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The epidermal growth factor receptor and the product of the *neu* protooncogene are members of a receptor tyrosine phosphorylation cascade

(oncogene/tyrosine kinase/*erbB2*)

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ABSTRACT **The protein product of the *neu* protooncogene, p185, is a tyrosine kinase with a high degree of sequence homology to the epidermal growth factor (EGF) receptor. Although p185 does not bind EGF, EGF stimulates tyrosine phosphorylation of p185. To determine the mechanism of this interaction we have used a vaccinia virus/bacteriophage T7-based transient gene expression system to induce production of normal and kinase-deficient forms of p185 in the absence and presence of EGF receptors. Tyrosine phosphorylation of kinase-deficient p185 was observed, but only in the presence of the EGF receptor. These findings strongly support the hypothesis that p185 is a substrate for the EGF receptor tyrosine kinase in a tyrosine kinase cascade.**

The product of the *neu* protooncogene (also called *erbB2* and HER2) is a recently identified member of the growth factor receptor family of protein-tyrosine kinases (1). It is closely related to, but distinct from, the epidermal growth factor (EGF) receptor (2–4). Although it is clear that the product of the *neu* gene, p185, does not bind EGF (3, 4), a physiological ligand for p185 has not been identified. The oncogenic potential of the rat *neu* oncogene product, p185*, is activated by a point mutation that substitutes a glutamic acid for valine at position 664 within the transmembrane domain (5). This mutation confers increased tyrosine kinase activity on p185 and thus implicates activation of the kinase in generation of the transformed phenotype (6, 7). Since amplification of the *neu* protooncogene is frequently found in breast and ovarian adenocarcinoma in humans and may correlate with severity of these diseases (8), the *neu* protein is an important target for study both as a prototype tyrosine kinase receptor and as a clinically important protein.

In efforts to identify the ligand for the *neu* protein, it was discovered that EGF, which does not bind to p185, stimulates tyrosine phosphorylation of p185 (3, 9). This result was subsequently confirmed and extended to other cell systems (10–12). Furthermore, incubation of cells with EGF increases the amount of p185-associated tyrosine kinase activity recovered in immune complexes, suggesting that EGF is able to regulate p185 (9). EGF might stimulate tyrosine phosphorylation of p185 through two different mechanisms: p185 could itself be a substrate for the EGF receptor kinase (or another EGF-stimulated tyrosine kinase); alternatively, the intrinsic p185 kinase activity could be activated by EGF (through other phosphorylations induced by EGF, or through formation of p185/EGF receptor heterodimers), resulting in greater p185 autophosphorylation. To distinguish between these possibilities we have expressed kinase-defective mutants of p185 in the absence or presence of the EGF receptor.

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MATERIALS AND METHODS

Antibodies 7.16.4 (13) and H4 are mouse anti-rat p185 monoclonal antibodies and were obtained from M. Greene (Univ. of Pennsylvania Medical School) and Y. Yarden (Weizmann Institute), respectively. Antibody 528 is a monoclonal anti-human EGF receptor antibody (14) that was obtained from H. Masui (Rockefeller University). Rabbit polyclonal anti-phosphotyrosine raised against poly(phosphotyrosine, glycine, alanine) was obtained from M. P. Kamps and B. M. Sefton (Salk Institute) or prepared as described (15).

The plasmid pXER, which carries a human EGF receptor cDNA, was obtained from M. G. Rosenfeld (Univ. of California at San Diego Medical School). pKS−/XER was produced by subcloning an *Xba* I–*Sal* I fragment from pXER containing the cDNA and a simian virus 40 polyadenylylation site into the polylinker of pBluescript KS(−) (Stratagene) so that a sense transcript would be produced from the bacteriophage T7 promoter. Plasmid AR/neuN was produced by subcloning a 4.1-kilobase *Nco* I fragment containing coding sequences from a rat *neu* cDNA clone (2) into the *Xho* I site of pARX (16), a plasmid derived from AR2529 (PET3). In the resulting plasmid, p185 coding sequences are under regulatory control of the bacteriophage T7 promoter.

Oligonucleotide-directed mutagenesis was used to change the codon for Lys-758 of p185 in AR/neuN to a codon for alanine. The *Nde* I–*Bgl* I fragment containing the mutated sequence was used to replace the normal sequence in AR/neuN, creating the plasmid AR/neuA758.

NR6 cells are a clone of Swiss mouse 3T3 fibroblasts selected for resistance to EGF (17). Stable cell lines NR-neuB8 and NR-neuN were produced by infection of NR6 cells with retrovirus vectors carrying the B8 mutant and wild-type *neu* cDNAs, respectively (18), and selection of G418-resistant cells.

Infections of the recombinant vaccinia virus vTF7-3 were performed as described (16) except that incubations were at 37°C. Lipofectin (Bethesda Research Laboratories Life Technologies) was used for liposome-mediated gene transfer. Cultures were incubated overnight before harvest for immunoprecipitations.

Procedures for metabolic labeling with [^{35}S]cysteine and $^{32}P_i$, immunoprecipitation, gel electrophoresis, and immunoblotting with anti-phosphotyrosine have been described (3, 9).

RESULTS

Coexpression of normal or mutant p185 and the EGF receptor was achieved using three different experimental proto-

Abbreviation: EGF, epidermal growth factor.

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cols. In the experiments in Fig. 1, stable cell lines were initially produced (by use of retrovirus vectors) that express normal or mutant *neu*, and the EGF receptor gene was subsequently introduced by a vaccinia virus-based transient-expression system. In the second series of experiments (Fig. 2), *neu* and EGF receptor genes were coexpressed by use of the transient system. Finally, in the third series of experiments (Fig. 3), the *neu* gene was introduced into cells that express endogenous EGF receptor. By using these systems we have produced all combinations of endogenous and transiently expressed *neu* and EGF receptor genes [both endogenous genes are expressed in Rat-1 fibroblasts (3)].

The first p185 mutant we used, neuB8-719, was a linker insertion at position 719 initially constructed and characterized by C. I. Bargmann (18). The mutant protein has no detectable kinase activity in immune complex assays, and when the insertion mutation is placed in cis with the activating glutamic acid at position 664, it does not induce cell transformation (18). We used the NR6 cell line as a recipient because NR6 cells do not produce the EGF receptor (17). We were unable to conclusively detect expression of p185 in NR6 cells (D.F.S., unpublished data); if NR6 cells express p185, they do so at levels considerably lower than mouse dermal fibroblasts.

NR6 cells were infected with retrovirus vectors encoding p185 (18). Stable infected cell lines NR-neuB8 and NR-neuN produced similar amounts of mutant and wild-type p185, respectively, as demonstrated by labeling with [^{35}S]cysteine and immunoprecipitation with anti-p185 antibody (Fig. 1*A*, lanes f and h). Previous work showed that the phosphotyrosine content of immunoprecipitated p185 correlates well with reactivity in immunoblots with anti-phosphotyrosine (9). Immunoprecipitation with anti-p185 followed by immunoblotting with anti-phosphotyrosine demonstrated that normal p185 (Fig. 1*A*, lane l) but not the kinase-deficient B8 mutant (lane j) contains basal phosphotyrosine. This suggested that the basal phosphotyrosine is contributed by autophosphorylation. When the cell lines were treated with EGF before preparation of immunoprecipitates, there was no stimulation of tyrosine phosphorylation of either wild-type or B8 mutant p185 (lanes m and k). Thus EGF does not stimulate tyrosine phosphorylation of p185 in the absence of the EGF receptor.

To determine whether production of a functional EGF receptor by these same cell lines would permit tyrosine phosphorylation of the mutant p185, we used a transient-expression vector encoding the EGF receptor. The plasmid KS-/XER contains human EGF receptor coding sequences under control of the bacteriophage T7 RNA polymerase promoter. To obtain expression of the receptor, cells transiently transfected with this clone were simultaneously infected with the recombinant vaccinia virus vTF7-3, which encodes T7 RNA polymerase (16, 19, 20). Transient expression of this vector system in NR6, NR-neuB8, and NR-neuN cells induced production of the human EGF receptor (Fig. 1*B*, lanes h, j, and l). In this experiment, transfected cells were incubated with EGF prior to lysis for immunoprecipitation with either anti-p185 antibody or anti-EGF receptor antibody (14) and the proteins were visualized by immunoblotting with anti-phosphotyrosine. Expression of the EGF receptor in NR-neuB8 cells induced tyrosine phosphorylation of the B8 mutant p185 (Fig. 1*B*, compare lanes c and d). This finding strongly supported the idea that p185 is itself a substrate for the EGF receptor kinase. The small stimulation of phosphorylation of normal p185 in NR6-neuN cells expressing the EGF receptor (Fig. 1*B*, lanes e and f) provided additional evidence for transphosphorylation.

Since the B8 linker insertion lies upstream from the putative ATP binding site of p185 (18), the mechanism of kinase inhibition by this mutation is uncertain. To confirm the

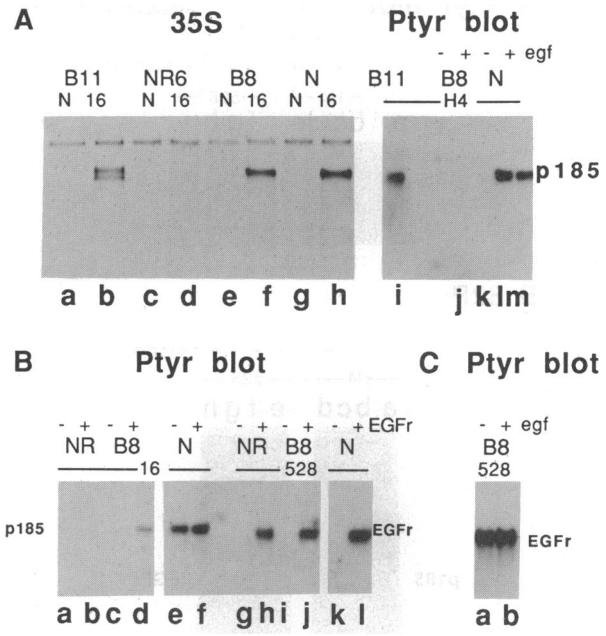

FIG. 1. (*A Left*) Expression of p185. Subconfluent cultures in 100-mm dishes were labeled metabolically with [^{35}S]cysteine for 16 hr. Cells were lysed and p185 was immunoprecipitated. Proteins were resolved by SDS/polyacrylamide gel electrophoresis (7.5% acrylamide/0.17% *N,N'*-methylenebisacrylamide gel) and the fluorographed gel was exposed to preflashed film to visualize p185. Lanes a, c, e, and g, immunoprecipitates prepared with normal mouse serum. Lanes b, d, f, and h, immunoprecipitates prepared with anti-p185 antibody 7.16.4. Precipitates were prepared from B104-1-1 cells (lanes a and b), which expresses transforming p185 (3); NR6 cells (lanes c and d); NR6-neuB8 cells (lanes e and f); and NR6-neuN cells (lanes g and h). (*A Right*) Effect of EGF on tyrosine phosphorylation of p185. Immunoprecipitates were prepared from nonlabeled cells by using monoclonal anti-p185 antibody H4, resolved by gel electrophoresis, transferred to nitrocellulose, and probed with polyclonal anti-phosphotyrosine (Ptyr) antibody. Immune complexes were detected using ^{125}I-labeled protein A (≈35 μCi/μg; ICN; 1 μCi = 37 kBq); lane i, B104-1-1 cells; lanes j and k, NR6-neuB8 cells; lanes l and m, NR6-neuN cells. Cells were mock-treated (lanes j and l) or incubated with 100 ng of EGF per ml (lanes k and m) for 10 min prior to lysis. (*B*) Effect of EGF receptor expression on phosphotyrosine content of p185. Cultures of NR6 (lanes a, b, g, and h), NR6-neuB8 (lanes c, d, i, and j), or NR6-neuN (lanes e, f, k, and l) cells on 35-mm plates were infected with recombinant vaccinia vTF7-3, which encodes T7 RNA polymerase. Plasmid KS−/XER was introduced by liposome-mediated gene transfer into cultures for lanes b, d, f, h, j, and l. All cultures were incubated with EGF (100 ng/ml) for 10 min prior to lysis. Lysates were divided into equal portions which were immunoprecipitated with anti-p185 antibody 7.16.4 (lanes a–f) or anti-human EGF receptor (EGFr) antibody 528 (lanes g–l). Precipitates were analyzed by gel electrophoresis and immunoblotting with anti-phosphotyrosine. All of the lanes in this composite were derived from a single original gel. (*C*) Effect of EGF on the phosphotyrosine content of the EGF receptor expressed in vaccinia-infected cells. Plasmid KS−/XER was expressed in vTF7-3-infected NR6-neuB8 cells as in *B*. Immunoprecipitates with antibody 528 were prepared from mock-treated cells (lane a) or EGF-treated (100 ng/ml; 10 min) cells (lane b). Phosphotyrosine was detected by immunoblotting.

results obtained with B8, we constructed a mutation that substitutes alanine for Lys-758, which is homologous to Lys-721 of the EGF receptor and similarly located lysines in other protein kinases that participate in ATP binding and are required for kinase activity (21–23). A T7 transient-expression vector, AR/neuA758, was constructed to permit expression of the mutant p185 in NR6 cells (Fig. 2*A*). Production of the appropriate protein was confirmed by immunoprecipitation of cells labeled metabolically with $^{32}P_i$

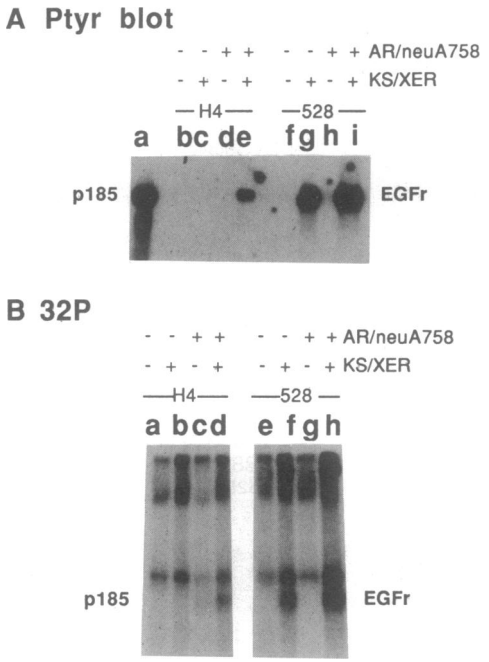

FIG. 2. Tyrosine phosphorylation of kinase-deficient p185 in the absence and presence of the EGF receptor. Cultures of NR6 cells in 60-mm dishes were infected with vTF7-3, and plasmids AR/neuA758 and/or KS−/XER were introduced by liposome-mediated gene transfer. After incubation for 3 hr at 37°C the medium was replaced with Dulbecco's modified Eagle's medium (DMEM) containing 10% calf serum (*A*) or phosphate-free DMEM containing 4% calf serum and $^{32}P_i$ (400 μCi/ml) (*B*) and the cultures were incubated overnight at 37°C. All cultures were incubated with EGF (100 ng/ml) for 10 min prior to lysis for immunoprecipitation. (*A*) Lysates from nonlabeled cultures were divided into two portions for immunoprecipitation with anti-p185 antibody H4 (lanes b–e) or anti-human EGF receptor antibody 528 (lanes f–i) and phosphotyrosine (Ptyr) was detected by immunoblotting. The filter was exposed for 1 day with an intensifying screen. Lane a, total protein lysate prepared from DHFR/G-8 cells, which express high levels of normal p185; the dense band is p185. Immunoprecipitates were prepared from cells transfected with no DNA (lanes b and f), KS−/XER (lanes c and g), AR/neuA758 (lanes d and h), or both plasmids (lanes e and i). (*B*) Lysates from ^{32}P-labeled cells were divided into two portions for immunoprecipitation with antibody H4 (lanes a–d) or antibody 528 (lanes e–h). The two portions of this composite are from the same original gel. The autoradiograph was exposed for 1 day with an intensifying screen. Immunoprecipitates were prepared from cells transfected with no DNA (lanes a and e), KS−/XER (lanes b and f), AR/neuA758 (lanes c and g), or both plasmids (lanes d and h).

(Fig. 2*B*, lanes c and d). This protein lacks kinase activity in immune complexes (P.A.C., unpublished data). The greater labeling of background proteins, the EGF receptor, and p185 in lysates from cells expressing the EGF receptor (Fig. 2*B*, lanes b, d, f, and h) is probably due to the activation of serine and threonine kinases by the vaccinia virus growth factor-activated EGF receptor (see below). The mutant p185 lacked phosphotyrosine when produced in the absence of the EGF receptor (Fig. 2*A*, lane d), confirming its lack of kinase activity, but contained phosphotyrosine when expressed in the presence of the EGF receptor (Fig. 2*A*, lane e). Thus tyrosine phosphorylation of A758 p185 was dependent on coexpression of the EGF receptor (Fig. 2*A*, lane i).

In a final experiment, we used transient expression of AR/neuA758 to determine whether an endogenous EGF receptor would phosphorylate the defective p185. Human HeLa cells contain EGF receptors that can be easily visualized by immunoblotting with anti-phosphotyrosine after EGF treatment (Fig. 3, lane b). In mock-infected cells the presence of phosphotyrosine in the HeLa EGF receptor was absolutely

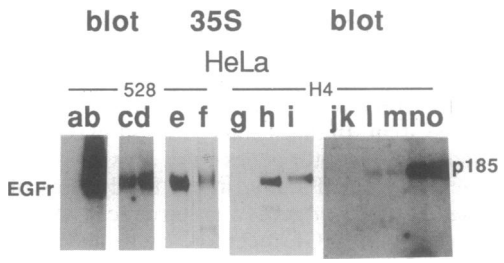

FIG. 3. Effect of vaccinia infection on HeLa EGF receptor (EGFr) activation and expression. Lanes a–d: HeLa cells were mock-infected (lanes a and b) or vTF7-3-infected (lanes c and d), mock-treated (lanes a and c) or EGF-treated (lanes b and d; 100 ng/ml, 10 min), and immunoprecipitated with anti-EGF receptor antibody 528; tyrosine phosphorylation was detected by immunoblotting with anti-phosphotyrosine. Lanes e and f: HeLa cells were mock-infected (lane e) or vTF7-3-infected and transfected with ARneuA758 (lane f), labeled metabolically with [^{35}S]cysteine overnight, and immunoprecipitated with antibody 528; radiolabeled proteins were detected by fluorography of the gel; these two lanes were derived from nonadjacent portions of a single gel. Lanes g–i: HeLa cells were infected with vTF7-3 and mock-transfected (lane g) or transfected with ARneuA758 (lane h) or ARneuN (lane i), labeled metabolically overnight with [^{35}S]cysteine, and immunoprecipitated with anti-p185 antibody H4; proteins were detected by fluorography. Lanes j–o: HeLa cells were infected with vTF7-3 and transfected with ARneuA758, (lanes l and m) or ARneuN (lanes n and o); cultures were mock-treated (lanes j, l, and n) or EGF-treated (lanes k, m, and o; 100 ng/ml; 10 min) and immunoprecipitated with anti-p185 antibody H4; phosphotyrosine was detected by immunoblotting.

dependent on the addition of EGF (Fig. 3, compare lanes a and b). However, in vaccinia-infected cells, a significant amount of phosphotyrosine was detected in the receptor without addition of EGF (Fig. 3, compare lanes c and d). Similarly, the tyrosine phosphorylation of the EGF receptor expressed in B8 cells was insensitive to the presence of EGF (Fig. 1*C*, lanes a and b). The vaccinia-induced EGF receptor phosphorylation is probably caused by production of vaccinia-encoded growth factor, which binds to the EGF receptor (24, 25). ^{35}S-labeling experiments showed that infection with vaccinia resulted in a lower steady-state level of EGF receptors (Fig. 3, lanes e and f), consistent with ligand-mediated down-regulation of the receptor.

Transient transfection of AR/neuA758 and AR/neuN into HeLa cells induced production of approximately equal levels of p185 (Fig. 3, lanes h and i). The protein encoded by AR/neuN contained substantial amounts of phosphotyrosine (Fig. 3, lanes n and o), consistent with the results obtained in stably transfected NR-neuN cells (Fig. 1). However, in contrast to the result obtained in transfected NR6 cells (Fig. 2*A*, lane d), the neuA758 protein did contain phosphotyrosine when expressed in HeLa cells, presumably as a result of the activity of the endogenous HeLa EGF receptor, although it is conceivable that an endogenous human p185 participated in the response. The phosphorylation was not stimulated by EGF (Fig. 3, lanes l and m), since the EGF receptor was already stimulated by vaccinia infection (lanes c and d).

DISCUSSION

Tyrosine phosphorylation of two distinct tyrosine kinase-deficient mutants of p185 was dependent on the expression of an active EGF receptor kinase. These experiments prove that the *neu* protein, p185, can be a substrate for the EGF receptor (or another EGF-activated tyrosine kinase) and therefore demonstrate the existence of a two-receptor tyrosine kinase cascade. It is possible that endogenous NR6 cell p185, expressed at levels undetectable in our assays, was activated by the EGF receptor and in turn phosphorylated the kinase-defective p185. This seems unlikely, not only because we

cannot detect an endogenous p185, but also because we have not observed tyrosine phosphorylation of the kinase-defective p185 in the absence of the EGF receptor.

Since these results show that p185, a tyrosine kinase, is itself a substrate for the EGF receptor kinase, they suggest that these two proteins are components in a tyrosine kinase cascade. Strictly speaking, such an interpretation requires that the tyrosine phosphorylation of p185 stimulates its intrinsic kinase activity. Since tyrosine phosphorylation of the EGF receptor weakly stimulates its activity (26, 27), the close homology of p185 and the EGF receptor suggests that tyrosine phosphorylation of p185 will stimulate p185, provided that the phosphorylation occurs on homologous sites. However, previous work failed to demonstrate a tyrosine phosphorylation-dependent stimulation of p185 by EGF (9). At least two other functions of tyrosine kinases, receptor down-regulation and binding of phosphatidylinositol-3-kinase, can require tyrosine phosphorylation (28–30). The latter result is especially interesting because it would provide a means for EGF to alter the substrate specificity of p185.

These experiments do not rule out the possibility that EGF also regulates the intrinsic kinase activity of p185 through other mechanisms. There is evidence for a tyrosine phosphorylation-independent regulation of p185 kinase activity by EGF (9). Thus the regulatory interplay between p185 and the EGF receptor may be quite complex.

Similar regulatory cascades are likely to be used by other tyrosine kinase receptors. No such interaction has been found between different receptors, but it is known that solubilized EGF receptor molecules can phosphorylate one another (31).

It has been reported that overexpression of the EGF receptor and p185, but not either separately, leads to transformation and tumorigenicity of cells (32). This demonstrates the physiological significance of the interaction and suggests that elucidation of the networks involved will have broad application in the study of the control of cellular proliferation and cancer.

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