Constitutive Tyrosine Phosphorylation of ErbB-2 via Jak2 by Autocrine Secretion of Prolactin in Human Breast Cancer*

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Overexpression of the oncogene for ErbB-2 is an unfavorable prognostic marker in human breast cancer. Its oncogenic potential appears to depend on the state of tyrosine phosphorylation. However, the mechanisms by which ErbB-2 is constitutively tyrosine-phosphorylated in human breast cancer are poorly understood. We now show that human breast carcinoma samples with ErbB-2 overexpression have higher proliferative and metastatic activity in the presence of autocrine secretion of prolactin (PRL). By using a neutralizing antibody or dominant negative (DN) strategies or specific inhibitors, we also show that activation of Janus kinase Jak2 by autocrine secretion of PRL is one of the significant components of constitutive tyrosine phosphorylation of ErbB-2, its association with Grb2 and activation of mitogen-activated protein (MAP) kinase in human breast cancer cell lines that overexpress ErbB-2. Furthermore, the neutralizing anti-PRL antibody or erbB-2 antisense oligonucleotide or DN Jak2 or Jak2 inhibitor or DN Ras or MAP kinase inhibitor inhibits the proliferation of both untreated and PRL-treated cells. Our results indicate that autocrine secretion of PRL stimulates tyrosine phosphorylation of ErbB-2 by Jak2, provides docking sites for Grb2 and stimulates Ras-MAP kinase cascade, thereby causing unrestricted cellular proliferation. The identification of this novel cross-talk between ErbB-2 and the autocrine growth stimulatory loop for PRL may provide new targets for therapeutic and preventive intervention of human breast cancer.

Breast cancer affects one in every eight women in the United States and is a significant cause of death. It was estimated that 181,600 new cases of breast cancer were diagnosed in the United States in 1997 and that 44,190 people would die of breast cancer during the same year (1, 2). Overexpression of the oncogene for ErbB-2 (3–6) is not observed in normal adult tissues but is present in 25–40% of human breast carcinoma samples (7), and has been correlated with nodal metastases (8), early relapse (9), and shortened survival (10) due to mechanisms that may be mediated by tyrosine phosphorylation of ErbB-2 (11–13).

ErbB-2, which belongs to the growth factor receptor superfamily (11), initiates its intracellular signal transduction by tyrosine-phosphorylating its intracellular domain and providing docking sites for signaling molecules. One of the signaling molecules known to be recruited to the tyrosine phosphorylation sites of ErbB-2 is Grb2 (growth factor receptor-bound protein 2). Association of tyrosine-phosphorylated proteins with Grb2 appears to be a crucial step in the activation of the Ras/mitogen-activated protein (MAP) kinase cascade (14–17), which has been reported to be associated with cell proliferation, tumor progression, and metastasis (12, 18). In the case of chemically induced rat neuroblastomas (19, 20), a single point mutation in the transmembrane domain of neu (the rat homolog of the human and mouse erbB-2), converting a valine residue to glutamic acid (21, 22), causes an increase in the tyrosine kinase activity of Neu and stimulates its tyrosine phosphorylation (23–25) by inducing ligand-independent receptor oligomerization (26). The same activating mutation, however, has not yet been identified in human breast tumors (27–29). Therefore, the mechanisms by which ErbB-2 is constitutively tyrosine-phosphorylated in human breast cancer (18, 30, 31), are poorly understood. Catalytic activation of ErbB-2 may be inducible by membrane overexpression itself. This mechanism alone, however, is unable to explain the variable net receptor tyrosine phosphorylation among cell lines with similar amount of ErbB-2 expression (30), suggesting that other mechanisms are likely to influence tyrosine phosphorylation of ErbB-2.

In the present study, to identify the factors that affect proliferative activity and clinical features of breast cancer in relation to overexpression of ErbB-2, we screened several factors by
using immunohistochemical analyses in human breast carcinoma tissues and found that breast cancers with ErbB-2 overexpression have higher proliferative and metastatic activity in the presence of prolactin (PRL) co-expression. PRL, whose receptor belongs to the cytokine receptor superfamily (32, 33), initiates its wide variety of biological effects by activating Janus kinase Jak2 tyrosine kinase. We show here in two reconstitute systems that PRL is able to stimulate tyrosine phosphorylation of ErbB-2, its association with Grb2, and activation of MAP kinase via Jak2, independent of the intrinsic tyrosine kinase activity of ErbB-2.

Recently, ectopic production of PRL by human breast cancer cells was reported (34–36) to induce proliferation of these cells (37, 38) in an autocrine fashion. By using a neutralizing antibody or dominant negative strategies or specific inhibitors, we also show that activation of Jak2 by autocrine secretion of PRL is one of the significant components of constitutive tyrosine phosphorylation of ErbB-2, its association with Grb2, and activation of MAP kinase in human breast cancer cell lines that overexpress ErbB-2. Moreover, the neutralizing anti-PRL antibody (39) or erbB-2 antisense oligonucleotide (40) or dominant negative (DN) Jak2 (41) or a Jak2 inhibitor (42) or DNRas (16) or MAP kinase inhibitor inhibits the proliferation of both untreated and PRL-treated cells.

Our results indicate that autocrine secretion of PRL stimulates tyrosine phosphorylation of ErbB-2 by Jak2, provides docking sites for Grb2, and stimulates Ras-MAP kinase cascades, thereby causing unrestricted proliferation of human breast cancer cells. These findings may explain, at least in part, the clinical effectiveness of monoclonal antibodies against the oncoprotein for erbB-2 (43–45), which cause down-regulation of ErbB-2 molecule, whose tyrosine phosphorylation by PRL-Jak2 pathway otherwise may cause unrestricted proliferation, as the novel treatment modality of breast cancer. Furthermore, the identification of this novel cross-talk between ErbB-2 and the autocrine growth stimulatory loop for PRL that significantly affects the growth of human breast cancer may provide new targets for therapeutic and preventive intervention.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Lines**—The polyclonal antibody against ErbB-2 (Neu) for immunoprecipitation and immunoblotting was from Santa Cruz Biotechnology, Inc. The polyclonal antibody against PRL (A569) for immunohistochemistry (IHC) from Dako (Carpinteria, CA). The monoclonal antibody against PRL receptor (PRLR) (U5) for immunostaining was from Affinity BioReagents, Inc. The polyclonal antibody against erbB-2 (A485) and monoclonal antibody against proliferating cell nuclear antigen (PCNA) (PC10) for immunostaining were from Dako (Glostrup, Denmark). The monoclonal antibody against epidermal growth factor receptor (EGFR) (E8F113) for immunostaining was from YLEM S.r.l. (Italy). The neutralizing anti-PRL antibody and the antibody against PRL for immunoblotting was the same one used for studies of immunoreactivity (39). PRL was from Sigma. MAP kinase kinase inhibitor PD98059 and Jak2 inhibitor AG490 were from Calbiochem. All other materials were obtained from the sources described previously (46, 47). SK-BR3, MDA-MB453, T47D, MC7, and HBL-100 cells were from ATCC.

DNRas and Adenovirus-mediated Gene Transfer—cDNA of DNRas (Ki-Ras with Ser-17 mutated to Asn by polymerase chain reaction method) was kindly provided by Dr. Takai (Osaka University, Osaka, Japan). The recombinant adenoviruses Adex1CADNRas and Adex1CADDNras were constructed by homologous recombination between the expression cosmid cassette, and the parental virus genome (48). The recombinants were transfected with or without 3 μg of each mutant ErbB-2 (49) plasmids under the SV40 promoter and with or without 3 μg of dominant negative Jak2 (ΔJak2) (41) plasmids in 6-cm dishes by the combination of LipofectAMINE and replication-deficient adenovirus, and the cells were stimulated as described (46). COS cells were cotransfected with 3 μg of human PRLR (47), and with or without 2 μg of WT-Jak2 or ΔJak2 at 60–80% confluence in 3 ml of Dulbeco’s modified Eagle’s medium in 6-cm dishes by the calcium phosphate precipitation method, and the cells were stimulated as described (46).

**Immunoprecipitation, Immunoblotting, MAP Kinase Assay, In Vitro Jak2 Kinase Assay, Generation of GST-ErbB-2 Fusion Protein, DNA Synthesis, and Proliferation**—The GST-cytoplasmic region of ErbB-2 (amino acids 1018–1260) was generated as described (46). Immunoprecipitation, immunoblotting, MAP kinase assay, and in vitro Jak2 kinase assay were performed as described (46) with modification. To determine whether Jak2 kinase is constitutively activated in SK-BR3 cells, we performed immunoprecipitation with limited quantities of anti-Jak2 antibody, thus ensuring that an equal amount of protein could be examined in both breast cancer cell line SK-BR3 and normal HBL-100 cells. The growth rates of breast cancer cells were determined as described (50) with modification. They were assessed by measuring relative increases in cell number using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (Dojindo). Cells were plated in 12-well flat-bottomed culture dishes in 0.5 ml of culture medium. The culture medium was changed after 48 h to fresh serum-free medium. Cells were treated with 20 μM PD98059 or for 16 h with or without PRL. The plates were analyzed 5 days later with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reagent according to the manufacturer’s instructions. Results were expressed as percentage growth of untreated cells with random oligonucleotides alone. DNA synthesis was determined as described (40) with modification. SK-BR3 cells were plated in 12-well dishes. Cells transfected with or without 0.15 μg of ΔJak2 or infected with or without adenovirus vectors (48) at the indicated multiplicity of infection were pretreated for 60 min with or without 30 μM PD98059 or for 16 h with or without the indicated doses of AG490. Then we determined incorporation of bromodeoxyuridine according to the manufacturer’s instructions (Roche Molecular Biochemicals). Each bar represents the mean ± S.E. of three independent experiments.

**Immunohistochemistry**—Immunohistochemical analyses were performed as described (51). For immunostaining of PRL, PRLR, and ErbB-2, respective antibodies were diluted and incubated as follows: PRL (1:50, A569), PRLR (30 μg/ml, U5) for 16 h at 4 °C, ErbB-2 (1:200, A485) for 30 min at room temperature in a moisture chamber. Positive controls for PRL included pituitary gland, and for ErbB-2 included breast carcinoma known to exhibit high levels of protein. The percentage of PCNA-positive cells (labeling index) was counted. All pathological features were evaluated separately by two investigators who were unaware of the clinical outcome of the patients. Statistical analyses were performed using unpaired t test and chi-square test for independence. The investigations were performed in accordance with the principles of the Declaration of Helsinki.

**RESULTS**

**Human Breast Carcinoma Samples with ErbB-2 Overexpression Have Higher Proliferative and Metastatic Activity in the Presence of Autocrine Secretion of PRL**—To identify the factors that affect proliferative activity and clinical features of breast cancer in relation to overexpression of ErbB-2, immunohistochemical analyses were performed in 76 human breast carcinoma tissues obtained at the time of surgery in Mitsui Memorial Hospital in 1994. We observed positive immunoreactivity for ErbB-2 in 29% of the specimens examined. PRL has been shown to stimulate proliferation of breast cancer cells (37, 38). Thus, we next examined immunohistochemically expression of PRL and PRLR. Immunoreactive PRL and PRLR were detected in 95% and 100% of the specimens, respectively, and 28% of the total displayed both ErbB-2 and PRL immunoreactivity, whereas 67% were positive for PRL immunostaining but negative for ErbB-2. Although growth hormone (GH) has also been reported to induce primate mammary epithelial proliferation (52), none of the specimens were positive for GH immunoreactivity. The expression of ErbB-2 or PRL was then correlated to that of PCNA, a marker of proliferation activity. As shown in
Table I

| PCNA | Lymph node metastasis |
|------|-----------------------|
|      | ErbB2-positive | ErbB2-negative | ErbB2-positive | ErbB2-negative |
| Prolactin-positive | 52.4 | 38.3 | 15/22 (68%) | 22/50 (44%) |
| Prolactin-negative | 5.9 | 4.1 | 0/1 (0%) | 0/3 (0%) |

Table I, the mean positivity percentage (labeling index) of PCNA (±S.E.) was 52.4 (± 4.2) in the group of patients with both ErbB-2- and PRL-positive staining, whereas the value (38.3 ± 3.2) was significantly lower (p = 0.013) in the patients with PRL-positive but ErbB-2-negative staining. In contrast, there was no significant difference in PCNA expression between the cancer tissues with and without expression of another growth factor receptor EGFR (EGFR-positive versus EGFR-negative; 45.3 (± 5.8) versus 41.9 (± 3.0); p = 0.618). Moreover, lymph node metastases were found more frequently in ErbB-2- and PRL-positive carcinomas compared with those with PRL-positive but ErbB-2-negative carcinomas (68% versus 44%; p = 0.058) (Table I). In addition, even in the presence of ErbB-2 overexpression, tumor negative for PRL was node-negative, and had a PCNA labeling index of only 5.9 (Table I left). These data suggest that breast cancers with ErbB-2 overexpression have higher proliferative and metastatic activity in the presence of autocrine or paracrine secretion of PRL.

PRL Is Able to Stimulate Tyrosine Phosphorylation of ErbB-2 and Its Association with Grb2 via Activation of Jak2 Kinase—PRL signals via its cell-surface receptor through Jak2 activation (32, 33). To explore the effects of the co-expression of PRL and ErbB-2, PRLR and Jak2 were cotransfected into COS cells which express endogenous ErbB-2. Since the level of expression of Jak2 in COS cells appears to be low, some level of co-expression of wild-type (WT)-Jak2 and PRLR has been reported to be required in order to detect activation of Jak2 by PRL (47). We confirmed that neither expression of WT-Jak2 alone nor coexpression of kinase-inactive Jak2 and PRLR, but coexpression of WT-Jak2 and PRLR resulted in PRL-induced tyrosine phosphorylation of Jak2 in COS cells (Fig. 1). Expression of WT-Jak2, but not kinase-inactive ΔJak2, which lacks the C terminus kinase domain (41), induced activation of Jak2 and interestingly, at the same time caused tyrosine phosphorylation of ErbB-2, and its association with Grb2 in a PRL-dose-dependent manner in COS cells (Fig. 1, A–D). These results suggest that PRL is able to stimulate tyrosine phosphorylation of ErbB-2 and its association with Grb2 via activation of Jak2 kinase. To clarify how PRL induces tyrosine phosphorylation of ErbB-2, we tested whether PRL could induce the association of ErbB-2 with Jak2 by co-immunoprecipitation experiments in COS cells transiently transfected with PRLR and WT-Jak2; we found that complex formation between Jak2 and ErbB-2 was induced in a PRL-dependent manner (Fig. 1E). To explore the mechanism by which Jak2 is recruited to ErbB-2, especially to clarify whether Jak2 tyrosine phosphorylation necessarily precedes binding of Jak2 to ErbB-2, we have performed the co-immunoprecipitation experiments and showed that coexpression of WT-Jak2 and PRLR or coexpression of kinase-inactive Jak2 and PRLR, but not expression of WT-Jak2 alone resulted in PRL-induced complex formation between ErbB-2 and WT-Jak2 (Fig. 1, F (lane 4) and H (lane 4)) or between ErbB-2 and ΔJak2 (Fig. 1, F (lane 6) and H (lane 6)), despite that ΔJak2 was not tyrosine-phosphorylated in response to PRL (Fig. 2, I, lane 6). However, WT-Jak2 was able to induce PRL-stimulated tyrosine phosphorylation of ErbB-2 (Fig. 1G, lane 4), whereas ΔJak2 failed to do so (Fig. 1G, lane 6), suggesting that Jak2 kinase activity was required for PRL-stimulated tyrosine phosphorylation of ErbB-2, but not for PRL-induced complex formation between ErbB-2 and Jak2.

In ErbB-2 and PRL, PRL-Induced Tyrosine Phosphorylation of ErbB-2, Its Association with Grb2, and Full Activation of MAP Kinase Did Not Depend on the Intrinsic Tyrosine Kinase Activity of ErbB-2, but on Jak2 Kinase Activity—To clarify the role and the mechanisms of PRL-induced tyrosine-phosphorylation of ErbB-2 in transmitting signals to downstream components, WT or kinase-negative (KN) ErbB-2 was introduced into CHO-PRLR cells (47), which do not express endogenous ErbB-2. In cells transfected with vector alone as a control, PRL failed to induce

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tyrosine phosphorylation of ErbB-2 (Fig. 2A, lane 2). In contrast, in CHO-PRLR cells expressing WT ErbB-2, PRL induced tyrosine phosphorylation of ErbB-2 (Fig. 2A, lane 4) and its association with Grb2 (Fig. 2B, lane 4). We next studied the PRL-induced MAP kinase activation, since the association of tyrosine-phosphorylated proteins with Grb2 appears to be a crucial step in the activation of the Ras/MAP kinase cascade (14–17), which has been reported to be associated with cell proliferation, tumor progression, and metastasis (12, 18). Expression of WT ErbB-2 in these cells was accompanied by PRL-induced MAP kinase activation, which was significantly enhanced compared with cells not expressing ErbB-2 (Fig. 2C). It is interesting to note that in cells expressing KN-ErbB-2, PRL induced tyrosine phosphorylation of ErbB-2, its association with Grb2 and concomitantly stimulated MAP kinase activity to an extent comparable to cells expressing WT ErbB-2 (Fig. 2, A–C, lanes 4 and 6), suggesting that tyrosine kinase activity of ErbB-2 is not required for PRL-induced MAP kinase activation. Furthermore, expression of ΔJak2, which inhibits tyrosine kinase activity of WT Jak2 in a dominant negative manner (41), almost completely abolished these responses (Fig. 2, A–C, lanes 4 and 8). This effect of dominant negative Jak2 was specific, since dominant negative Jak2 had no effect on tyrosine phosphorylation of ErbB-2 induced by mutation (V659E) that causes constitutive activation of intrinsic tyrosine kinase activity of ErbB-2 in the absence of ligand (glutamic acid instead of valine at position 659 within the transmembrane domain) (28, 49) (Fig. 2D), nor induced by EGF stimulation through EGFR/ErbB-2 heterodimerization (31, 59) (Fig. 2E). These data indicated that PRL-induced tyrosine phosphorylation of ErbB-2, its association with Grb2, and full activation of MAP kinase did not depend on the intrinsic tyrosine kinase activity of ErbB-2, but on Jak2 kinase activity. To determine whether ErbB-2 could be directly phosphorylated by Jak2 in response to PRL, we carried out immune complex phosphorylation experiments in vitro. The GST-cytoplasmic region of ErbB-2 was indeed phosphorylated by Jak2 immunoprecipitated from CHO-PRLR cell extracts treated with PRL (Fig. 2F, lane 4).

**Activation of Janus Kinase Jak2 by Autocrine Secretion of PRL Is One of the Significant Components of Constitutive Tyrosine Phosphorylation of ErbB-2, Its Association with Grb2, and Activation of MAP Kinase in Human Breast Cancer Cell Lines**—We next studied the mechanisms by which ErbB-2 is constitutively tyrosine-phosphorylated in human breast cancer cell lines (18, 30, 31). SK-BR3 cells have 4–8-fold amplification of erbB-2 and express 2-fold more PRLR than normal cells (37). In these cells, ErbB-2 was indeed constitutively tyrosine-phosphorylated without exogenously added PRL (Fig. 3, A and B, lane 3). Interestingly, Jak2 was also constitutively tyrosine-phosphorylated (Fig. 3, C and D, lane 3) and activated (Fig. 3G, lane 2), and expression of dominant negative Jak2 (Fig. 3D, lanes 1 and 2) reduced the endogenous Jak2 kinase activity (Fig. 3C, lanes 1, 3), the amount of tyrosine phosphorylation of ErbB-2 (Fig. 3A, lanes 1 and 3), its association with Grb2 (Fig. 3E, lanes 1 and 3), and MAP kinase activation in untreated cells (Fig. 3F, lanes 1 and 3). We also observed that exogenously added PRL further increased the amount of tyrosine phosphorylation of ErbB-2 via Jak2-dependent fashion (Fig. 3, A and C). Very similar findings were also obtained in MDA-MB453 cells, another human breast cancer cell line with ErbB-2 overexpression (37) (data not shown). These results indicated that, in both untreated and PRL-treated human breast cancer cell lines that overexpress ErbB-2, tyrosine phosphorylation of ErbB-2, its association with Grb2 and MAP kinase activation can be induced, at least in part, by Jak2.

Recently, ectopic expression of PRL was reported in human breast carcinoma (34–36). Thus, we next determined whether PRL secreted from human breast cancer cell lines was able to stimulate tyrosine phosphorylation of ErbB-2. Immunoblot
analysis revealed that the amounts of expression of PRL at the protein level within SK-BR3 and MDA-MB453 cells were comparable to that within T47D or MCF7 cells (Fig. 4A), in which PRL synthesis and secretion were reported previously (34, 35). The addition of a neutralizing anti-PRL antibody (39), which blocked the PRL-induced increase in tyrosine phosphorylation of the Jak2 (Fig. 4B), significantly reduced the level of tyrosine phosphorylation of ErbB-2 (Fig. 4C) and its association with Grb2 (Fig. 4D) and MAP kinase activation (Fig. 4F) in untreated SK-BR3 cells. It is therefore likely that activation of Jak2 by autocrine secretion of PRL is one of the significant components of constitutive tyrosine phosphorylation of ErbB-2, its association with Grb2 and MAP kinase activation in SK-BR3 cells. Consistent with this, ErbB-2 was constitutively recruited into the complex where it could be tyrosine-phosphorylated by the constitutively activated Jak2, and exogenously added PRL further increased the amount of ErbB-2 associated with Jak2 (Fig. 4, G and H). It is also possible that, under basal conditions, other mechanisms relevant to MAPK activation are also important.

Tyrosine Phosphorylation of ErbB-2 and Its Association with Grb2 via Jak2 in Response to Autocrine Secretion of PRL Activates Ras-MAP Kinase Cascade, Thereby Stimulating Proliferation of Cells Overexpressing ErbB-2—To determine the importance of PRL-induced tyrosine phosphorylation of ErbB-2 in proliferation of breast carcinoma cells, we studied the effect of erbB-2 antisense oligonucleotides (40) on proliferation of cells that overexpress ErbB-2. Although erbB-2 antisense oligonucleotides had no significant effect on Jak2 tyrosine phosphorylation (Fig. 5A, middle), they reduced ErbB-2 protein levels and concomitantly induced a significant inhibition of proliferation of both untreated and PRL-treated SK-BR3 (Fig. 5A) and MDA-MB453 cells, which overexpress ErbB-2, but not in MCF-7 cells, which have low levels of ErbB-2 (37) (data not shown). Furthermore, the neutralizing anti-PRL antibody (Fig. 5B) or dominant negative (DN) Jak2 (Fig. 5C) or DNras (Fig. 5D) or the MAP kinase kinase inhibitor PD98059 (Fig. 5E) significantly inhibited DNA synthesis both in untreated and PRL-treated SK-BR3 (Fig. 5, B–E) and MDA-MB453 cells, but not in untreated HBL-100 normal cells (data not shown). These data indicate that tyrosine phosphorylation of ErbB-2 and its association with Grb2 via Jak2 in response to autocrine secretion of PRL activate Ras-MAP kinase cascade, thereby stimulating proliferation of cells overexpressing ErbB-2. To further confirm these observations, we analyzed whether a Jak2 inhibitor AG490 had an inhibitory effect on the DNA synthesis of SK-BR3 cells. AG490, which indeed inhibited Jak2 kinase activity and constitutive tyrosine phosphorylation of ErbB-2 (Fig. 5F), blocked DNA synthesis of both untreated and PRL-treated SK-BR3 cells in a dose-dependent manner (Fig. 5G).

**DISCUSSION**

**The Mechanisms by Which ErbB-2 Is Constitutively Tyrosine-phosphorylated in Human Breast Cancer—**ErbB-2 is amplified and overexpressed in 25–40% of primary breast cancers, which correlates with poor patient prognosis (8, 27, 54–57). ErbB-2...
belongs to the type I subclass of receptor tyrosine kinases (11), and its oncogenic potential appears to depend on the state of tyrosine phosphorylation (12, 13). In the present study, by using a neutralizing antibody (Fig. 4) or a dominant negative strategy (Fig. 3) or a specific inhibitor (Fig. 5F), we demonstrate that activation of Jak2 by autocrine secretion of PRL is one of the significant components of constitutive tyrosine phosphorylation of ErbB-2 in human breast cancer cell lines. These data clarify the mechanisms by which ErbB-2 is constitutively tyrosine-phosphorylated in human breast cancer cell lines. To the best of our knowledge, interactions between ErbB-2 and its ligands or mutations that trigger autophosphorylation of ErbB-2 have not been documented in human cancer (27–29).

The Roles of Tyrosine Phosphorylation of ErbB-2 via Jak2 by Autocrine Secretion of PRL in Human Breast Cancer—In the present study, we demonstrated that complementation with ErbB-2 receptor expression plasmid into ErbB2-deficient cell line, even if it lacks its intrinsic tyrosine kinase activity, resulted in a significant increase in PRL-stimulated MAP kinase activity (Fig. 2C). Moreover, we provided the evidence that erbB-2 antisense oligonucleotides or DN Jak2 or the Jak2 inhibitor or the neutralizing anti-PRL antibody reduced the amounts of tyrosine phosphorylation of ErbB-2 and its association with Grb2 (Figs. 3 [A and E], 4 [C and D], and 5F; data not shown) and induced a significant inhibition of MAP kinase activation (Figs. 3F and 4F; data not shown) or cell proliferation in both untreated and PRL-treated human breast cancer cell lines which overexpress ErbB-2 (Fig. 5, A–C and G; data not shown). These data indicated that tyrosine phosphorylation of ErbB-2 via Jak2 by autocrine secretion of PRL provides docking sites for Grb2 and stimulates Ras-MAP kinase cascade, thereby causing unrestricted cellular proliferation, independent of the intrinsic tyrosine kinase activity of ErbB-2, in human breast cancer cell lines.

Cross-talk between Growth Factor Receptor and Cytokine Receptor Superfamily—We have recently shown that another cytokine family member GH stimulates tyrosine phosphorylation of EGFR/ErbB-1 and MAP kinase activity via Jak2 in liver, an important target tissue of GH (46). Together with these observations, our findings provide the novel paradigm that tyrosine phosphorylation of ErbBs via Jak2 kinase by cytokine receptor superfamily proteins, independently of the intrinsic kinase activity of ErbBs, may play pivotal roles in activation of MAP kinase and vital phenomena such as proliferation of normal tissue and neoplasm.

Interleukin-6 (IL-6) was recently reported to induce tyrosine phosphorylation of ErbB-2 and stimulate MAP kinase in a prostate cancer cell line (58). However, there may be important differences in the mechanisms of cytokine-induced tyrosine phosphorylation of ErbB-2 between our and their reports. First, PRL-induced tyrosine phosphorylation of ErbB-2 and activation of MAP kinase are dependent on functional Jak2 kinase, but independent of the intrinsic kinase activity of ErbB-2 (Fig. 1 [A–D, G, and I], 2 [A–C and F], 3 [A–G], and 5F); on the contrary, IL-6-induced tyrosine phosphorylation of ErbB-2 and activation of MAP kinase were dependent on the

![Diagram](Image)

**Fig. 4.** Ectopic production of prolactin by breast cancer cells increased the amount of tyrosine phosphorylation of ErbB-2, its association with Grb2, and MAP kinase activity via Jak2 kinase. A, the amount of expression of PRL at the protein level within each breast cancer cell lines was determined by probing with an anti-PRL antibody in whole-cell lysates. B, the neutralizing anti-PRL antibody blocked the PRL-induced increase in tyrosine phosphorylation of the Jak2. Quiescent COS cells were treated with 10⁻³ mL PRL for 5 min. Precipitated Jak2 from cells in the presence of control IgG or neutralizing anti-PRL antibody (20 µg mL⁻¹) was immunoblotted with anti-PY. C–F, the neutralizing anti-PRL antibody significantly reduced the amount of tyrosine phosphorylation of ErbB-2 and Jak2, ErbB-2 association with Grb2, and MAP kinase activity in human breast cancer cell lines that overexpress ErbB-2. Precipitated ErbB-2 or Grb2 (Fig. 3) or Jak2 (E) from quiescent SK-BR3 cells in the presence of control IgG (C) versus cells in the presence of anti-PRL IgG (D) or Grb2 (Fig. 3) or Jak2 (E) or anti-ErbB-2 (D). F, MAP kinase activity was determined by immune complex assay as described (46). Results are expressed as the percentage of the value of cells in the presence of control IgG. Each bar represents the mean ± S.E. of three independent experiments. (*, p < 0.05; cells in the presence of control IgG versus cells in the presence of anti-PRL). G and H, quiescent SK-BR3 cells were stimulated for 5 min with PRL (10⁻³ M). Upon lysis, precipitates with control rabbit IgG (C) (lane 1) or anti-Jak2 (G, lanes 2 and 3) or anti-ErbB-2 (H, lanes 2 and 3) were immunoblotted with anti-ErbB-2 (G) or anti-Jak2 (H).
ErbB-2 intrinsic kinase activated by an unidentified mechanism. Second, presumably in relation to the first difference, ErbB-2 forms a complex with the Jak2 in a PRL-dependent manner (Figs. 1 (E, F, and H) and 4 (G and H)); in contrast, IL-6 induced association of ErbB-2 with gp130 subunit of the IL-6R, but not with Jak family kinases in prostate cancer cell line.

**New Targets for Therapeutic and Preventive Intervention of Human Breast Cancer**—We show here that human breast carcinoma samples with ErbB-2 overexpression have higher proliferative and metastatic activity in the presence of autocrine secretion of PRL (Table I). Recently, the clinical effectiveness of recombinant humanized monoclonal antibodies against the oncoprotein for erbB-2/HER-2/neu (43–45) was shown, as the novel treatment modality of breast cancer. These results may be explained at the molecular level, at least in part, by down-regulation of ErbB-2 molecule whose tyrosine phosphorylation by PRL-Jak2 pathway otherwise may cause unrestricted proliferation. Furthermore, the identification of this novel cross-talk between ErbB-2 and the autocrine growth stimulatory loop for PRL that significantly affects the growth and dissemination of human breast cancer may provide new targets for therapeutic and preventive intervention. In fact, the PRLR antagonist was reported to be more effective at inhibiting the growth of SK-BR3 cells when added in the presence of an inhibitory antibody to the ErbB-2 than for just the antibody alone (37). On the basis of our data showing the involvement of the PRL/Jak2 and ErbB-2/Ras/MAP kinase signaling pathway in the development of breast cancer, the combination of the intervention to each of the components in this pathway may improve the clinical effectiveness of breast cancer therapy.

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