Increased Oncogenic Potential of ErbB Is Associated with the Loss of a COOH-terminal Domain Serine Phosphorylation Site*

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Steven J. Theroux§§, Cherie Taglienti-Sian¶, Nandini Nair¶, Janice L. Countaway**, Harriet L. Robinson¶, and Roger J. Davis§§‡

From the §Howard Hughes Medical Institute, **Program in Molecular Medicine, ¶Department of Biochemistry and Molecular Biology, and ¶Department of Pathology, University of Massachusetts Medical School, Worcester, Massachusetts 01605

The erbB oncogene encodes an altered form of the epidermal growth factor (EGF) receptor that lacks the extracellular ligand binding domain. This oncogene is exclusively leukemogenic. However, an increase in oncogenic potential and a broadening of the tissue specificity of tumor formation occurs after retroviral transduction of erbB. The increased oncogenic potential correlates with structural alterations within the erbB gene. One common event is the deletion of a serine phosphorylation site located within the COOH-terminal domain. This site of phosphorylation has been demonstrated to be required for EGF-induced desensitization of signaling by the EGF receptor (Countaway, J. L., Nairn, A. C., and Davis, R. J. (1992) J. Biol. Chem. 267, 11289–1140). Here we show that the mutation of erbB at this negative regulatory site causes fibroblast transformation in vitro and is associated with an increased oncogenic potential in vivo.

Retroviruses can cause erythroleukemia after a long latent period by inserting within the gene that encodes the epidermal growth factor (EGF)* receptor (1). This insertion results in the expression of a truncated EGF receptor with constitutive protein-tyrosine kinase activity (1). The insertionally activated erbB oncogene stimulates the self-renewal of erythroid progenitor cells and is found to be exclusively leukemogenic (1–4). In contrast, acute transforming viruses that carry the erbB oncogene are associated with an increased oncogenic potential of ErbB.

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‡‡ To whom correspondence should be addressed: Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation St., Worcester, MA 01605.

The abbreviations used are: EGF, epidermal growth factor; CEF, chicken embryo fibroblasts; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazinethesulfonic acid.

be attributed to structural alterations in the ErbB protein (5–10). Deletion of residues within the COOH-terminal domain correlates with the increased oncogenic potential of ErbB (5–8). This domain is an important effector region of ErbB that contains all the sites of tyrosine autophosphorylation and binds to Src homology regions of signaling molecules (11, 12). It is therefore likely that the mutation of this protein domain may be significant for the expansion of the disease potential of ErbB.

Comparison of the COOH-terminal domain deletions present within a series of viruses that exhibit an expanded disease spectrum indicates that the deletion of a serine phosphorylation site is a common event (Ser477 and Ser100 of the chicken EGF receptor; Ser477 and Ser477 (Ser477) of the truncated erbB sequence) (1, 5–8). Recently, it has been demonstrated that the phosphorylation of the EGF receptor at this site accounts for the desensitization of EGF receptor signal transduction observed in EGF-treated cells (13). Phosphorylation of the ErbB protein at Ser477 therefore represents a potential mechanism of negative regulation of ErbB function.

The purpose of the present experiments was to test the hypothesis that the Ser477 phosphorylation site functions as a negative regulatory element that influences the oncogenic potential of ErbB. The experimental approach that we employed was to replace Ser477 with Ala or Glu residues and to investigate the effect of this mutation on transformation caused by ErbB. We report that the mutation of the erbB phosphorylation site Ser477 causes fibroblast transformation in vitro and is associated with an increase in the oncogenic potential of ErbB in vivo.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The retroviral expression vector REBC containing a wild-type (insertionally activated) erbB gene has been described previously (2). Site-directed mutagenesis was performed using a procedure employing the polymerase chain reaction (14). The structure of the mutated erbB genes was confirmed by dideoxynucleotide sequencing.

Tissue Culture—Chicken embryo fibroblasts (CEF) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum and 2% chicken serum (GIBCO/BRL). NR6–93 fibroblasts were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum (GIBCO/BRL). The cells were co-transfected with the wild-type (Ser477) or the mutated (Ala477) and GIA (Ser477) erbB expression vector (REBC) together with pRSVNeo and selected for resistance to Geneticin (GIBCO/BRL) as described (15). The growth of cells in soft agar was investigated using a procedure described previously (15).

Protein-tyrosine Kinase Assays—Protein-tyrosine kinase assays were performed using the exogenous protein substrate TK6 (16). CEF cells from a confluent 100-mm plate were lysed in 1 ml of 50 mM Hepes pH 7.4, 0.5 mM CaCl2, 5 mM EGTA, 10% glycerol, 1% Triton X-100, 200 mM sodium orthovanadate, 50 mM NaF, and 10 μg/ml leupeptin. Cell extracts were centrifuged at 100,000 g for 20 min at 4 °C, and the supernatant was incubated at 4 °C for 90 min with 10 μl of rabbit anti-ErbB antiserum (NN1) prebound to 20 μl of protein A-Sepharose. The immune precipitate was washed two times with lysis buffer and two times with HNTG (50 mM Hepes pH 7.4, 150 mM NaCl, 0.1% Triton X-100, 10% glycerol, 50 mM NaF, 200 mM sodium orthovanadate). Kinase assays were performed using 40 μl of HNTG supplemented with 15 mM MnCl2, 1 mM diithiothreitol, 5 μl of TK6, and 5 μl of [γ-32P]ATP (20 μCi/nmol). The effect of different concentrations of ATP (5–100 μM) and TK6 (0.74–5.6 μM) was investigated. Control experiments demonstrated that the time course of TK6 phosphorylation was linear for 30 min. To determine the
initial rate of phosphorylation, the reactions were terminated after 15 min of incubation at 22 °C. The phosphorylated TK6 protein was resolved by polyacrylamide gel electrophoresis and the incorporation of [32P]phosphate was determined using a Phosphorimager and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA). The relative levels of ErbB protein were determined by Western blot analysis using the monoclonal anti-ErbB antibody 20.3.6 (provided by Dr. M. Hayman, SUNY). Kinetic constants of TK6 phosphorylation (per assay) were calculated by fitting to the Michaelis-Menten formalism.

To determine the state of tyrosine phosphorylation of the ErbB proteins in situ, CEF cells expressing [Ser477/8] ErbB, [Ala477/8] ErbB, and [Glu477/8] erbB were cultured in 100-mm dishes. The ErbB proteins were isolated by immunoprecipitation from detergent extracts of the cells as described above. The immunoprecipitates were subjected to polyacrylamide gel electrophoresis and then transferred to a polyvinylidene difluoride membrane (Immobilon-F, Millipore Corp.). The blots were probed using: 1) a monoclonal mouse antiphosphotyrosine antibody (PY20, ICN Biomedicals Inc.) and 125I-goat anti-mouse Ig (Du Pont-New England Nuclear); and 2) a monoclonal rat anti-ErbB antibody (20.3.6 from Dr. M. Hayman), a rabbit anti-rat Ig secondary antibody, and 125I-protein A (Du Pont-New England Nuclear). The blots were analyzed using a Phosphorimager and ImageQuant software (Molecular Dynamics Inc., Sunnyvale, CA), and the relative levels of ErbB tyrosine phosphorylation were calculated.

Oncogenic Potential of ErbB—Oncogenicity tests were performed by direct injection of the ErbB expression vectors as plasmid DNA (17). One-day-old Spafas chicks were injected with 100 μg of plasmid DNA dissolved in 200 μl of saline at each of three sites: 1) intramuscularly in the wing web; 2) intraperitoneally; and 3) intravenously.

FIG. 2. Effect of ErbB proteins on anchorage-independent growth of CEF in soft agar. CEF cells (vector control) and CEF cells expressing ErbB proteins were suspended in soft agar. The colony morphology (panel A) and cloning efficiency (panel B) were determined after 21 days of growth.

RESULTS AND DISCUSSION

The ErbB phosphorylation site Ser477/8 was mutated by replacing the Ser residues with other amino acids. Two mutant constructs were prepared: 1) a phosphorylation-defective ErbB protein (Ala477/8); and 2) an ErbB protein with constitutive negative charge at the phosphorylation site (Glu477/8). The structure of the ErbB proteins created is illustrated in Fig. 1. In initial experiments, the ErbB proteins were expressed in CEF. Western blot analysis indicated that the level of expression of each of the three ErbB proteins in CEF was similar (data not shown). The transformed phenotype of the CEF was examined by investigating the anchorage-independent growth of the cells in soft agar. A low cloning efficiency was found for cells expressing the wild-type [Ser477/8] ErbB protein, and the colonies observed were very small (Fig. 2). These data are consistent with previous reports that the wild-type ErbB protein does not cause significant fibroblast transdifferentiation (2). In contrast, cells expressing the mutated [Ala477/8] ErbB protein exhibited a marked increase in soft agar cloning efficiency and formed large colonies (Fig. 2). An increase in the anchorage-independent growth of CEF expressing the [Glu477/8] ErbB protein was also observed (Fig. 2). Together, these data indicate that activation of the in vitro transformation of CEF by ErbB is caused by mutation of the phosphorylation site Ser477/8. Thus, phosphorylation at Ser477/8 may suppress the ability of the wild-type ErbB protein to cause transformation of CEF. However, negative charge (Glu) is not able to functionally substitute for this phosphorylation site.

The experiments using CEF cells (Fig. 2) indicate that in vitro fibroblast transformation can result from the mutation of ErbB at the phosphorylation site Ser477/8. To confirm this conclusion, further experiments were performed using murine NR6 3T3 fibroblasts (which do not express EGF receptors). Fig. 3 shows that these 3T3 fibroblasts are contact-inhibited in monolayer culture and do not grow in soft agar. Expression of the wild-type ErbB protein in the 3T3 cells caused no significant change in the growth properties of the cells (Fig. 3). Modest changes in the growth of the 3T3 cells were observed after expression of the [Glu477/8] ErbB protein (Fig. 3). However, 3T3 fibroblasts expressing the [Ala477/8] ErbB protein were found to exhibit a transformed phenotype characterized by the formation of numerous large colonies in soft agar and the lack of contact growth inhibition in monolayer culture (Fig. 3).

The observation that cultured fibroblasts are transformed by ErbB mutated at Ser477/8 (Figs. 2 and 3) suggests that this
proteins expressed in CEF cells were compared using an phosphorilation. It was observed that the mutation of the type and mutated ErbB proteins on fibroblast transformation web tumors was detected in birds injected with the wild-type expression vectors caused a high incidence of wing web tumors after 21 days of growth of the fibroblasts was determined after 21 days of growth in soft agar.

mutation may increase the oncogenic potential of ErbB. To test this hypothesis, we examined whether the mutation of ErbB at Ser477/8 was associated with increased tumor formation in chickens. Direct injection of the plasmid DNA expression vectors into 1-day-old chicks caused a 100% incidence of erythroblastosis (data not shown). A low incidence of wing web tumors was detected in birds injected with the wild-type (Ser477/8) erbB expression vector (Fig. 1). In contrast, injection of the birds with the mutated (Ala477/8 and Glu477/8) erbB expression vectors caused a high incidence of wing web tumors after 18–42 days (Fig. 1). This increased formation of neoplasms demonstrates that mutation of the phosphorylation site Ser477/8 is associated with an increase in the ErbB disease potential in vivo.

To investigate the basis for the different effects of wild-type and mutated ErbB proteins on fibroblast transformation (Figs. 2 and 3), the protein-tyrosine kinase activities of these proteins expressed in CEF cells were compared using an in vitro assay employing an exogenous substrate for tyrosine phosphorylation. It was observed that the mutation of the Ser477/8 phosphorylation site did not cause a significant increase in protein-tyrosine kinase activity (Table I). Similarly, no increase in the in situ tyrosine phosphorylation of the mutated ErbB proteins was found by Western blot analysis using a monoclonal anti-phosphotyrosine antibody (Fig. 4). Thus, the mutation of the ErbB phosphorylation site Ser477/8 causes an increase in oncogenic potential without increasing the apparent ErbB protein-tyrosine kinase activity. In previous studies, a correlation between increased kinase activity and oncogenic potential has been observed (9). However, several examples of ErbB with increased oncogenic potential that do not exhibit increased kinase activity have also been described (10), and the mutated (Ala477/8) ErbB appears to represent one example of this class of erbB oncogene.

The mutation of the Ser477/8 phosphorylation site causes transformation of both CEF and murine 3T3 fibroblasts in vitro and results in the formation of wing web tumors in vivo (Figs. 1–3). These observations are consistent with the hypothesis that the negative regulatory phosphorylation site Ser477/8 acts to suppress the transforming effects of the wild-type ErbB protein in fibroblasts. The weakly transforming properties of the [Glu477/8] ErbB protein indicate that negative charge may be insufficient to account for the effects of phosphorylation at Ser477/8 and suggest a specific functional requirement for phosphate at this site. Previous studies of the EGF receptor indicate that mutation of this negative regulatory serine phosphorylation site alters the subcellular localization (cell surface expression) and the interaction of the receptor with other cellular proteins (13). It is possible that these functional alterations may be significant for fibroblast transformation caused by ErbB (18).

We conclude that the oncogenic potential of ErbB is increased by the mutation of a negative regulatory site of phosphorylation, Ser477/8. The removal of a negative regulatory element has previously been implicated as a mechanism of oncogenic conversion of normal genes (19). In the present study we have extended this concept by demonstrating that the deletion of a negative regulatory phosphorylation site can cause an increase in the oncogenic potential.

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| Table 1 | Summary of kinetic rate constants for ErbB protein-tyrosine kinase activity |
|---------|---------------------------------------------|
| Protein | $K_m$ | $V_{max}$ |
| Vector  | 1.2 ± 0.5 | 7.3 ± 3.0 | 18 ± 5 |
| [Ser477/8] ErbB | 2.3 ± 0.7 | 2.7 ± 2.0 | 10 ± 4 |
| [Glu477/8] ErbB | 3.1 ± 0.8 | 1.5 ± 0.5 | 21 ± 3 |

Fig. 3. Effect of ErbB proteins on the growth of murine NR6 3T3 fibroblasts. Panel A, the morphology of confluent monolayer cultures of NR6 fibroblasts is presented; panel B, the cloning efficiency of the fibroblasts was determined after 21 days of growth in soft agar.

Fig. 4. Tyrosine phosphorylation of ErbB proteins in situ. Wild-type (Ser477/8) and mutated (Ala477/8, Glu477/8) ErbB proteins expressed in CEF were immunoprecipitated and analyzed by Western blotting using monoclonal antibodies specific for ErbB and phosphotyrosine. The relative state of ErbB tyrosine phosphorylation is presented.

Fig. 5. Tyrosine phosphorylation of ErbB proteins in CEF. Wild-type and mutated (Ala477/8) ErbB proteins expressed in CEF were immunoprecipitated and analyzed by Western blotting using monoclonal antibodies specific for ErbB and phosphotyrosine.
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