The EMBO Journal vol.7 no.4 pp.995-1001, 1988

EGF-stimulated tyrosine phosphorylation of p185^{neu}: a potential model for receptor interactions

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Communicated by D.Baltimore

p185^{neu} is a receptor-like protein encoded by the *neu/erbB*-2 proto-oncogene. This protein is closely related to the epidermal growth factor (EGF) receptor, but does not bind EGF. We report here that incubation of Rat-1 cells with EGF stimulates tyrosine phosphorylation of p185. This effect is specific to EGF since neither platelet derived growth factor (PDGF) nor insulin, which also bind to receptors with ligand-stimulated tyrosine kinase activity, induced tyrosine phosphorylation of p185. The EGF-stimulated tyrosine phosphorylation of p185 and of the EGF receptor occurred with similar kinetics and EGF dose-responses, and both phosphorylations were prevented by down-regulation of the EGF receptor with EGF. Since p185 does not bind EGF, these results suggested that p185 is a substrate for the EGF receptor kinase. Incubation of cells with EGF before lysis stimulated the tyrosine phosphorylation of p185 in immune complexes. This suggested that EGF, acting through the EGF receptor, can regulate the intrinsic kinase activity of p185.

Key words: EGF/*erbB*-2/*neu*/p185^{neu}/phosphotyrosine

Introduction

Peptide growth factors regulate cell proliferation and differentiation. These factors act on cells by binding to cell surface receptors, which in turn transmit signals to the cell interior. At least five growth factors, epidermal growth factor (EGF), platelet-derived growth factor (PDGF), macrophage colony-stimulating factor-1 (CSF-1), insulin and insulin-like growth factor-1 (IGF-1) bind to receptors that are protein tyrosine kinases. The tyrosine kinase activity of the receptors is regulated by ligand binding and is likely to be the major signalling mechanism of these receptors. Understanding the cascade of events that immediately follows binding of growth factors to their receptors has been hampered by the difficulty in identifying physiologically relevant substrates for the receptor kinases (Hunter and Cooper, 1986).

In addition to their importance as regulators of cell division, growth factor receptors have recently been implicated in carcinogenesis. The viral oncogenes v-*erbB* and v-*fms* encode mutated versions of the receptors for EGF and CSF-1 (Downward *et al.*, 1984; Ullrich *et al.*, 1984; Sherr *et al.*, 1985). These altered receptors probably transform cells by constitutively transmitting growth stimulatory signals normally produced only after activation by ligand binding. Other studies have shown that genes encoding growth factor receptors are amplified in certain human tumors, with concomitant increases in the abundance of the gene products (Libermann *et al.*, 1985; Ozanne *et al.*, 1986). Receptor amplification may result in aberrant growth stimulation, either because it multiplies a basal receptor signalling activity (DiFiore *et al.*, 1987) or because it amplifies the response of the cell to limited amounts of ligand.

We have been studying the mechanism of transformation by the *neu* oncogene, which, like v-*erbB* and v-*fms*, appears to encode a mutated growth factor receptor. The *neu* oncogene was originally identified using the NIH-3T3 transfection assay. *Neu* has acquired oncogenic activity in a series of chemically-induced rat neuro-ectodermal tumors (Bargmann *et al.*, 1986a; Shih *et al.*, 1981). The normal progenitor of the *neu* oncogene is closely related to, but distinct from, *erbB*, the gene encoding the EGF receptor (Schechter *et al.*, 1984, 1985). The *neu* proto-oncogene and *erbB* are substantially more closely related to one another than either is to any other genes (Bargmann *et al.*, 1986b; Coussens *et al.*, 1985; Yamamoto *et al.*, 1986). This has led to the use of the alternative designation *erbB*-2 to denote *neu*. Both genes encode 140 kd core polypeptides with single putative membrane-spanning domains, two homologous cysteine-rich extracellular domains and intracellular tyrosine kinase domains.

The product of the *neu* proto-oncogene is a 185 kd glycoprotein designated p185 which, like the EGF receptor, is a transmembrane protein with tyrosine kinase activity (Akiyama *et al.*, 1986; Padhy *et al.*, 1982; Stern *et al.*, 1986). These properties and the close structural homology of p185 and the EGF receptor suggest that p185 is itself a growth factor receptor. However, p185 does not bind EGF and efforts to identify a ligand for p185 have been unsuccessful thus far (Akiyama *et al.*, 1986; Stern *et al.*, 1986).

In attempting to detect the ligand for p185 we screened known growth factors and tissue extracts for the ability to stimulate tyrosine phosphorylation of p185. Surprisingly, these experiments revealed that EGF stimulates tyrosine phosphorylation of p185 in the Rat-1 fibroblast cell line (Stern *et al.*, 1986). Since this suggested that p185 might itself be a substrate for the EGF receptor kinase, we have further examined the EGF-stimulated tyrosine phosphorylation of p185 as a possible model for receptor-receptor interactions mediated by tyrosine phosphorylation.

Results

EGF stimulates tyrosine phosphorylation of p185

Rat-1 cells express both p185 and the EGF receptor (Stern *et al.*, 1986). We examined the effects of EGF on the phosphoamino acid content of these two proteins by labeling cells metabolically with $^{32}P_i$, purifying the proteins by immunoprecipitation and gel electrophoresis and analyzing

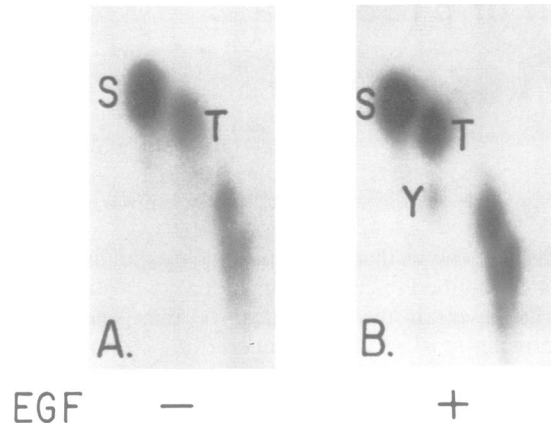

Fig. 1. Effect of EGF on phosphoamino acid composition of p185. Rat-1 cells were labeled with $^{32}P_i$, incubated with EGF (33 μg/ml) for 5 min at 37°C, and p185 was immunoprecipitated, purified by gel electrophoresis and subjected to partial acid hydrolysis. Phosphoamino acids were resolved by two-dimensional electrophoresis on thin-layer cellulose plates. Electrophoresis in the first dimension was at pH 1.9 (anode at the left) and that in the second dimension was at pH 3.5 (anode at the top). Non-labeled phosphoamino acid standards mixed in with the samples were located by ninhydrin staining. S, the position of phosphoserine; T, the position of phosphothreonine, Y, the position of phosphotyrosine. Thin layer plates were exposed to preflashed film (Kodak X-Omat R) for 10 days at −70°C using an intensifying screen. Phosphoamino acid analysis of p185 from mock-treated (**Panel A**) and EGF-treated (**Panel B**) Rat-1 cells.

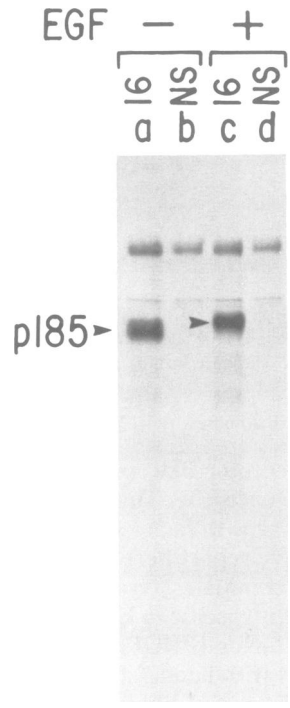

Fig. 2. Effect of EGF treatment on the electrophoretic mobility of p185. Dense cultures of Rat-1 cells in 100 mm dishes were labeled metabolically with L-[^{35}S]cysteine (100 μCi/ml; ~1000 Ci/mmol; Amersham) for 29 h at 37°C as described (Stern *et al.*, 1986). The labeling medium was then replaced with DMEM−0.1% CS without or with EGF (10 ng/ml) and incubation continued for 8 min. Cells were lysed and p185 was immunoprecipitated using antibody 7.16.4 as described (Stern *et al.*, 1986) except that phosphate-buffered RIPA contained sodium orthovanadate at a concentration of 1 mM and lacked ATP and EDTA. Fixed *Staphylococcus aureus* (IgGSorb; The Enzyme Center Inc.) was suspended in 2% SDS−1% 2-mercaptoethanol and incubated at 100°C for 15 min, concentrated by sedimentation, and this procedure repeated. *S.aureus* was then washed five times in 20 mM Tris HCl, pH 8.0, 1 M NaCl, 1% Triton X-100, 0.02% NaN_3 and stored at −20°C before use as described (Stern *et al.*, 1986). Proteins were resolved by SDS−PAGE (Sefton *et al.*, 1978; Stern *et al.*, 1986) and the fluorographed gel exposed to preflashed film for 2 days at −70°C. Immunoprecipitates from untreated (**lanes a** and **b**) or EGF-treated (**lanes c** and **d**) Rat-1 cells prepared with antibody 7.16.4 (lanes a and c) or normal mouse serum (lanes b and d).

their phosphoamino acids. p185 in unstimulated cells contained phosphoserine and phosphothreonine, but little or no phosphotyrosine (Figure 1A). p185 isolated from cells incubated briefly with EGF before lysis contained more phosphoserine and phosphothreonine, and substantially increased phosphotyrosine relative to the amounts of these phosphoamino acids in p185 from non-treated cells (Figure 1B). The increased phosphorylation of p185 induced by EGF treatment was accompanied by a decrease in the electrophoretic mobility of the protein (Figure 2a and c), indicating that most p185 molecules were modified as a result of EGF treatment, probably by phosphorylation. In parallel experiments we confirmed that EGF stimulates serine, threonine, and tyrosine phosphorylation of the EGF receptor, as had been observed by others (Cochet *et al.*, 1984; Iwashita and Fox, 1984).

Detection of phosphotyrosine in p185 by immunoblotting

Because of the low abundance of p185 in Rat-1 cells, further characterization of the EGF-stimulated increase in tyrosine phosphorylation of p185 was fraught with technical difficulties. We therefore sought an alternative method for detecting this phosphorylation. Several laboratories have employed anti-phosphotyrosine antibodies to recognize phosphotyrosine-containing proteins (Comoglio *et al.*, 1984; Frackelton *et al.*, 1983; Huhn *et al.*, 1987; Kadowaki *et al.*, 1987; Kamps and Sefton, 1988; Ohtsuka *et al.*, 1984). One such antiserum, raised against a synthetic copolymer of phosphotyrosine, alanine, and glycine, recognizes a number of substrates for viral tyrosine kinases in Western immunoblots (Kamps and Sefton, 1988). Preliminary studies showed that this antiserum reacts with the transforming variant p185* protein (Stern *et al.*, 1986) expressed at high levels in B104-1-1 cells (Figure 3, lane i). The presence of phosphotyrosine in p185* was confirmed by metabolic labeling with $^{32}P_i$ and phosphoamino acid analysis (not shown).

To determine if the anti-phosphotyrosine antiserum recognizes p185 in EGF-stimulated cells, extracts of EGF-treated Rat-1 cells were analyzed by Western blotting. EGF treatment induced the appearance of two bands (Figure 3, lane b). The electrophoretic mobilities of these proteins (M_r ~175 kd and 185 kd) suggested that they were the EGF receptor and p185. The identity of these bands was confirmed by immunoprecipitation with monoclonal antibody 7.16.4 (which recognizes rat p185) or anti-human EGF receptor antiserum [which recognizes the rat EGF receptor and has some reactivity with rat p185 (Schechter *et al.*, 1984; Stern *et al.*, 1986)]. When the immunoprecipitates were analyzed by Western blotting with the anti-phosphotyrosine antibody, no signal was detected in immunoprecipitates prepared from non-treated cells (Figure 3, lanes c and d), or in immunoprecipitates using control sera (not shown). In contrast, precipitates prepared from EGF-stimulated cell lysates with antibody 7.16.4 yielded a band (Figure 3, lane e) that comi-

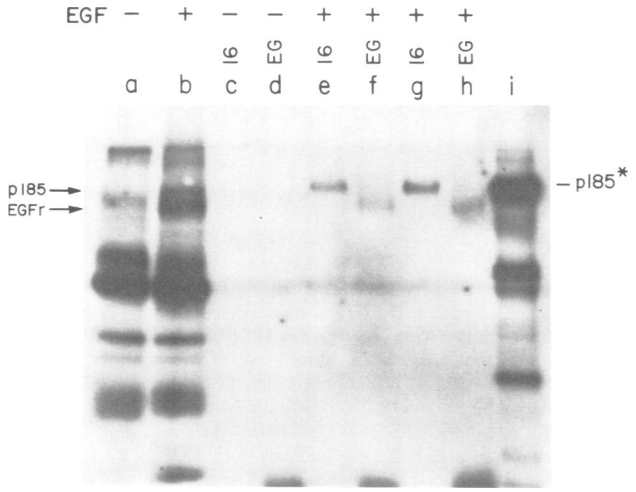

Fig. 3. Detection of p185 and EGF receptor with anti-phosphotyrosine immunoblots. Dense cultures of Rat-1 cells were incubated overnight in DMEM−0.1% CS, and then for 5 min in DMEM−0.1% CS without or with EGF (10 μg/ml) as indicated. Total protein extracts were prepared using boiling electrophoresis sample buffer (Kamps and Sefton, 1988). RIPA lysates were prepared and immunoprecipitated as described in Materials and methods. Proteins were resolved by SDS−PAGE, transferred to nitrocellulose and probed with anti-phosphotyrosine antibodies. The autoradiograph was exposed to preflashed film at −70°C overnight using an intensifying screen. Extracts of untreated (**lane a**) and EGF-treated (**lane b**) Rat-1 cells. Immunoprecipitates prepared from untreated (**lanes c** and **d**) or EGF-treated (**lanes e−h**) Rat-1 cells using antibody 7.16.4 (lanes c,e,g) or anti-EGF receptor antibody (lanes d,f,h). Extract of B104-1-1 cells (**lane i**).

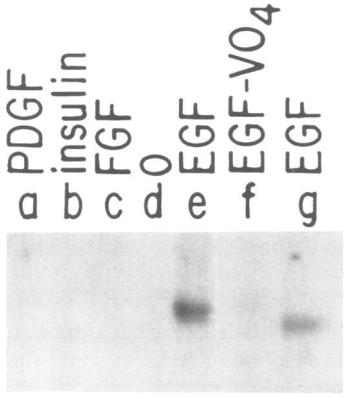

Fig. 4. Effects of PDGF, insulin, FGF and sodium orthovanadate on p185. Dense 35 mm cultures of Rat-1 cells were incubated in 200 μl of DMEM−0.1% CS containing PDGF (25 ng/ml), insulin (50 μg/ml), pituitary FGF (210 ng/ml) or EGF (10 ng/ml) at 37°C for 6 min. Cells were lysed, and immunoprecipitates prepared using antibody 7.16.4. The lysis buffer used to prepare the sample for lane f lacked sodium orthovanadate. The proteins were separated by gel electrophoresis, transferred to nitrocellulose, and probed using anti-phosphotyrosine antibody. The autoradiograph was exposed to preflashed film at −70°C for 2 days. **Lanes f** and **g** are from a different section of the same gel as **lanes a−e**. Anti-phosphotyrosine immunoblot analysis of 7.16.4 immunoprecipitates of Rat-1 cells incubated in DMEM−0.1% CS (lane d) or DMEM−0.1% CS containing PDGF (lane a), insulin (lane b), FGF (lane c), or EGF (lanes e,f,g). Lane f, immunoprecipitate prepared from EGF-treated cells in the absence of sodium orthovanadate.

grated with the transforming variant (p185*) of rat p185 (Figure 3, lane i). Precipitation with anti-EGF receptor antibody yielded an EGF-induced band (Figure 3, lane f) with

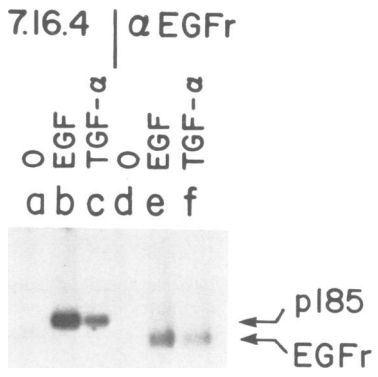

Fig. 5. Effect of TGF-α on p185. Dense 60 mm cultures of Rat-1 cells were incubated in 1.0 ml of DMEM−0.1% CS containing EGF (100 ng/ml) or TGF-α (100 ng/ml) at 37°C for 6 min. Cell lysates were divided into two portions and immunoprecipitated using antibody 7.16.4 (**lanes a−c**) or anti-EGF receptor antibody (**lanes d−f**). Proteins were resolved by gel electrophoresis, transferred to nitrocellulose and probed using anti-phosphotyrosine antibodies. The autoradiograph was exposed to preflashed film at −70°C for 16 h. Anti-phosphotyrosine immunoblot analysis of immunoprecipitates of Rat-1 cells incubated in DMEM−0.1% CS (lanes a and d), EGF (lanes b and e) or TGF-α (lanes c and f).

the appropriate mobility for the 170 kd EGF receptor. These results confirmed the identity of the two EGF-stimulated bands seen in Figure 3, lane b. Since isolation of the EGF receptor or p185 by immunoprecipitation before Western blotting reduced the background, this procedure was used in subsequent experiments. The greater recovery of p185 than the EGF receptor in this and subsequent immunoprecipitation experiments resulted from the use of limiting amounts of anti-EGF receptor antibody; Rat-1 cells contain a few-fold more EGF receptors than p185 molecules (Stern *et al.*, 1986).

Antisera raised against phosphotyrosine analogs do not recognize phosphotyrosine-containing proteins exclusively (Frackelton *et al.*, 1983). To verify that the anti-phosphotyrosine immunoblots were detecting tyrosine phosphorylated forms of p185 we compared the signals obtained from lysates prepared under conditions favoring or inhibiting the activity of tyrosine phosphatases. Lysates for the experiments described above were prepared using buffers containing 1 mM sodium orthovanadate, an inhibitor of tyrosine phosphatases. Preparation of lysates in the absence of vanadate and the presence of EDTA, conditions which spare tyrosine phosphatase activity (Kamps and Sefton, 1988), nearly eliminated the EGF-stimulated signal [compare Figure 4, lane f (no VO_4) to Figure 4, lane g (with VO_4)]. This result and the good correlation between results of immunoblotting experiments with anti-phosphotyrosine antibodies and phosphate labeling experiments supported the interpretation that the signal detected in Western blots resulted from tyrosine phosphorylation of p185. This experiment also demonstrated that it would be possible to manipulate the phosphotyrosine content of p185 by choosing appropriate lysis conditions.

Specificity of EGF response

In earlier metabolic labeling experiments, stimulation of p185 tyrosine phosphorylation by TGF-α was not detected (Stern

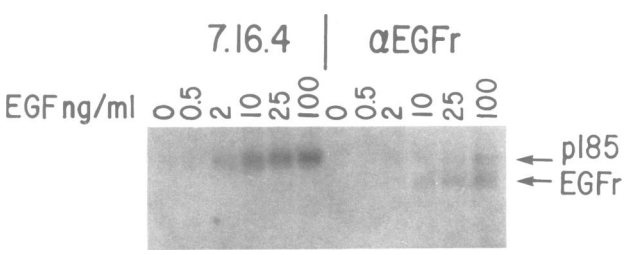

Fig. 6. EGF dose–response of tyrosine phosphorylation of p185 and the EGF receptor. Dense 35 mm cultures of Rat-1 cells were incubated in DMEM–0.1% CS containing the indicated amounts of EGF for 6 min at 37°C. Lysates were divided into two portions and immunoprecipitated using antibody 7.16.4 or anti-EGF receptor antibody. Proteins were resolved by gel electrophoresis, transferred to nitrocellulose and probed using anti-phosphotyrosine antibodies. The autoradiograph was exposed to preflashed film at −70°C for 2 days using an intensifying screen. Anti-phosphotyrosine immunoblot analysis of Rat-1 cells incubated in varying amounts of EGF and immunoprecipitated using antibody 7.16.4 or anti-EGF receptor.

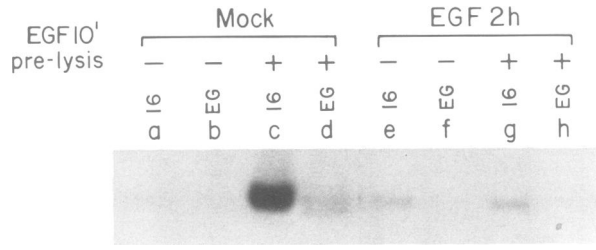

Fig. 7. Effect of EGF pre-treatment on EGF-stimulated tyrosine phosphorylation of p185 and EGF receptor. Dense cultures of Rat-1 cells on 35 mm dishes were incubated in DMEM–0.1% CS in the absence (**lanes a–d**) or presence (**lanes e–h**) of 100 ng/ml EGF for 2 h at 37°C. The medium was replaced with DMEM–0.1% CS without (lanes a,b,e,f) or with EGF (10 ng/ml; lanes c,d,g,h) and incubated for an additional 6 min. Cells were lysed and immunoprecipitates prepared using antibody 7.16.4 (lanes a,c,e,g) or anti-EGF receptor antibody (lanes b,d,f,h). The samples were analyzed by anti-phosphotyrosine immunoblotting. Lanes a,b,c,d: lysates prepared from mock-treated cells that were incubated without (lanes a and b) or with (lanes c and d) EGF for 6 min and immunoprecipitated using antibody 7.16.4 (lanes a and c) or anti-EGF receptor (lanes b and d). Lanes e,f,g,h: lysates prepared from cells incubated with EGF for 2 h followed by mock-treatment (lanes e and f) or EGF treatment (lanes g and h), and immunoprecipitated using antibody 7.16.4 (lanes e and g) or anti-EGF receptor (lanes f and h).

et al., 1986). This was surprising since TGF-α acts by binding to the EGF receptor and has similar biological activities. In an effort to verify this result we used the anti-phosphotyrosine antibody to compare the tyrosine phosphorylation of p185 and the EGF receptor isolated from TGF-α-treated cells. TGF-α stimulated tyrosine phosphorylation of both p185 and the EGF receptor (Figure 5, lanes c and f), although the conditions chosen led to a sub-optimal phosphorylation of both proteins relative to EGF-induced phosphorylation (Figure 5, lanes b and e). Our previous failure to detect TGF-α-stimulated tyrosine phosphorylation of p185 was probably due to the poorer sensitivity of the metabolic labeling assay.

Since receptors for PDGF, insulin and IGF-1 have ligand-stimulated tyrosine kinase activity, we determined whether the cognate growth factors would stimulate tyrosine phosphorylation of p185. Neither PDGF, insulin (at concentrations sufficient for binding to both insulin and IGF-1 receptors), nor pituitary FGF stimulated tyrosine phosphorylation of p185 as detected by immunoblotting (Figure 4, lanes a–c) or in the less-sensitive metabolic labeling experiments (Stern *et al.*, 1986). Since fibroblasts generally bear PDGF and IGF-1 receptors this demonstrated that the tyrosine phosphorylation of p185 is stimulated specifically by EGF, and not by all factors that bind to tyrosine kinase receptors.

Role of the EGF receptor in the EGF response

The EGF dose responses of tyrosine phosphorylation of the EGF receptor and p185 were closely parallel (Figure 6). This indicated that the EGF-responsive component of the tyrosine phosphorylation of p185 was mediated by a receptor with EGF binding properties similar to those of the EGF receptor. Since EGF does not bind with high affinity to p185 (Akiyama *et al.*, 1986; Stern *et al.*, 1986), it appeared that this response is initiated via EGF binding to the EGF receptor.

We had previously reported that incubation of cells for 2 h with EGF results in loss of immunoprecipitable EGF receptor but does not affect the stability of p185 (Stern *et al.*, 1986). Since this procedure permits preferential elimination of the EGF receptor from cells, we determined whether it affected the EGF-stimulated tyrosine phosphorylation of p185. Rat-1 cells were pre-treated for 2 h with EGF, challenged with an additional dose of EGF, and analyzed by phosphotyrosine immunoblotting after immunoprecipitation (Figure 7). Incubation of cells with EGF followed by immunoprecipitation of p185 or the EGF receptor induced the appearance of p185 and EGF receptor bands recognized by anti-phosphotyrosine immunoblotting (Figure 7, lanes a–d) . In this experiment p185 and the EGF receptor were not resolved well because the proteins were fractionated on a shorter gel than usual. Pre-incubation of cells for 2 h with EGF obliterated the induction of these bands by subsequent EGF treatment (Figure 7, lanes e–h). The loss of the EGF-stimulated EGF receptor band caused by EGF pretreatment is probably due to the turnover of the EGF receptor documented previously (Stern *et al.*, 1986). EGF pre-treatment also inhibited the EGF-stimulated tyrosine phosphorylation of p185. Since this treatment does not down-regulate p185, this result suggested that EGF pre-treatment reduced EGF-induced phosphorylation of p185 by other means, and was consistent with the model that this tyrosine phosphorylation requires the presence of the EGF receptor.

Since EGF-induced phosphorylation of p185 apparently involved the intercession of the EGF receptor, we compared the kinetics of tyrosine phosphorylation of p185 to phosphorylation of the EGF receptor itself (Figure 8). EGF-stimulated tyrosine phosphorylation of p185 was detectable when cells were lysed within 30 s of EGF addition. The kinetics of phosphorylation of the EGF receptor and p185 were similar, indicating a close coupling between EGF binding to the EGF receptor and phosphorylation of p185. Comparably rapid kinetics of phosphorylation have been observed when other proteins are phosphorylated on tyrosine in EGF-treated cells (Hunter and Cooper, 1981).

EGF regulates p185 phosphorylation in vitro

Phosphorylation of protein-tyrosine kinases in some cases stimulates and in other cases attenuates their kinase activities

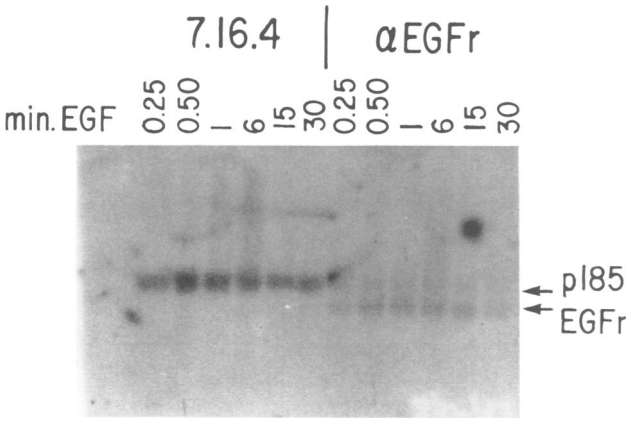

Fig. 8. Kinetics of tyrosine phosphorylation of p185 and the EGF receptor. Dense cultures of Rat-1 cells in 35 mm dishes were incubated in DMEM−0.1% CS containing 10 ng/ml EGF for the indicated times. The shortest EGF incubation period was 0.25 min but lysis buffer was not added until 0.5 min after EGF addition because of the time required for the intervening washes. Lysates were immunoprecipitated using antibody 7.16.4 or anti-EGF receptor antibody and samples were analyzed by anti-phosphotyrosine immunoblotting. The autoradiograph was exposed to preflashed film for 2 days at −70°C. Anti-phosphotyrosine immunoblots of Rat-1 cells incubated for various amounts of time with EGF and immunoprecipitated using antibody 7.16.4 or anti-EGF receptor.

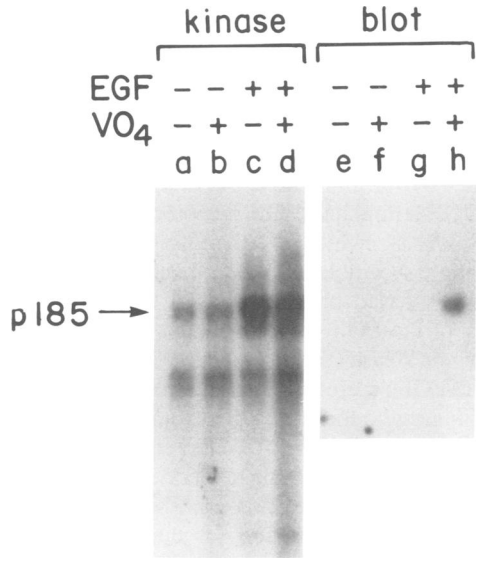

Fig. 9. Effect of EGF pretreatment and sodium orthovanadate on phosphorylation of p185 in the immune complex kinase assay. Dense 100 mm cultures of Rat-1 cells were incubated for 6 min at 37°C in 2.0 ml DMEM−0.1% CS containing no EGF or 10 ng/ml EGF. Cultures were washed twice with cold PBS, lysed and p185 was immunoprecipitated using antibody 7.16.4 as described (Stern *et al.*, 1986) except that all buffers contained 100 μM sodium orthovanadate and samples were washed four times in TG. After washing, a portion of the immune complexes was added directly to one volume of 2-fold concentrated electrophoresis sample buffer for analysis by immunoblotting using anti-phosphotyrosine antibodies (**lanes e−h**). The remainder (**lanes a−d**) was labeled *in vitro* (in the presence of 100 μM sodium orthovanadate) as described (Stern *et al.*, 1986) but for 15 min at 30°C. For samples analyzed in lanes a,c,e and g, sodium orthovanadate was omitted from the lysis buffer only. Exposures were for 2 days (lanes a,b,c,d) or 18 h. (lanes e,f,g,h) using preflashed film at −70°C. Lanes a,b,c,d: immune complex kinase assays of samples prepared from untreated (lanes a and b) or EGF-treated (lanes c and d) using samples prepared in the absence (lanes a and c) or presence (lanes b and d) of sodium orthovanadate. Lanes e,f,g,h: anti-phosphotyrosine immunoblotting analysis of samples prepared from untreated (lanes e and f) or EGF-treated (lanes g and h) using samples prepared in the absence (lanes e and g) or presence (lanes f and h) of sodium orthovanadate.

(Bertics and Gill, 1985; Cochet *et al.*, 1984; Cooper *et al.*, 1986; Courtneidge, 1985; Friedman *et al.*, 1984; Rosen *et al.*, 1983; Yu and Czech, 1986). Since the EGF-stimulated phosphorylation of p185 may have regulatory significance, we determined whether the p185 kinase activity, detected in an immune complex assay (Akiyama *et al.*, 1986; Stern *et al.*, 1986), is affected by EGF (Figure 9). p185 isolated from EGF-treated cells, but not mock-treated cells, contained phosphotyrosine, as expected (Figure 9, lanes f and h). EGF treatment of cells prior to lysis stimulated labeling of p185 in the immune complex assay (Figure 9, lanes b and d). The predominant ^{32}P-labeled phosphoamino acid in p185 isolated from untreated or EGF-treated cells and labeled *in vitro* was phosphotyrosine, although some phosphoserine was also present (not shown). This indicated that the bulk of increased phosphorylation of p185 was due to greater activity of a tyrosine kinase, probably p185, rather than one of the serine or threonine kinases stimulated by EGF.

A possible mechanism for the EGF-stimulated phosphorylation of p185 in the immune complex assay was that one or more of the EGF-induced *in vivo* phosphorylations enhances the self-phosphorylating activity of p185 *in vitro*. We determined the role of *in vivo* phosphorylation of p185 on tyrosine by examining the cell-free phosphorylation of p185 immunoprecipitated using lysis buffer lacking sodium orthovanadate. p185 isolated from EGF-treated cells in the absence of this tyrosine phosphatase inhibitor contained no phosphotyrosine (Figure 9, lane g). However, this protein was labeled to approximately the same extent *in vitro* as p185 prepared in the presence of orthovanadate and which contained phosphotyrosine (Figure 9, lane c) and once again the predominant labeled phosphoamino acid was phosphotyrosine. Thus the presence of phosphotyrosine in p185 did not correlate with the extent of cell-free tyrosine phosphorylation of the protein.

Discussion

Exposure of Rat-1 cells to EGF rapidly stimulated tyrosine phosphorylation of p185, and the *in vitro* tyrosine phosphorylation of p185 in an immune complex kinase assay. Since EGF does not bind to p185 (Akiyama *et al.*, 1986; Stern *et al.*, 1986), it appears that both the tyrosine phosphorylation and enzymatic activation of p185 are mediated by EGF binding to the EGF receptor.

The inhibition of EGF-stimulated tyrosine phosphorylation of p185 by down-regulation of the EGF receptor with EGF provides further evidence that EGF-stimulated tyrosine phosphorylation of p185 requires the presence of the EGF receptor. However an alternative explanation arises from the possible activation of serine/threonine kinases by EGF. The presence in p185 of a potential site for phosphorylation homologous to a kinase C acceptor site on the EGF receptor (Hunter *et al.*, 1984; Bargmann *et al.*, 1986b; Coussens *et al.*, 1985; Yamamoto *et al.*, 1986) suggests that the p185 kinase may, like the EGF receptor kinase, be inhibited by threonine phosphorylation (Cochet *et al.*, 1984; Downward

et al., 1985; Friedman *et al.*, 1984; King and Cuatrecasas, 1982)

If the EGF receptor is a necessary participant in the EGF-induced phosphorylation of p185, three possible mechanisms can be envisaged: (i) p185 is a substrate for the EGF receptor kinase; (ii) p185 is a substrate for a tyrosine kinase that is activated by the EGF receptor in a kinase cascade; and (iii) the EGF-stimulated EGF receptor activates the intrinsic p185 kinase, which then phosphorylates itself or a neighboring p185 molecule. One mechanism for the latter interaction is based upon the model that EGF stimulates clustering of the EGF receptor which in turn activates the EGF receptor kinase (Schlessinger, 1986). The close homology of the EGF receptor and p185 raises the possibility that EGF stimulates the formation of clusters between the EGF-activated EGF receptor and p185, thereby activating the p185 kinase. At present we have no evidence either for or against the existence of such mixed clusters. The recognition of p185 by anti-EGF receptor antibody is due to cross-reactivity rather than co-precipitation of the two proteins since this antibody quantitatively precipitates p185 from B104-1-1 and DHFR/G-8 cells, which contain p185 in vast stoichiometric excess over the EGF receptor (Stern *et al.*, 1986). The presence of the EGF receptor is probably not obligatory for *neu* protein function since the oncogenic allele of the rat *neu* gene transforms the NR6 variant of Swiss 3T3 cells, which lacks functional EGF receptors (M.-C.Hung and C.Bargmann, personal communication). However, this does not mean that the EGF receptor and p185 are incapable of forming a complex when both are present.

The specificity and potential regulatory role of p185 tyrosine phosphorylation in response to EGF indicate that p185 is likely to be a physiologically significant substrate of the EGF receptor. The interaction between the EGF receptor and p185 is unique in representing a circuit in which one receptor appears to be directly phosphorylated by another *in vivo* and is the first example of a tyrosine kinase cascade in which both enzymes have been identified. Although we were unable to detect an effect of tyrosine phosphorylation on p185 kinase activity using the relatively crude immune complex assay, precedent in the insulin and EGF receptor systems suggests that the EGF-stimulated phosphorylation will turn out to play some regulatory role (Bertics and Gill, 1985; Rosen *et al.*, 1983; Yu and Czech, 1986). Kadowaki and co-workers reported that EGF, but not IGF-1 nor insulin, stimulates the rapid tyrosine phosphorylation of a 190 kd protein in KB cells (Kadowaki *et al.*, 1987). Our findings support their speculation that this 190 kd protein is p185.

Incubation of cells with EGF before lysis stimulated the cell-free tyrosine phosphorylation of p185 in immune complexes. This tyrosine phosphorylation was almost certainly a result of increased p185 kinase activity, although the remote possibility exists that the EGF-stimulated tyrosine kinase activity was actually the EGF receptor carried non-specifically through the immunoprecipitation. The modification of p185 kinase activity induced by EGF treatment survived mild detergent solubilization and incubation for 2 h, and may, therefore, result from a covalent alteration such as phosphorylation. Since the increased kinase activity does not correlate with tyrosine phosphorylation of p185 it seems probable that one of the EGF-stimulated serine or threonine phosphorylations is responsible for this increased enzyme activity. Stimulation of a tyrosine kinase by serine or threonine phosphorylation is an unusual, but not unprecedented form of regulation, since serine phosphorylation of v-*src* may stimulate its kinase activity (Roth *et al.*, 1983).

We initially observed the EGF-stimulated phosphorylation of p185 in experiments designed to identify the ligand for p185. These experiments, in which the phosphoamino acid content of metabolically-labeled p185 was analyzed, were laborious, required the use of substantial amounts of $^{32}P_i$, and yielded only a poor signal-to-noise ratio. The immunoblotting assay described here is significantly more rapid and is sensitive enough to detect sub-nanomolar concentrations of EGF. Recent studies have shown that *neu* is frequently amplified in human adenocarcinoma, particularly breast carcinoma (King *et al.*, 1985; Kraus *et al.*, 1987; Yokota *et al.*, 1986; Slamon *et al.*, 1987; Venter *et al.*, 1987). These findings provide a strong impetus for identifying the p185 ligand: if autocrine stimulation through p185 is involved in these diseases, antagonists to ligand binding would have therapeutic potential. The Western blot assay described here should prove to be a powerful tool in the search for the p185 ligand.

Materials and methods

Cells

Cells were grown in Dulbecco–Vogt modified Eagle medium (DMEM) supplemented with 10% calf serum (CS) under an atmosphere of 5% CO_2 at 37°C. Rat-1 cells are a continuous rat fibroblast line; B104-1-1 cells are NIH-3T3 transformed by transfection with a *neu* oncogene originally derived from B104 cells (Padhy *et al.*, 1982).

Growth factors

Receptor-grade mouse EGF was obtained from Collaborative Research, Inc. PDGF was from A.R.Frackelton, Jr, (Roger Williams Hospital) and L.T.Williams (University of California at San Francisco), TGF-α was from Rik Derynck (Genentech, Inc.), insulin was from Sigma Chemical Co., and pituitary fibroblast growth factor was from D.Gospodarowicz (University of California at San Francisco).

Antibodies

Monoclonal antibody 7.16.4, which recognizes rat p185, (Drebin *et al.*, 1984) was a gift from Jeff Drebin and Mark Greene (University of Pennsylvania Medical School). Polyclonal anti-human EGF receptor antiserum (Decker, 1984) was obtained from Stuart Decker (Rockefeller University). Anti-phosphotyrosine antibodies were prepared as described (Kamps and Sefton, 1988).

Phosphoamino acid analysis

Dense cultures of Rat-1 cells in 60 mm dishes were washed once in serum-free DMEM and incubated overnight in DMEM–0.1% CS. The next day cultures were washed once with phosphate-free DMEM and incubated for 4 h in 1.0 ml phosphate-free DMEM containing 1.0 mCi carrier-free $^{32}P_i$ (New England Nuclear). The medium was replaced with 0.1 ml of DMEM–0.1% CS containing 3.3 μg EGF and cultures were incubated for an additional 5 min at 37°C. Cells were washed with ice-cold PBS, lysed in phosphate-buffered RIPA containing 1 mM ATP, 2 mM EDTA, 20 mM sodium fluoride and 100 μM sodium orthovanadate, and p185 was immunoprecipitated using antibody 7.16.4 as described (Stern *et al.*, 1986) p185 was purified by gel electrophoresis, eluted, and phosphoamino acids analyzed as before (Stern *et al.*, 1986).

Immunoblotting

Total cell protein extracts were prepared by lysing cells in hot electrophoresis sample buffer as described (Kamps and Sefton, 1988). For experiments in which immunoprecipitates were analyzed by immunoblotting, dense cultures of Rat-1 cells in 35 or 60 mm dishes were incubated for 6-8 min at 37°C in DMEM–0.1% CS containing growth factors, then washed twice in PBS, and lysed in phosphate-buffered RIPA containing 20 mM sodium fluoride and 1 mM sodium orthovanadate (Kamps and Sefton, 1988; Stern *et al.*,

1986). Immunoprecipitates were prepared exactly as described (Stern *et al.*, 1986) except that the immune complexes were not centrifuged through a sucrose cushion, and were washed once in RIPA−fluoride−vanadate and once in PBS. Proteins were resolved by electrophoresis in 7.5% acrylamide−0.17% bis-acrylamide Laemmli gels. The gels were washed twice for 5 min in water, twice in transfer buffer containing SDS and sodium orthovanadate, and proteins transferred to nitrocellulose by electrophoresis for 2 h at 50 V, 400−600 mA in the cold. The transfer buffer and procedures for blocking and washing the filter have been described (Kamps and Sefton, 1988). Blots were developed by incubation in blocking buffer containing 1 μCi/ml ^{125}I Protein A (ICN, ~35 μCi/μg).

Acknowledgements

We thank Jeff Drebin, Mark Greene, and Stuart Decker for anti-sera, and Ray Frackelton, Rik Derynck and D.Gospodarowicz for growth factors. Maria Andrews provided excellent technical assistance. We thank Carolyn Machamer, Debby Brown and Bart Sefton for criticism of the manuscript. This work was supported by Public Health Service Grants CA14195, CA17289, T32CA09370 and CA45708-01, and BRSG Grant RR 05358 from the National Institutes of Health, and by the Argall L. and Anna G.Hull Fund.

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Received on October 27, 1987; Revised on January 12, 1988