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EGF binding to its receptor triggers a rapid tyrosine phosphorylation of the *erb*B-2 protein in the mammary tumor cell line SK-BR-3

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The epidermal growth factor receptor (EGF-R) and the *erb*B-2 proto-oncogene product protein are closely related by their structural homology and their shared enzymatic activity as autophosphorylating tyrosine kinases. We show that in mammary tumor cells (SK-BR-3) EGF causes a rapid increase in tyrosine phosphorylation of the *erb*B-2 protein. Phosphorylation of *erb*B-2 does not occur in cells lacking the EGF-R (MDA-MB-453). Phosphorylation of *erb*B-2 in SK-BR-3 cells is blocked if EGF is prevented from interacting with its receptor by specific monoclonal antibodies. While EGF induces the down-regulation of its receptor in SK-BR-3 cells, EGF has no effect on the stability of the *erb*B-2 protein. This result suggests that the *erb*B-2 protein is a substrate of the EGF-R and indicates the possibility of communication between these two proteins early in the signal transduction process.

Key words: EGF receptor/oncogenes/tyrosine phosphorylation

Introduction

Many cells can be influenced to grow by the presence of epidermal growth factor (EGF) both *in vitro* and *in vivo* (reviewed in Cohen, 1983). This extracellular agent has its effect by binding a specific cell surface receptor, the EGF receptor (EGF-R) (reviewed in Carpenter and Cohen, 1979; Schlessinger *et al.*, 1983; Schlessinger, 1986). The structural features of this molecule include a cysteine-rich extracellular ligand binding domain, a transmembrane sequence and an intracellular portion which has tyrosine kinase enzymatic activity (Ullrich *et al.*, 1984). The same structural features, as well as significant sequence homology, are shared by a protein variously termed *erb*B-2 (Sembar, 1985), HER2 (Coussens *et al.*, 1985) or *neu* (Bargmann *et al.*, 1986a). The common structural characteristics suggest that the *erb*B-2 protein is itself a growth factor receptor for an unidentified ligand. Abnormalities at the gene level for both EGF-R and *erb*B-2 are linked with the growth disorders of neoplastically transformed cells. The truncation of the EGF receptor gene which occurred during the formation of the avian erythroblastosis virus results in the dominant oncogene gene v-*erb*B (Yamamoto *et al.*, 1983; Downward *et al.*, 1984). A chemically induced rat neuroblastoma mutation of the *erb*B-2 gene results in the creation of a dominant transforming gene (Bargmann *et al.*, 1986b). Gene amplification and accompanied mRNA overexpression of the EGF-R gene or *erb*B-2 gene are also found in human epidermoid cells (Ullrich *et al.*, 1984; Merlino *et al.*, 1984), mammary carcinomas (King *et al.*, 1985a,b; Filmus, 1985; Slamon *et al.*, 1987; van der Vijuer *et al.*, 1987) and glioblastomas (Libermann *et al.*, 1985). The resulting abnormally high levels of EGF-R or *erb*B-2 protein are thought to be important in driving either the initiation or progression of these neoplasias. Evidence for this idea comes from studies indicating that the artificial overexpression of the *erb*B-2 mRNA and protein in NIH-3T3 cells is sufficient to cause neoplastic transformation (DiFiore *et al.*, 1987; Hudziak *et al.*, 1987).

EGF triggers a number of rapid changes in its receptor. EGF causes a rapid aggregation of receptors (Yarden and Schlessinger, 1987a,b) and transport to the coated pits on the cell surface (reviewed by Carpenter and Cohen, 1979; Schlessinger *et al.*, 1983). The protein is then internalized and degraded. In an event thought to be central to the transduction of the growth signal, EGF binding leads to an increase of the tyrosine kinase activity inherent in the cytoplasmic domain of the protein (Ushiro and Cohen, 1980; Livneh *et al.*, 1987; Honegger *et al.*, 1987a,b). A major substrate of the kinase activity of EGF-R is the receptor molecule itself. The *erb*B-2 protein is also an active autophosphorylating tyrosine kinase (Akiyama *et al.*, 1986; Stern *et al.*, 1986). Although EGF cannot apparently bind the *erb*B-2 protein (Stern *et al.*, 1986), EGF has been reported to have effects on *erb*B-2 phosphoryiation on serine and threonine residues (Akiyama *et al.*, 1986) and on *erb*B-2 phosphorylation on tyrosine residues (Stern *et al.*, 1986; Kadowaki *et al.*, 1987).

We investigated the interaction of the *erb*B-2 protein and EGF-R *in vivo* using human mammary carcinoma cell lines which have high levels of *erb*B-2 protein. We find that a rapid increase in tyrosine phosphorylation of the *erb*B-2 protein occurs in response to treatment of cells with EGF. We report that this phosphorylation is dependent on direct interaction of EGF with its receptor, and thus may represent a mechanism for receptor communication.

Results

EGF stimulates the phosphorylation of erbB-2

Previous studies indicate that human mammary tumor cell lines differ widely in their content of *erb*B-2 and EGF-R mRNA (Filmus *et al.*, 1985; King *et al.*, 1985a,b). Quantitative mRNA analysis indicates that the cell line SK-BR-3 has *erb*B-2 mRNA 100-fold above the normal levels (Kraus *et al.*, 1987). RNA blot analysis indicates the presence of EGF-R mRNA in substantial amounts in these cells, although at a level that is at least 10-fold below that of the *erb*B-2 mRNA. Previous studies using radioactive EGF have indicated the presence of ~90 000 EGF receptors on the surface

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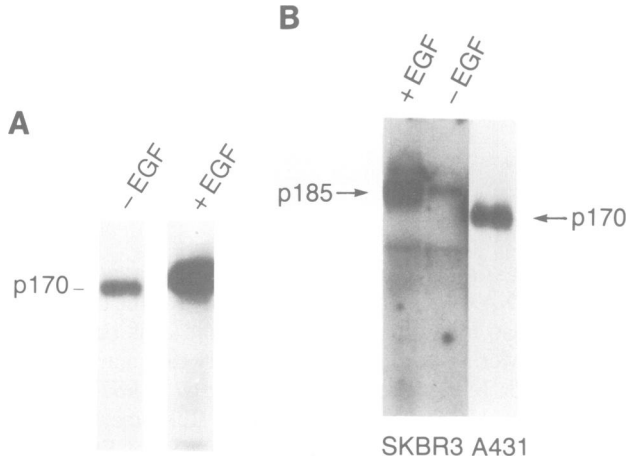

Fig. 1. Detection of phosphotyrosinylated proteins of A431 and SK-BR-3 cells. (**A**) A431 cells unstimulated or stimulated by EGF (100 ng/ml) lysed in SDS, separated by polyacrylamide electrophoresis, transferred to nitrocellulose and visualized using antiphosphotyrosine antibody and ^{125}I-labeled staphylococcal protein A. (**B**) SK-BR-3 cells were unstimulated or stimulated with EGF (100 ng/ml). Using immunoblot analysis for comparison, a sample of A431 proteins unstimulated with EGF was analyzed in parallel. The autoradiograms of SK-BR-3 analysis were exposed for 2 days, that for A431 for exposed for 1 day.

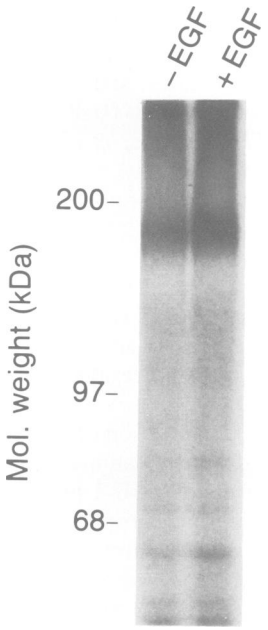

Fig. 2. Immunoprecipitation of *erb*B-2 protein from SK-BR-3 cells. Cells were labeled with [^{32}P]orthophosphate and stimulated with EGF (100 ng/ml) where indicated. Cells were lysed and *erb*B-2 proteins immunoprecipitated with antipeptide antisera directed against amino acids position 1240–1255 (Coussens *et al.*, 1985). After immunoprecipitation, proteins were analyzed by SDS–PAGE.

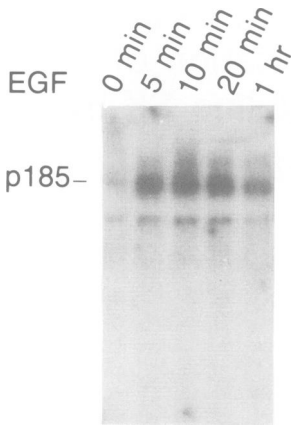

Fig. 3. Time course of tyrosine phosphorylation of *erb*B-2 in response to EGF. SK-BR-3 cells were treated with EGF (100 ng/ml) for the indicated times and lysed in SDS. Immunoblot analysis using antiphosphotyrosine antibody was as described in Figure 1 and Materials and methods.

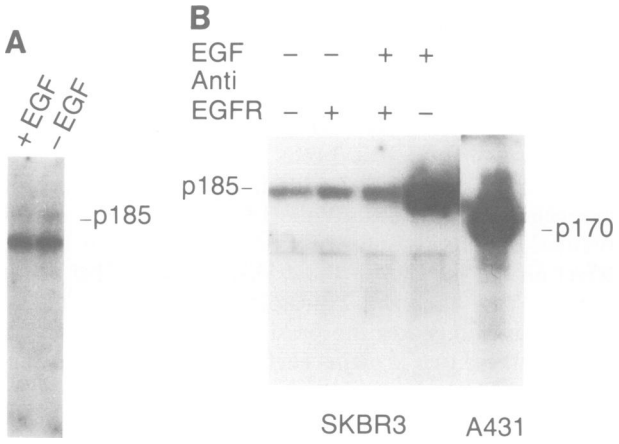

Fig. 4. Phosphorylation of *erb*B-2 proteins requires the presence of EGF-R. (**A**) MDA-MB-453 cells which lack EGF-R were either unstimulated or stimulated with EGF (100 ng/ml). The immunoblot analysis with antiphosphotyrosine antibody was as described in Figure 1 and Materials and methods. (**B**) SK-BR-3 cells were untreated or treated by a monoclonal antibody against EGF-R which blocks EGF binding for 1 h followed by stimulation with EGF (100 ng/ml) for 20 min. Immunoblot detection with antiphosphotyrosine antibody was as described in Figure 1 and Materials and methods.

of SK-BR-3 cells (Imai *et al.*, 1982). These receptors show high and low affinity binding sites characteristic of EGF-R (Imai *et al.*, 1982), react with anti-EGF-R monoclonal antibodies and can undergo autophosphorylation *in vitro* (data not shown).

In the present study, two methods were used to monitor the tyrosine phosphorylation of *erb*B-2 in response to EGF, both of which are essentially *in vivo* assays. First, in an immunoblot assay, cells stimulated with EGF are lysed for application to gel electrophoresis, transferred to nitrocellulose and the phosphotyrosinylated proteins are detected using polyclonal antisera directed against phosphotyrosine (Zippel *et al.*, 1986). The procedure efficiently detects the increased autophosphorylation of the EGF-R of A431 cells in response to EGF. Figure 1A shows a more intense band recognized by the antiphosphotyrosine antibody at 170 kd in size when cells were treated with EGF.

We then examined the phosphotyrosinylated proteins of the SK-BR-3 cell. Using the antiphosphotyrosine immunoblot analysis a result similar to the A431 situation was observed. As shown in Figure 1B, a major phosphotyrosinylated protein was detected by the antiphosphotyrosine antibody. This phosphotyrosinylated protein co-migrates with the 185 kd *erb*B-2 protein as detected by specific anti-*erb*B-2 antisera (data not shown). There was a significant intensification of the band detected by antiphosphotyrosine antibodies when

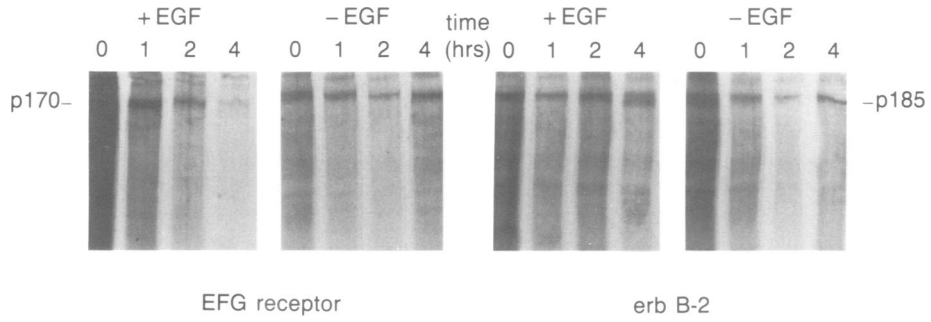

Fig. 5. Stability of *erb*B-2 protein in SK-BR-3 cells treated with EGF. SK-BR-3 cells were metabolically labeled with [^{35}S]methionine and then incubated in media containing non-radioactive methionine and with or without EGF (100 ng/ml). At the indicated times following the chase immunoprecipitation analysis was performed using antisera specific for *erb*B-2 or EGF-R.

cells had been stimulated by EGF (Figure 1B). Also shown is the position of the 170 kd protein of the EGF-R detected in extracts of A431 cells using antiphosphotyrosine antibodies at the exposure of the autoradiograms used to detect *erb*B-2. This is very likely due to the significantly lower amount of EGF receptor when compared with the amount of *erb*B-2 protein in these cells. To assure ourselves that the 185 kd protein detected in SK-BR-3 cells indeed represents the *erb*B-2 protein we labeled SK-BR-3 cells *in vivo* with [^{32}P]orthophosphate and stimulated with EGF. As shown in Figure 2, immunoprecipitation using antisera specific for the *erb*B-2 protein again allows the detection of a 185 kd phosphoprotein and the intensity is enhanced when cells are treated with EGF. Moreover, antibodies specific for the EGF-R did not recognize the 185 kd protein, but did recognize the EGF-R expressed in these cells (data not shown). Figure 3 shows a time course of EGF stimulation using immunoblot analysis and antiphosphotyrosine antibody. The effect is rapid, occurring within the first 5 min. Thus, these results indicate that the *erb*B-2 protein is subject to rapid increases in the amount of phosphate incorporated on tyrosine when SK-BR-3 cells are treated with EGF.

The EGF-R mediates increases in erbB-2 phosphorylation

Since the natural ligand for the *erb*B-2 protein is not yet known we wanted to be sure that the stimulation of *erb*B-2 phosphorylation was actually due to the interaction of EGF with its receptor and not due to contaminants in the EGF preparation or to binding of EGF with the *erb*B-2 protein. The mammary tumor cell line MDA-MB-453 is similar to the SK-BR-3 cell in that it contains an amplification of the *erb*B-2 gene and ~100-fold overexpression of the *erb*B-2 mRNA. The two lines differ in that MDA-MB-453 contains undetectable levels of the EGF-R mRNA (Kraus *et al.*, 1987). When total cellular protein from MDA-MB-453 cells is analyzed for the presence of phosphotyrosine containing proteins using immunoblot analysis a prominent 185 kd band is detected. The intensity of this protein band is consistent with the *erb*B-2 protein of the overexpression of *erb*B-2 mRNA. Interestingly, the *erb*B-2 protein is not the predominant phosphotyrosinylated protein in these cells; in addition to the 185 kd polypeptide we detected an intense band corresponding to 150 kd in size. This protein may represent a degradation product of the *erb*B-2 protein or an unidentified polypeptide. As shown in Figure 4A, the intensity of the 185 kd band does not change when cells are treated with EGF. This strongly suggests that the EGF preparation used in this study does not contain ligands that can stimulate the phosphorylation of *erb*B-2 in these cells.

In order to directly address the question of whether the interaction of EGF with its receptor is required for the increased levels of *erb*B-2 phosphorylation observed in SK-BR-3 cells, we sought to block EGF interaction to its receptor using monoclonal antibodies. The antibody 96 is able to block the binding of [^{125}I]EGF to its receptor and does not recognize the *erb*B-2 protein (F.Bellot *et al.*, in preparation). SK-BR-3 cells were preincubated with this antibody and EGF was then added to the media. As shown in Figure 4B, when total cellular protein was analyzed by immunoblotting using antiphosphotyrosine antibodies no difference in the intensity of the 185 kd *erb*B-2 protein band resulted from the addition of 100 ng/ml EGF to the media. This indicates that EGF interaction with its receptor is required for the increases in tyrosine phosphorylation of *erb*B-2.

EGF does not induce degradation of the erbB-2 protein

The increased tyrosine phosphorylation of the *erb*B-2 protein which occurs in response to EGF indicates that EGF can initiate some of the early events thought to be important in the process of signal transduction for many growth factor receptors. We investigated whether the later event of protein down-regulation also occurs. SK-BR-3 cells were labeled with [^{35}S]methionine and subjected to a chase in cold methionine either in the presence or absence of EGF. As shown in Figure 5, when the EGF-R is immunoprecipitated (Kris *et al.*, 1985) from cell extracts there is a rapid loss of protein with a half-life of about 1 h only when cells are treated with EGF. In the absence of EGF the half-life of the EGF-R is 10−12 h (Honegger *et al.*, 1987a). By contrast, when the *erb*B-2 protein is immunoprecipitated no difference in stability is detectable between EGF treated or untreated cells.

Discussion

The protein tyrosine kinase activity of many growth factor receptors and proteins encoded by oncogenes is widely held to be the key mechanism by which these proteins transmit their growth stimulus. Binding of EGF (reviewed by Carpenter and Cohen, 1979; Schlessinger, 1983), platelet-derived growth factor (Frackelton *et al.*, 1984; Pike *et al.*, 1983), insulin (Kasuga *et al.*, 1982), IGF_1 (Jacobs *et al.*, 1983; Rubin *et al.*, 1983; Zick *et al.*, 1984) and colony stimulating factor (Sherr *et al.*, 1985) all stimulate the kinase

activity of their respective receptors. Mutations which abrogate the tyrosine kinase activity of EGF-R also abolish its ability to transmit a growth stimulus (Livneh *et al.*, 1987; Honegger *et al.*, 1987a,b). The quantitative increase in either EGF-R or *erb*B-2 in some human tumor cells also results in the accumulation of tyrosine kinase activity in these cells. In spite of the importance of this activity, it is not yet clear which proteins are the critical targets for tyrosine phosphorylation. The results presented in this study suggest the involvement of the tyrosine kinase of the EGF-R not only in signal transduction, but also in the modification of a second putative receptor, the *erb*B-2 protein.

Our results suggest a biochemical interaction between two growth factor receptors. We have studied this interaction in mammary tumor cell lines which contain an amplification of the *erb*B-2 gene and overexpressed *erb*B-2 protein. This large amount of protein facilitates experiments *in vivo*; however, it should be cautioned that the large amount of *erb*B-2 protein in these lines is clearly abnormal and may in fact contribute to their neoplastic properties. Our data indicate that when cells containing the EGF-R are treated with EGF there is a rapid increase in tyrosine phosphorylation of the *erb*B-2 protein. This phosphorylation requires the interaction of EGF with its receptor. Recent studies also indicate EGF receptor dependent phosphorylation of *erb*B-2 in kB cells (Kadowaki *et al.*, 1987). Two models are possible for the mechanism of this increased phosphorylation. First, the *erb*B-2 protein may be a substrate of the EGF-R tyrosine kinase, and stimulation of the EGF receptor kinase by EGF would then result in the observed increases in phosphorylation of the *erb*B-2 protein. Second, and less likely, is that the interaction between the EGF-R and the *erb*B-2 protein may allosterically activate the endogenous tyrosine autophosphorylation of the *erb*B-2 protein by formation of receptor heterodimers. Both models are consistent with our results suggesting EGF dependent direct association between EGF-R and *erb*B-2 proteins. In any case, it is tempting to speculate that the interaction between *erb*B-2 and EGF-R reflects the transmission of a signal modulating the activity of the *erb*B-2 protein.

Previous studies have indicated a flow of information from one growth factor receptor system to another. In the case of fibroblasts stimulated by PDGF the EGF-R becomes phosphorylated on Thr-654 leading to loss of high affinity EGF binding sites (Wrann *et al.*, 1980). This effect is likely mediated by protein kinase C (Davis and Czech, 1985). This kind of receptor 'cross-talk' can be viewed as part of a general mechanism in which all receptors effecting the intracellular diacylglycerol levels can communicate with receptors which are regulated by protein kinase C. This mechanism is also probably used as part of a feed-back regulation system for the EGF receptor. The kind of receptor interaction investigated in the present study is significantly different in two important ways. First, there is the possibility for specificity of affected receptors. Stimulation of one growth factor system could communicate with only particular classes of other receptors allowing the cell to fine-tune its growth responses directly at the cell membrane. In fact, at present we cannot exclude the possibility that tyrosine kinase interaction is limited to very closely related receptor proteins like the EGF receptor and *erb*B-2. A second difference with protein kinase C-mediated receptor cross-talk is the specificity of the interaction for tyrosine phosphorylation. The importance of tyrosine autophosphorylation of the EGF receptor either increases or does not affect the tyrosine kinase activity of the receptor (Betrics and Gill, 1985; Downward *et al.*, 1985). It should be possible to explore whether phosphorylation has any effect on kinase activity of the *erb*B-2 protein *in vitro*. In spite of the similarity of EGF-induced phosphorylation for both the *erb*B-2 and EGF receptor, we report that the two proteins do not follow identical fates in subcellular trafficking. There is a clear absence of degradation of *erb*B-2 in response to EGF. In this regard our results are in agreement with those obtained in studies of Rat-1 fibroblasts (Stern *et al.*, 1986). In any case, changes in tyrosine phosphorylation on the *erb*B-2 protein provide a potential means by which the mitogenic signals transmitted by this protein could be regulated in normal cell growth or neoplasia.

Materials and methods

Immunoblot analysis

Cells were grown to ~90% saturation in 24-well dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and then starved in DMEM containing 25 mM Hepes, pH 7.4 for 16 h. EGF (Toyobo Co., Inc.) stimulation (100 ng/ml) was conducted in 200 μl of DMEM–Hepes, pH 7.4 for 20 min at 37°C. Cells were lysed in 100 μl of electrophoresis buffer containing 50 mM Tris–HCl, pH 6.8, 1% SDS, 5% glycerol. Following protein determination, each was brought to 0.1% BME, heated to 95°C for 2 min, sonicated for 5 s to decrease viscosity and subjected to gel electrophoresis in 5% polyacrylamide gels. Proteins were then transferred to nitrocellulose. The antigen–antibody reaction was conducted using previously described procedures for both anti-*erb*B-2 receptor antibody (Kraus *et al.*, 1987) and antiphosphotyrosine (Zippel *et al.*, 1986), and detected using [^{125}I]protein A and autoradiography.

Immunoprecipitation of phosphoprotein

Cells were grown to ~90% saturation in 80 mm dishes in DMEM and 10% FBS. They were washed twice with DMEM lacking phosphate and starved for 45 min in the same medium. Cells were labeled for 6 h in phosphate-free medium containing 5 mCi ^{32}P-labeled *o*-phosphate. Cells were treated with 200 ng/ml EGF for the last 20 min of the labeling period. Cells were lysed in lysing buffer containing 50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 2 mM Na orthovanadate 1 mM. Lysates were centrifuged 12 000 *g* for 5 min to remove debris and anti-*erb*B-2 antibody was added for incubation of 1 h at 4°C. Immunocomplexes were collected using protein A-Sepharose (Sigma), washed four times with lysing buffer and resuspended in electrophoresis sample for separation on 7% polyacrylamide gels.

Down-regulation studies

Cells were grown to 90% confluence in 35 mm dishes in DMEM and 10% FBS. Cells were then starved in DMEM lacking methionine for 45 min and labeled with 0.5 mCi [^{35}S]methionine (NEN) for 1 h. Following labeling cells were washed with DMEM and a chase of medium DMEM containing 25 mM methionine, with or without EGF, was added. At the indicated times plates of cells were lysed in lysing buffer (lacking phosphatase inhibitors). The lysate was divided in half and the immunoprecipitation procedure above was used with either anti-*erb*B-2 or anti-EGF receptor antibodies (Kris *et al.*, 1985). Immunoprecipitates were analyzed on 7% polyacrylamide gels.

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