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C-Src Activation by ErbB2 Leads to Attachment-Independent Growth of Human Breast Epithelial Cells

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Nontumorigenic human mammary epithelial cells (184.A1 line) were stably transfected with ErbB2 or with Ha-Ras. Transformation with ErbB2, but not ras, resulted in a 5-6 fold increase in c-src activity without affecting c-src content of cells. Similar activation of c-src by ErbB2 was also observed in other non-tumorigenic mammary epithelial cells, including the human line MCF10A and the mouse line NMuMG. Activation of c-src appeared to be dependent on active ErbB2 tyrosine kinase, as the ErbB2 inhibitor tyrphostin AG 825 blocked the induction of c-src kinase activity, as well as the ability of transformed cells to grow on soft agar, but not plastic. The src-selective inhibitor PP1 effectively reduced c-src activity, as well as growth of ErbB2-transformed cells on soft agar, but not on plastic. These results indicate that activation of c-src is a consequence of ErbB2 kinase activity in human breast cancer cells overexpressing ErbB2, and that increased activity of c-src may be responsible for attachment-independent growth of the cells.

ErbB2 (neu) is overexpressed in 10-30% of human breast cancers (1). ErbB2 expression has been associated with aggressively growing tumors and poor prognosis (2, 3). Such effects are thought to be a result of ErbB2 activity, rather than correlatory effect, since ErbB2 expression in rodent mammary tissue by either in situ gene transfer or transgenic technology results in the development of mammary carcinoma at young ages (4-7). Furthermore, nontumorigenic human mammary epithelial cells can be tumorigenically transformed by expression of ErbB2 (8, 9).

The product of the ErbB2 gene is a tyrosine kinase in the same family as EGF receptor, but with no known ligand (10). A variety of signal transduction events mediated by ErbB2 have been described, including activation of ras-MAPK and PI_3 Kinase pathways (11, 12). In mice transgenic for ErbB2, tumor tissue contains elevated c-src activity relative to non-tumor tissue (13). These results suggest that ErbB2 overexpression causes an activation of c-src, which may be at least partly responsible for the phenotype of ErbB2 transformed cells. Therefore, the objective of this study was to determine if overexpressing ErbB2 results in activation of c-src in non-tumorigenic human mammary epithelial cells and if c-src activation may be responsible for tumor phenotype.

MATERIALS AND METHODS

Cell lines. 184.A1 human mammary epithelial cells (14), MCF10A human mammary epithelial cells (15) and NMuMG mouse mammary epithelial cells (16) were obtained from ATCC (Rockville, MD). Cells maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 ng/ml EGF. pJRneu (17) and pJRras (18) were kindly provided by Dr. M. Gould (University of Wisconsin, Madison). pJRneu is based on the plasmid pJR containing tumorigenic human neu, driven by CMV promoter and containing a neomycin resistance marker. pJRras is similar, except that v-Ha-ras is present insead of neu. Transfected cell lines were selected in media containing 400 μg/ml G418 sulfate.

Western blot analysis. To assess expression of ErbB2 and c-src, cells were grown to near confluency and lysed with SDS loading buffer (19) lacking 2-mercaptoethanol and bromophenol blue. Protein content of samples was determined by BCA assay (Pierce Chemical Co., Rockford, IL), 2-mercaptoethanol and bromophenol blue added to the samples and 50 μg protein separated by SDS-PAGE. Proteins were transferred to PVDF membranes and western blots probed with antibodies against c-src (UBI, Lake Placid, NY) essentially as described by Fenton and Sheffield (20).

c-src activity. Cells grown to approximately 80% confluency were lysed with lysis buffer (50 mM HEPES, pH 7.0 containing 30 mM sodium pyrophosphate, 10 mM EDTA, 50 mM NaCl, 50 mM NaF, 1% Triton X-100,m 0.1% BSA, 1 mM sodium orthovanadate and 1 mM PMSF) and centrifuged (15,000 g for 15 minutes). Protein content of the supernatant was determined by BCA assay, equalized among cell lines and c-src immunoprecipitated from equal protein amounts essentially as previously described (21) using anti-c-src (UBI) and agarose conjuated protein A and G (Santa Cruz Biotechnology, Santa Cruz, CA). Beads were washed 4 times with lysis buffer and c-src activity determined by incubating immunoprecipitated enzyme in 15 μl of assay buffer (200 mM HEPES, pH 7.0 containing 125 mM $MgCl_2$, 25 mM $MnCl_2$, and 0.25 mM sodium orthovanadate) with or without substrate peptide ([lys^{19}]cdc2(6-20)) or control pep-

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tide ([phe^{15}lys^{19}]cdc2(6-20)) (1 mM for standard assays). Reactions were started by adding 5 μM of a 0.5 mM γ^{32}P-ATP solution (approximately 1 Ci/mmole, Dupont, Boston, MA). Standard reactions were continued for 5 minutes and stopped by adding 10% trichloroacetic acid and 100 μg bovine serum albumin. Samples were centrifuged (3000 g for 5 minutes) and supernatant spotted onto Whattman P81 phosphocellulose paper. Paper was washed 5 times with 100 mM phosphoric acid, dried and counted by liquid scintillation. c-src content in immunoprecipitates was determined by western blot analysis as described above, quantitated by computer-assisted densitometry (Collage, Fotodyne, New Berlin, WI) and activity adjusted for c-src content.

Cell growth. Control or ErbB2 transfected cells were plated onto petri dishes (10^4 cells/cm^2), treated as described and allowed to grow for 3 days with media changed each day. Cells were counted by hemocytometer counting and population doubling time estimated as an index of growth rate.

Soft agar growth. Wild-type or ErbB2 transformed cells were suspended in DMEM containing 10% fetal bovine serum and 0.3 % agar and plated over 0.5 % bottom agar at a density of 10^3 cells/100 mm petri dish. Cells were then treated as described and colony forming efficiency determined after 7 days of growth.

Statistical analysis. All experiments were replicated on at least 3 occasions. Quantitative data were analyzed by analysis of variance (ANOVA) and transformed cell lines compared with parental cells by Dunnett's t-test (22). Unless otherwise states, all differences noted were $P<0.05$.

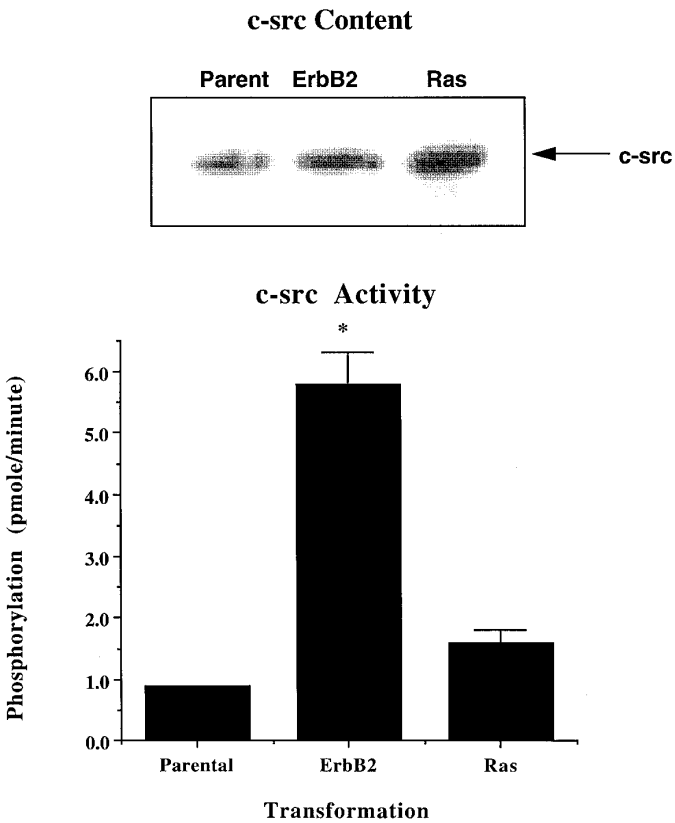

FIG. 1. c-src content and activity of parental, ErbB2 transformed and ras-transformed 184.A1 human mammary epithelial cells. For both studies, parental, ErbB2 and ras transformed cells were generated and cultured as described in Materials and Methods. A. Cells were lysed with SDS loading buffer, proteins separated by SDS PAGE and western blot analysis was performed as described in Materials and Methods. Representive of 3 experiments. Densitometry analysis indicated no significant differences in c-src expression. B. c-src was immunoprecipitated, c-src content of immunoprecipitates assessed by western analysis and kinase activity determined as described in Materials and Methods. c-src activity was reported prior to normalization to c-src content of immunoprecipitates. Mean ± SEM of 3 experiments. * = Significantly different than parental line, $P<0.05$.

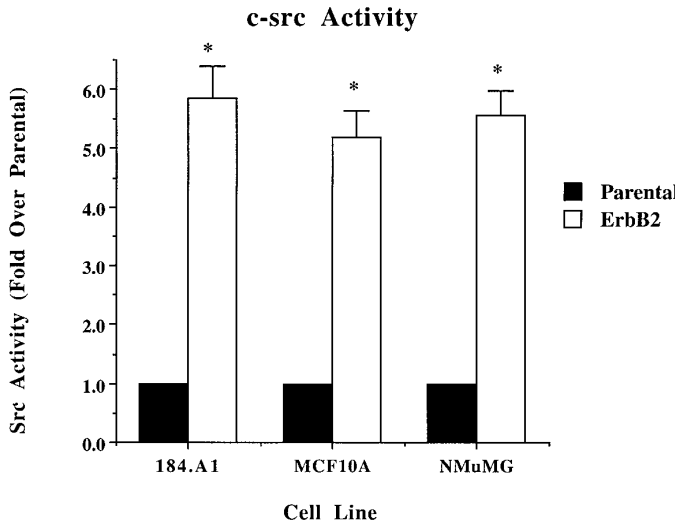

FIG. 2. Comparison of cell lines. Parental, ErbB2 and ras transformed 184.A1, MCF-10A and NMuMG cells were generated as described in Materials and Methods. c-src was immunoprecipitated, c-src content of immunoprecipitates determined by western blot analysis and kinase activity of immunoprecipitates determined as described in Materials and Methods. Kinase activity was reported after normalization to c-src content of immunoprecipitates. Mean ± SEM of 3 determinations. * = Significantly different than parental line, $P<0.05$.

RESULTS

The c-src content of parental, ErbB2 transformed and ras transformed cells did not vary significantly among the 3 lines (Figure 1A). Despite similar levels of c-src, phosphorylation of a c-src substrate peptide was increased approximately 5-6 fold in ErbB2 expressing cells compared with control cells (Figure 1B). The c-src activity in ras-transformed cells was not significantly different than parental cells, suggesting that this effect was not a general effect of transformation but was likely due to ErbB2 expression. In preliminary studies (not shown), c-src kinase activity was found to be dependent on the presence of substrate peptide, linear over time and linear over the amount of cell extract used for immunoprecipitation.

The effect of ErbB2 transformation on c-src activity was not confined to 184.A1 cells. The nontumorigenic human mammary epithelial cell line MCF-10A and the mouse line NMuMG also exhibited substantial increases in c-src activity upon transfection with ErbB2, but not with ras (Figure 2).

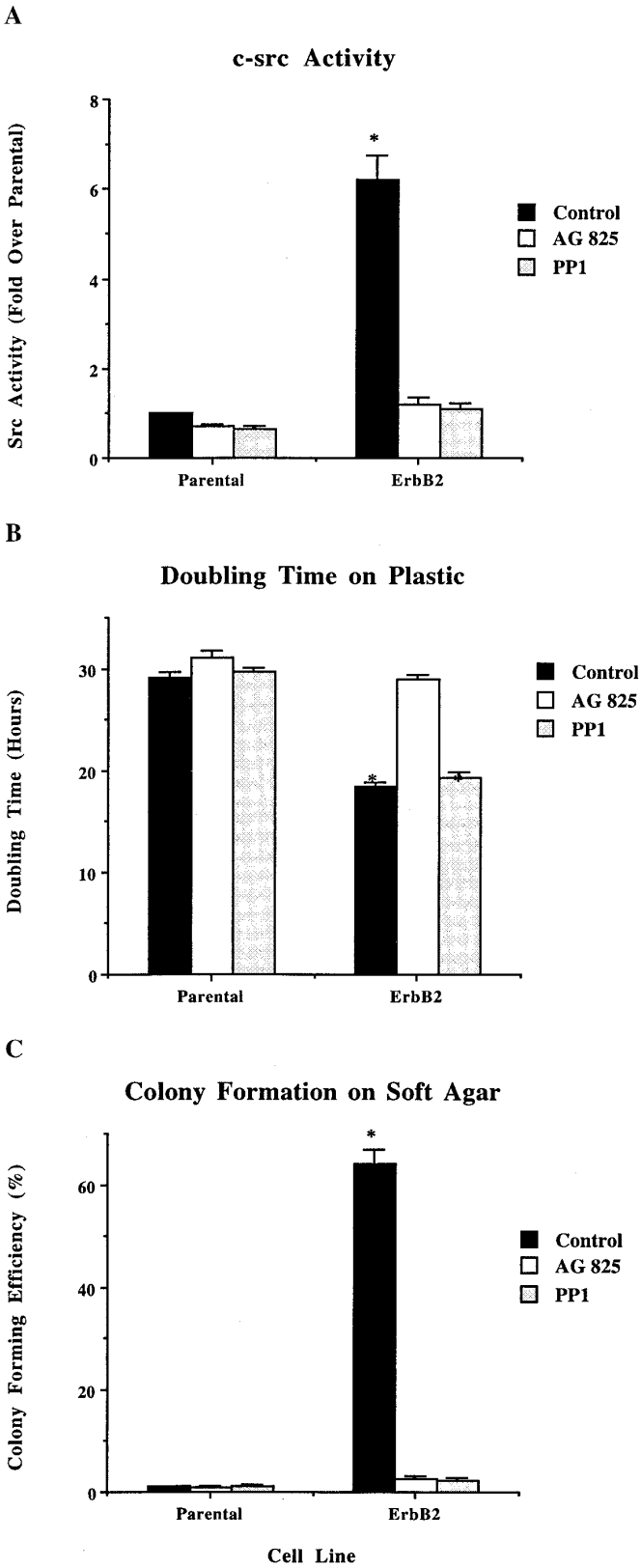

FIG. 3. Influence of Tyrphostin AG 825 (ErbB2 inhibitor) or PP1 (src inhibitor) on c-src activity (A), growth on plastic (B) and growth on soft agar (C). Parental and ErbB2 transformed 184.A1 cells were generated as described in Materials and Methods, cultured on plastic (A and B) or soft agar (C) and treated with Tyrphostin AG 825 (10 μM, AG) or PP1 (100 nM). A. c-src was immunoprecipitated, c-src content determined by western blot analysis, kinase activity determined as described in Materials and Methods and kinase activity normalized to c-src content of immunoprecipitates. B. Cells were plated, cultured for 3 days and growth rate was expressed as population doubling time. C. Cells were plated onto soft agar, cultured for 7 days and colony forming efficiency was estimated. Mean ± SEM of 4 experiments. * = Significantly different than control treated parental cells, $P<0.05$.

In order to further verify that the observed change in c-src activity was due to ErbB2 activity, the effects of the selective ErbB2 inhibitor Tyrphostin AG 825 on c-src activation, cell growth and attachment independent growth was assessed. AG 825 (10 μM) dramatically decreased c-src activity in ErbB2 transformed cells, to levels approximately equal to control levels (Figure 3A). This concentration had no effect on cell viability, as measured by trypan blue exclusion (not shown). Cell proliferation on plastic was slightly reduced by AG 825. Untreated ErbB2 transformed cells grew significantly faster than parental cells. However, AG 825 reduced the growth rate of ErbB2 transformed cells to rates that were not different than parental cells (Figure 3B). Parental cells were largely incapable of forming colonies on soft agar, while ErbB2 transformed cells readily formed colonies on soft agar. AG 825 dramatically inhibited the ability of ErbB2 transformed cells to form colonies on soft agar (Figure 3C).

In order to evaluate possible roles of c-src in the ErbB2 tumor phenotype, the ability of the putative c-src inhibitor PP1 to reverse tumor phenotype was examined. PP1 reduced c-src activity in ErbB2 transformed cells (Figure 3A). At a dose that resulted in c-src activity similar to control levels (100 nM), PP1 had little effect on cell proliferation on plastic and ErbB2 transformed cells continued to grow faster than parental cells (Figure 3B). However, PP1 dramatically inhibited growth of ErbB2 transformed cells on soft agar (Figure 3C). These results suggest that c-src activation by ErbB2 may have little effect on mitogenesis of human breast cancer, but may play an important role in other tumor phenotypes, such as attachment independent growth.

DISCUSSION

The present study indicates that overexpression of ErbB2 in a non-tumorigenic human mammary epithelial cell line, which has previously been shown to result in tumorigenic transformation of the cells (9), results in dramatically increased c-src activity, in the absence of altered c-src content. These results indicate that previously observed associations between ErbB2 expression and c-src activity (23-25) are likely to be a result of ErbB2 expression, and not a coincidental correlation.

Furthermore, the results indicate that previously reported increases in c-src activity in transgenic mice expressing ErbB2 in mammary tissue (13) is also observed in human mammary epithelium. The lack of effect of ras transformation suggests that the observed effect is not a general result of transfection, selection or transformation, but is likely due to specific signaling pathways.

Because ErbB2 is known to function as a tyrosine kinase (10), we also examined the effects of the ErbB2 selective kinase inhibitor Tyrphostin AG 825 on c-src activity. This inhibitor appears to have substantial selectivity for ErbB2 over other tyrosine kinases, including c-src and ErbB1 (26). However, as with all inhibitor studies, possible non-specific effects cannot be completely eliminated. Nonetheless, these studies suggest ErbB2-mediated c-src activation is likely to depend on the tyrosine kinase activity of ErbB2. However, the exact pathway involved is not elucidated in these studies. Presumably, the pathway could involve activation of phosphotyrosine phosphatases (9, 27), inhibition of CSK-like activities (28) or direct association with ErbB2 (29).

Functionally, c-src activation could mediate a variety of tumorous phenotypes. Guy et al. (30) observed that c-src ablation decreased Polyoma virus middle-T antigen-induced mammary tumorigenesis in transgenic mice. Other researchers have reported that c-src mediated signaling may be important in cell cycle progression (31). In addition, src is well known as a mediator of cytoskeletal architecture, cell adhesion and motility (32, 33). Such processes are clearly implicated in tumor metastasis (34). Members of the c-src family have also been associated with integrin signaling complexes, which may be involved in tumor development and metastasis (35). Since ErbB2 is associated with increased tumor invasiveness and poor prognosis, the hypothesis that c-src activation by ErbB2 contributes to the highly invasive phenotype of ErbB2 expressing tumors is attractive, but as yet unproven.

In order to evaluate the possible role of c-src in tumor development, the src family selective inhibitor PP1 was used. Originally, PP1 was described as an inhibitor of Lck and Fyn (36), but is also capable of inhibiting c-src (37). In contrast to recent studies on ErbB2-induced mouse mammary tumors, which found that PP1 did not affect attachment independent cell growth (38), the present study found that proliferation of ErbB2-transformed human mammary epithelium on plastic was unaffected by PP1, but soft agar growth was dramatically inhibited by PP1. These results suggest that c-src activation by ErbB2 may not be important in mediating cell cycle progression, but may be critical for mediating other aspects of the transformed phenotype.

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