A, cells were incubated in serum-free medium overnight prior to stimulation with nothing (ns), EGF (10 nM), or IGF-I (10 nM) for 5 min. The cell lysates were then prepared and immunoprecipitated with antibodies to EGFR. The resulting immunoprecipitates were probed with antibodies to phosphotyrosine (upper blot). Equivalent amounts of lysate protein were also processed for direct immunoblotting with antibodies to phospho-EGFR (lower blot). B, following overnight incubation in serum-free medium, the cells were pretreated with Me2SO (0.1%; control), ZD1839 (1 μM), CRM197 (10 μg/ml), or GM6001 (10 μM) for 10 min. The cells were then left with no further treatment (ns) or stimulated with IGF-I (10 nM) for 5 min. The cells were then lysed, and the samples containing equivalent amounts of protein were processed for immunoblotting with antibodies to phospho-ERK. The blots shown are representative of three independent experiments.

DISCUSSION

The results presented in this paper demonstrate that IGF-I requires the EGFR both to activate ERKs and to induce mitogenesis in normal human breast epithelial cells. This requirement does not extend to the activation of Akt by IGF-I, which is unaffected by EGFR inhibition. A divergence in the mechanisms leading to activation of ERK and Akt by IGF-I has been noted in two recent studies (14, 15). Because our conclusions are drawn mainly from experiments employing a small molecule EGFR tyrosine kinase inhibitor, it could be argued that the effects on IGF-I-induced ERK activation are due to nonspecific effects of ZD1839. However, several lines of evidence argue against this. Firstly, ZD1839 selectively blocked ERK activation without affecting AKT activation by IGF-I. This, together with the lack of effect of ZD1839 on IGF-I receptor tyrosine kinase activity, demonstrates that the inhibitor does not target the IGF-IR. Secondly, the activation of ERK induced by IGF-I was more sensitive to ZD1839 than that induced by EGF. This implies an exquisite sensitivity of IGF-I-induced ERK activation to EGFR blockade and further argues against nonspecific effects of the drug, which would be expected to occur at higher concentrations. Finally, the EGFR requirement in IGF-I signaling was also demonstrated using the independent approach of inhibiting the receptor with a function-blocking monoclonal antibody. The antibody used, clone 225, is thought to induce receptor dimerization without concomitant autophosphorylation and to down-regulate the receptor from the cell surface (24). Although it is not clear which of these effects of EGFR-directed monoclonal antibodies is responsible for preventing the activation of ERKs by IGF-I, their ability to prevent EGFR autophosphorylation and activation lends further support to a role for the EGFR tyrosine kinase activity in this pathway.

Activation of Akt is dependent on phosphatidylinositol 3-kinase, which, in the case of the IGF-I signaling, is stimulated by
binding to either IRS-1 or -2, two major physiological substrates of the activated IGF-IR. IRS can also mediate activation of the ERK pathway, through its ability, when tyrosine-phosphorylated, to bind the growth factor receptor-binding protein 2-son of sevenless complex. Other studies have shown that IGF-I can induce ERK activation via IGF-IR-associated SHC proteins (25). It is possible that one or the other of these more direct mechanisms is used by IGF-I to stimulate ERKs in MCF-7 and CAL-51 cells, where there was no dependence upon EGFR. The use of different mechanisms to activate the same downstream signaling pathway in different cellular contexts is well documented, but the reasons are not always clear. One potential explanation could involve the relative levels of expression of components of alternative pathways. In comparing the relative expressions of the IGF-IR and EGFR, we observed that the ratio of EGFR:IGF-IR is higher in HB4A cells compared with MCF-7 cells. Thus, because of its higher level of expression, the EGFR may be available for cross-talk with selected IGF-I signals in HB4A cells. Arguing against this simple explanation, however, is our finding that, in other breast cancer cell lines with relatively high EGFR expression (e.g. MDA-MB231 and SKBr3), IGF-I was incapable of inducing ERK activation, despite being able to activate PKB/Akt. This suggests instead that the involvement of the EGFR in IGF-I-induced ERK activation may be a phenomenon of normal breast epithelial cells that is lost during tumorigenesis. The fact that the cross-talk mechanism occurred in primary mammary epithelial cells, as well as in two cell lines derived from normal tissue (HB4A and MCF-10A; latter data not shown), but was absent in the seven tumor cell lines examined supports this conclusion. In this context it is also worth mentioning that down-regulation of IGF-IR expression and associated signaling is thought to be a feature of more aggressive, metastasis-competent cells (26, 27). This may explain why ERK activation by IGF-I was uncommon in the panel of breast cancer lines tested, most of which are derived from metastatic lesions. The lack of EGFR/IGF-IR cross-talk in MCF-7 and CAL-51 cells, where IGF-I is capable of ERK activation, may be due to elevated expression of molecules that provide a more direct route to the ERK pathway. Supporting this idea, multiple components of IGF-I signaling, including the IGF-IR and IRS-1, are overexpressed in MCF-7 cells, and recent studies suggest that their overexpression is associated with enhanced activation of ERKs (28).

As well as demonstrating the dependence of the EGFR tyrosine kinase in IGF-I signaling in mammary epithelial cells, our data provide some insight into the possible mechanisms involved. Using specific blockers, IGF-I was shown not to transactivate the EGFR via matrix metalloproteinase-mediated cleavage of cell surface-bound HB-EGF. This mechanism has been invoked in a large number of recent studies as the means by which heterologous receptors activate ERKs via the EGFR. Indeed studies in transfected COS cells showed that the activation ERKs by IGF-I occurred through such a mechanism (14). Instead our experiments have revealed what appears to be a novel mechanism of communication between the IGF-IR and EGFR, involving the formation of a complex between the two receptors. The EGFR co-precipitated with the IGF-IR when the latter was immunoprecipitated from HB4A cells. Further studies will be necessary to establish whether the two receptors directly interact or if this occurs via intermediate “bridging” proteins. Our data indicate that the amount of tyrosine-phosphorylated EGFR present within these complexes increased following treatment of cells with IGF-I. Interestingly EGFR tyrosine kinase activity is not essential to complex formation per se, because the amount of EGFR complexed with the IGF-IR was not affected when the cells were treated with ZD1839. An unresolved question is whether ligand-induced IGF-IR activation leads to an increase in the tyrosine kinase specific activity of the EGFR. Direct analysis of global EGFR tyrosine phosphorylation by two different methods failed to

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G. Farnie and N. G. Anderson, unpublished observations.
detect any increase in response to IGF-I, arguing that IGF-I does not increase EGFR tyrosine phosphorylation. However, because the proportion of total cellular EGFR complexed with the IGF-IR at any given time is likely to be low, because of the relative stoichiometry of the two receptors, any effect of IGF-I on a small fraction of the total EGFR population would be difficult to detect. IGF-I-dependent EGFR phosphorylation within IGF-IR-EGFR complexes remains a possibility.

Although, to our knowledge, this study provides the first evidence for complex formation between the IGF-IR and EGFR, previous studies have reported an apparent functional interplay between the two receptors. Studies by Baserga and co-workers (29–31) using cells derived from mice with targeted ablation of the IGF-IR demonstrated that the mitogenic action of EGF on these cells and its ability to induce prolonged ERK activation required re-expression of the IGF-IR. These workers also showed activation of the IGF-IR by EGF, although they did not report whether this involved direct interaction between the EGFR and IGF-IR (30). Evidence for a direct interaction between the IGF-IR and ErbB2, another member of the ErbB receptor family, was provided recently (32). In this study, the IGF-IR was shown to be necessary for the activation of ErbB2 by heregulin family ligands.

Although the physiological significance of our findings remains to be determined, a functional interplay between the receptors of two important peptide growth factors for mammary epithelial cells is in line with observations made in previous studies. For example, the proliferation of mammary epithelial cells in culture invariably depends upon both EGF and IGF-I (33, 34), and studies by Ethier and co-workers (35) indicate that stimulation of both EGFR and IGF-IR is necessary for proliferation of these cells. Our studies provide a possible molecular explanation for these findings and further imply that, because it is ERK signaling that relies on the EGFR, this pathway may be necessary for proliferation in mammary epithelial cells.

Finally, the involvement of the EGFR in IGF-I signaling became apparent from our use of the EGFR tyrosine kinase inhibitor, ZD1839. This agent has recently been approved clinically for the treatment of lung cancers with EGFR overexpression and is undergoing trials for other human malignancies with similar characteristics (36). The present study, together with numerous recent reports demonstrating an involvement of the EGFR in signaling by a diverse range of heterologous receptors, indicate that EGFR-specific agents may exert unexpected effects and, furthermore, that such effects may be cell context-dependent.

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