Autocrine-mediated Activation of STAT3 Correlates with Cell Proliferation in Breast Carcinoma Lines*

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The intracellular signals driving the proliferation of breast carcinoma (BC) cells have been widely studied. Both the mitotic and metastatic potential of BC cells have been linked to the frequent overexpression of ErbB family members. Other signaling molecules, including the estrogen receptor, the tyrosine kinases c-Src and Syk, and STAT proteins, especially STAT3, have also been implicated in BC tumor growth. Here we have examined ErbB and STAT protein expression and activation in six BC-derived cell lines. ErbB expression and tyrosine phosphorylation varied considerably among the six cell lines. However, STAT protein expression and activation were more consistent. Two levels of STAT3 activation were distinguished in DNA-binding assays: an epidermal growth factor-inducible, high level that requires both ErbB1 and Janus kinase (JAK) activity and an elevated serum-dependent level that is maintained by autocrine/paracrine signaling and requires JAK activity but is independent of ErbB1 kinase activity. BC cell growth could be inhibited by dominant-negative versions of STAT3 and the JAK inhibitor AG490 but not by PD153035 or PD168393, inhibitors of ErbB1 kinase activity. This indicates that BC cell proliferation may be a consequence of STAT3 activation by autocrine/paracrine signals.

Overexpression of ErbB2 is sufficient to cause its hyperphosphorylation, which may trigger signaling and transformation (6). Alternatively, ErbB2 overexpression may enhance the binding affinities of both EGF1 and neu differentiation factor for their ligands, thereby amplifying subsequent downstream signals. Thus, ErbB2 overexpression may allow tumor cells to respond to low concentrations of mitogenic growth factors (7). However, in vitro assays indicate that although low levels of neu differentiation factor increase the growth rate of cancer cell lines overexpressing ErbB2, higher levels result in anti-proliferative and differentiating effects (2).

Analyses of transgenic mice carrying ErbB2 have indicated that its overexpression alone is insufficient to cause malignancies, since those detected could be attributed to somatic activating mutations in the extracellular domain of ErbB2 (8). Subsequent work revealed a splice variant of ErbB2 in human BC samples with in vitro transforming potential (9). However, other factors may contribute to tumorigenesis mediated by ErbB2 overexpression. For example, c-Src, which is also overexpressed in BC (10), is able to synergize with ErbB1 to transform cells (11), possibly by c-Src-mediated receptor phosphorylation (12). Conversely, it has recently been shown that loss of Syk tyrosine kinase expression correlates with invasive breast carcinoma (13). Thus, although the association of ErbB2 overexpression with BC is compelling, its role in malignant progression is not completely understood. An alternative explanation for the strong association between BC and ErbB protein overexpression may therefore be the recent finding that ErbB2 is critical for carcinoma cell migration and invasion rather than for cell proliferation (14).

Activated ErbB family members are tyrosine-phosphorylated and recruit signaling molecules to their intracellular domains (15). As well as direct activation of Ras, phosphotydilinositol 3-kinase, and phospholipase C, ErbB1 has recently been implicated in the activation of these molecules by G protein-coupled receptors (16–18). ErbB family proteins are also capable of activating, directly and indirectly, signal transducers and activators of transcription (STAT) proteins, originally identified as downstream mediators of cytokine receptor signaling (19). When activated by tyrosine phosphorylation, STAT proteins, of which seven have been identified, dimerize and translocate to the nucleus, where they bind to enhancer elements in cytokine-responsive gene promoters (20).

STAT3 appears to play critical role in the determination of cell fate (21). The differentiation of PC12 cells induced by nerve growth factor was found to require the inhibition of STAT3,

* This work was supported by a grant from the Association for International Cancer Research. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: EGF, epidermal growth factor; STAT, signal transducers and activators of transcription; JAK, Janus kinase; EMSA, electrophoretic mobility shift assay; PVDF, polyvinylidene fluoride; PBS, phosphate-buffered saline; FCS, fetal calf serum; SIE, v-Sis-inducible element; GFP, green fluorescent protein.
implying that constitutive STAT3 activity prevents differentiation and maintains cells in a state of continual proliferation. Indeed, mouse ES cells are sustained in an undifferentiated state by activated STAT3 (22). Conversely, activation of STAT3 is required for cell transformation by oncogenic Src and by a constitutively active form of Gαo, a heterotrimeric G-protein subunit (23, 24). In addition, STAT3 is found to be active in fibroblasts transformed by a selection of oncoproteins and in human BC cell lines (25, 26). The routes by which STAT3 is activated under these circumstances remain obscure. However, all of the data implicating STAT3 in cell transformation received further support when a form of STAT3 modified to dimerize spontaneously was shown to be oncogenic (27, 28).

Here we have examined a panel of six BC cell lines for ErbB and STAT protein expression and activity. We first observed marked variations in ErbB protein expression and tyrosine phosphorylation. In comparison, expression of STAT1 and STAT3 was more consistent. DNA binding assays distinguished two levels of STAT activity: acute induction of STAT3 was more consistent. DNA binding within minutes, suggesting the involvement of an autocrine/paracrine signaling pathway. Since proliferation of these BC cells was inhibited by dominant negative versions of STAT3 and the JAK inhibitor AG490, but not by PD153035 or PD168393, inhibitors of ErbB1 kinase activity, we infer that a serum-dependent autocrine/paracrine activation of STAT3 may be involved in BC cell proliferation.

**FIG. 1.** ErbB and STAT protein expression in BC cell lines. Lysates were prepared from BT20 (lane 1), MCF-7 (lane 2), T47D (lane 3), MDA-MB-231 (lane 4), MDA-MB-468 (lane 5), and BR293 (lane 6) cells. For ErbB1 200 μg of protein, for ErbB2 and ErbB3 400 μg of protein, and for STAT1 and STAT3 200 μg of protein from each cell lystate was separated by SDS-PAGE, transferred to PVDF membranes, and probed with anti-ErbB (upper panel) or anti-STAT (lower panel) antibodies as indicated. One set of lysates was used throughout.

**FIG. 2.** Tyrosine phosphorylation of ErbB proteins in BC cell lines. Lysates were prepared (see “Materials and Methods”) from BT20 (lanes 1 and 2), MCF-7 (lanes 3 and 4), T47D (lanes 5 and 6), MDA-MB-231 (lanes 7 and 8), MDA-MB-468 (lanes 9 and 10) and BR293 (lanes 11 and 12) cells that had been serum-starved (-) or starved and treated with EGF (5 nm) for 15 min (-). ErbB proteins were collected as immune complexes, separated by SDS-PAGE, transferred to PVDF membrane, and probed first with an anti-phosphotyrosine antibody and subsequently with the corresponding anti-ErbB antibody, as indicated. ND, indicates that the protein is not expressed at detectable levels by the cell line (see Fig. 1). The numbers below each panel show the level of tyrosine phosphorylation, quantified with AIDA software (Fuji) and expressed as the ratio α-PY/α-ErbB, whereby the unstimulated value for each protein in each cell line is set as 1. The results shown are compiled from several experiments in which ErbB proteins from each cell line were analyzed at least three times with similar results.

**MATERIALS AND METHODS**

**Cell Culture and Extract Preparation—**Breast cancer cell lines (BR293, BT20, MCF-7, MDA-MB-231, MDA-MB-468, and T47D) were maintained in Eagle’s minimum essential medium (Sigma) supplemented with 10% fetal calf serum (FCS), 1% nonessential amino acids, 1% glutamine, and 1% penicillin/streptomycin at 37 °C under 5% CO₂. MCF-10F cells, one of a series of nontumorigenic lines derived from benign breast epithelial tissue, were grown as adherent cells in a 2:1 mixture of Eagle’s minimum essential medium and Ham’s F-12 medium (Sigma) supplemented with 5% horse serum, 2 mM glutamine, 10 μg ml⁻¹ insulin, 20 ng ml⁻¹ EGF, 100 ng ml⁻¹ cholaer toxin, 0.5 μg ml⁻¹ hydrocortisone, and 1% gentamycin.

For preparation of nuclear extracts for electrophoretic mobility shift assays (EMSA), cells were seeded in 10-cm dishes and cultured until confluent. Thereafter, the cells were maintained in serum-free medium overnight before application of appropriate stimuli. Nuclear extracts were prepared as described previously (29) in high salt hypertonic buffer (20 mM HEPES, pH 7.9, 420 mM NaCl, 20% glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM Na₃VO₄, 1 mM Na,P₂O₇, 2 mM benzamidin, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin).

For immunoprecipitation and immunoblotting experiments, cells were grown to confluence in 10-cm dishes and maintained in full medium or starved in serum-free medium overnight before the application of appropriate stimuli. Lysates were prepared in TBSN buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40) supplemented with protease inhibitors (1 mM Na₃VO₄, 10 mM Na,P₂O₇, 10 mM NaF, 5 mM EGTA, 10 mM benzamidin, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin).
Lysates were cleared by centrifugation at 16,000 × g for 10 min and used directly for immunoprecipitations or stored at −20 °C for further use. AG490 was purchased from Sigma, PD153035 was provided by Glaxo-Smith-Kline, and PD188393 was purchased from Calbiochem.

**Immunoprecipitation and Immunoblotting**—Equal amounts of lysates were incubated with the appropriate antibody for 2 h at 4 °C. Immune complexes were then allowed to bind to protein-A-Sepharose beads for 1 h at 4 °C and collected by centrifugation. Immunoprecipitations were washed three times in 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 0.5% Nonidet P-40. Thereafter, samples were taken up in SDS loading buffer and boiled for 5 min.

Samples were separated by electrophoresis through 6% polyacrylamide-SDS gels. Proteins were then transferred to polyvinylidene difluoride (PVDF) membranes with a semidry electroblotting apparatus. The membranes were incubated with appropriate primary antibodies at room temperature for 1 h or 4 °C overnight according to suppliers’ instructions, washed, and stained with horseradish peroxidase-coupled secondary antibodies. The membranes were developed with an enhanced chemiluminescence kit (Amersham Biosciences).

**EMSA**—DNA binding assays were carried out as previously described (29). Briefly, DNA binding by STAT proteins was analyzed with a [32P]-labeled oligonucleotide duplex (M67SIE) (30). Extracts were incubated with the DNA probe, and protein-DNA complexes were separated by electrophoresis on 5% polyacrylamide gels containing 2.5% glycerol in 0.5× Tris-borate-EDTA buffer. After separation, the gels were fixed, dried, and analyzed with a phosphor imager (Fuji). For supershift analyses of STAT-DNA complexes, extracts were preincubated with α-STAT1 or α-STAT3 antisera at room temperature for 1 h. The oligonucleotide probe was then added, and the EMSA was performed as described above.

**Plasmids and Oligonucleotides**—The expression vectors for wild type and dominant-negative STAT3 proteins (STAT3-EV and STAT3-Y705F) were generous gifts of Drs. Curt Horvath (Mount Sinai School of Medicine, New York) and James E. Darnell, Jr. (Rockefeller University, New York) and have been characterized previously (see Ref. 24 and references therein).

The sequences of the oligonucleotides used to generate the M67 EMSA probe, which was derived from the v-Sis-inducible element (SIE) of the human c-fos promoter, are as follows: upper strand, 5′-CTAG-CATTCCCGTAAAT; lower strand, 5′-CTAGATTACCGGAAAATG.

**Cell Proliferation Assays**—Equal numbers of MCF-10F, MCF-7, BR293, and MDA-MB-468 cells were seeded in the appropriate growth medium into 10-cm dishes. Cells were allowed to grow in the presence of the JAK inhibitor AG490 or the ErbB1 inhibitors PD153035 (100 nM) and PD188393 (2 μM) for 24 or 48 h. For the 48 time points, fresh medium containing the appropriate inhibitor was applied to the cells after 24 h. Controls were allowed to grow for 48 h in the absence of inhibitor. Thereafter, all of the cells were washed twice with ice-cold PBS, harvested, and counted under a phase-contrast microscope. Values are expressed as averages ± S.D. (n = 3).

For proliferation assays with wild type and dominant-negative STAT3 mutants (24), 2.5 × 10^5 MDA-MB-468 or MCF-7 cells, maintained in Eagle’s minimum essential medium supplemented with 10% FCS, were transfected with LipofectAMINE with 3.5 μg of pEGFP-CLONTECH and 4 μg of the corresponding STAT3 expression vector or the control vector (pRc/CMV). After 24 h, green fluorescent protein (GFP)-positive cells (3% of MDA-MB-468 and 11% of MCF-7 cells) were selected by fluorescence-activated cell sorting and seeded to 96-well plates at 2 × 10^3 cells/well. After recovery overnight, cells were incubated with [3H]thymidine for 24 h prior to harvesting. Incorporation of [3H]thymidine was measured after trichloroacetic acid precipitation and NaOH solubilization by liquid scintillation counting. Significant differences between groups were determined by Student’s t test (two-tailed). p values of <0.05 (∗) are considered significant. p values of <0.01 are indicated with a double asterisk.

**RESULTS**

**ErbB and STAT Protein Expression in BC Cell Lines**—Initially, the expression levels of ErbB proteins in six BC-derived cell lines were compared by immunoblotting. As shown in Fig. 1 (upper panel), ErbB1 was strongly expressed in MDA-MB-468 cells, moderately expressed in BT20 cells, weakly expressed in MDA-MB-231 cells, and undetectable in the other
three cell lines (MCF-7, T47D, and BR293). However, MCF-7 and T47D cells have been shown previously to express low levels of surface ErbB1, indicating that the limit of detection must lie above 10,000 receptors/cell (31). ErbB2 was expressed at a similar level in all of the cell lines, with the exception of MDA-MB-468, in which it was undetectable. Expression of ErbB3 was also analyzed and found to be moderate in MCF-7 and T47D, weak in BT20 and MDA-MB-468, and absent from MDA-MB-231 and BR293 cells. In contrast, the expression of STAT1 and STAT3 proteins in these cells showed much less variation (Fig. 1, lower panel). BR293 cells alone express low levels of STAT1 proteins (lane 6). Both isoforms of STAT3 (STAT3α and -β) are expressed in all of the cell lines,2 but the β isoform is expressed at a lower level in BT20 and BR293 cells (lanes 1 and 6). Thus, these six BC-derived cell lines exhibit five different profiles of ErbB expression, whereby only those exhibited by MCF-7 and T47D cells are similar. However, they express comparable levels of STAT1 and STAT3 proteins.

Tyrosine Phosphorylation of ErbB Proteins in BC Cell Lines—The activity of ErbB proteins is a consequence of their tyrosine phosphorylation status. Accordingly, tyrosine phosphorylation of ErbB proteins was analyzed, in those cells in which they could be detected (Fig. 1), by immunoprecipitation and subsequent detection with a phosphotyrosine-specific antibody (PY20). In BT20, MDA-MB-231, and MDA-MB-468 cells, tyrosine phosphorylation of ErbB1 was weak or undetectable in normally growing cells (Fig. 2, upper panel), but, as expected, it was induced (5.9-, 10.8-, and 8.3-fold, respectively) upon treatment of cells with EGF. Tyrosine phosphorylation of ErbB2 is detectable in normally growing MCF-7 and T47D cells but not in the other cell lines. In MDA-MB-231 cells, EGF treatment does not elicit an increase in ErbB2 tyrosine phosphorylation, although ErbB1 is ex-

\[2\] L. Li and P. E. Shaw, unpublished observations.

Fig. 5. Inhibition of EGF-induced phosphorylation of ErbB1 and DNA binding of STAT proteins. a, serum-starved MDA-MB-468 cells were pretreated with PD153035 (100 nM) for the times indicated and then treated with EGF (5 nM) for 15 min (+). Lysates were prepared, from which ErbB1 proteins were collected as immune complexes, separated by SDS-PAGE, transferred to PVDF membrane, and probed with an anti-phosphotyrosine antibody as indicated. b, equal amounts of each nuclear extract were incubated alone (lanes 1, 3, 5, 7, 9, 11, and 13) or with an antibody specific for STAT3 (lanes 2, 4, 6, 8, 10, 12, and 14). Complexes were then formed on a radiolabeled oligonucleotide duplex corresponding to the M67 sequence derived from the c-fos SIE. In this and subsequent figures only the upper parts of the EMSA gels are shown. c, BT20 (lanes 1–3) and MDA-MB-468 cells (lanes 4–6) were serum-starved (–), treated with EGF (5 nM) for 15 min, or pretreated with AG490 (100 nM) for 30 min and then treated with EGF (5 nM) for 15 min (+). STAT3 DNA complexes were analyzed as described for b.

Fig. 6. Delayed activation of STAT3 by serum. a, extracts were prepared from serum-starved BR293 cells (lanes 1 and 6) or starved cells stimulated directly with 10% FCS for the times indicated (lanes 2–5), or after pretreatment with AG490 (lane 7). STAT3 and JAK2 proteins were collected as immune complexes, separated by SDS-PAGE, transferred to PVDF membrane, and probed first with an anti-phosphotyrosine antibody and subsequently with anti-STAT3 or anti-JAK2 antibodies as indicated. b, serum-starved BR293 cells were stimulated with 10% FCS (lanes 3 and 4) that had been serum-starved (–) or starved and stimulated with serum for 2 h. Proteins (200 µg) were separated by SDS-PAGE, transferred to PVDF membrane, and probed first with an anti-phospho-STAT3 antibody and subsequently with an anti-STAT3 antibody as indicated.
pressed (see Fig. 1) and becomes phosphorylated itself. However, in T47D and BR293 cells, which both lack ErbB1 (see Fig. 1), stimulation of ErbB2 tyrosine phosphorylation by EGF is apparent (5.7- and 3.2-fold, respectively).

ErbB3 tyrosine phosphorylation is also observed under normal growth conditions in all four cell lines in which it is expressed. Moreover, in those cell lines in which ErbB1 is co-expressed, tyrosine phosphorylation of ErbB3 is induced by EGF (6.6- and 6.3-fold). In summary, although the variations in ErbB protein expression among the cell lines preclude direct quantitative comparison, those cells expressing ErbB1 display low levels of tyrosine phosphorylation on ErbB2 and ErbB3 proteins that become elevated following stimulation by EGF.

Conversely, cell lines that lack ErbB1 show constitutive levels of tyrosine phosphorylation on ErbB2 and ErbB3 that remain unchanged or increase only marginally when cells are treated with EGF.

**STAT Activation in BC Cell Lines**—The phosphorylation of STAT1 and STAT3 proteins was also examined in all six cell lines with phosphospecific antibodies for each protein. As shown in Fig. 3 (upper panel), tyrosine phosphorylation of STAT1 was undetectable in serum-starved cells but was stimulated in BT20 and MDA-MB-468 cells following EGF treatment (lanes 2 and 10). As already seen in Fig. 1, BR293 cells express low levels of STAT1. By comparison, a low level of STAT3 tyrosine phosphorylation could be seen in serum-starved BR293 cells (lower panel, lane 11), whereas in STAT3 immunoprecipitates probed with an anti-phosphotyrosine antibody, we detected phosphorylated STAT3 in all six cell lines (result not shown). Following EGF stimulation, however, tyrosine phosphorylation of STAT3 also increased in BT20 and MDA-MB-468 cells (lanes 2 and 10), mirroring the behavior of STAT1. Because EGF-induced tyrosine phosphorylation of ErbB1 also occurs in MDA-MB-231 cells (see Fig. 2), the failure to induce STAT3 tyrosine phosphorylation is likely to be a consequence of the lower level of ErbB1 expression in this cell line (see Fig. 1).

The function of STAT proteins depends on their DNA binding ability, for which tyrosine phosphorylation and dimerization are prerequisites. Initially, nuclear extracts prepared from BC cells were analyzed for STAT binding activity with a cognate binding element derived from the c-fos SIE (30). In extracts of serum-starved MDA-MB-468 cells, in which ErbB1 is highly expressed, a low level of DNA binding was detected (Fig. 4, lane 1), which could be attributed, by supershift assay with anti-STAT antibodies, predominantly to STAT3 (lane 3). Control experiments confirmed that the anti-STAT3 antibody does not generate the supershifted complex (3SS), seen here and in subsequent figures, in the absence of DNA-binding by STAT3 (data not shown). After stimulation of the cells with EGF, DNA binding was much enhanced, and several additional complexes were detected (lane 4) that contained STAT1 and STAT3, as evidenced by supershift assay with specific antibodies (lanes 5 and 6). In parallel experiments with BT20 cells, which also express ErbB1, EGF induced the formation of a similar set of complexes (Fig. 4, lanes 10–12). However, we did not detect the induction of STAT complexes by EGF in MDA-MB-231 cells, which express less ErbB1 (result not shown). When this experiment was carried out with cells lacking ErbB1 (BR293), weak DNA binding by STAT3 was again detected in extracts of serum-starved cells (Fig. 4, lanes 13–15), but EGF failed to stimulate the formation of additional STAT-DNA complexes (lanes 16–18). Thus, acute stimulation of STAT1 and STAT3 DNA binding activity in response to EGF correlates directly with ErbB1 expression in BC cells.

**Acute STAT Activation Requires ErbB1 and JAK Kinase Activity**—To confirm that the acute activation of STAT DNA-binding in response to EGF was dependent upon ErbB1 kinase activity, EGF stimulation was repeated in the presence of the quinazoline inhibitor PD153035 (32). Pretreatment of MDA-MB-468 cells with 100 nM PD153035 for 30 min inhibited tyrosine phosphorylation of ErbB1 (Fig. 5a) and abrogated the induction of SIE-bound STAT complexes by EGF (Fig. 5b). Thus, the acute activation of STAT DNA-binding by EGF requires ErbB1 kinase activity. However, PD153035 had no effect on the weak DNA binding by STAT3 detected by supershift
FIG. 8. Inhibition of STAT3 blocks BC cell growth. a, equal numbers (2.5 × 10⁶) of MDA-MB-468 and MCF-7 cells were co-transfected with a GFP expression plasmid and a control vector or expression vectors for wild type or dominant-inhibitory versions of STAT3 (Y/F and E/V; see “Materials and Methods”). Cells positive for GFP were selected by fluorescence-activated cell sorting, and their proliferation was measured by [³H]thymidine incorporation. Values are expressed as averages ± S.E.; for MDA-MB-468 cells, n = 3; for MCF-7 cells, n = 4. b, equal numbers (2 × 10⁶) of MCF-10F, MCF-7, MDA-MB-468, and BR293 cells were plated in full medium and cultured in the absence or presence of increasing STAT3 Activity and Tumor Cell Growth.
STAT3 Activity and Tumor Cell Growth

assay in extracts from unstimulated cells (lane 2 and lanes 6, 8, 10, 12, and 14).

As the involvement of JAKs in cellular responses to EGF is controversial (33, 34), the acute induction of STAT DNA binding activity was examined in cells treated with the JAK inhibitor AG490 (35). As shown in Fig. 5c, 100 μM AG490 abolished STAT activation by EGF in BT20 and MDA-MB-468 cells. Thus, acute stimulation of STAT DNA binding by EGF requires both ErbB1 and JAK kinase activity.

Serum Induces Elevated STAT3 Activity via an Autocrine Signal—When serum-starved BR293 cells, which lack ErbB1, were returned to full medium, we observed an increase in STAT3 tyrosine phosphorylation over a 2-h time course (Fig. 6a, upper panels). Tyrosine phosphorylation of JAK2 was also stimulated by serum over the same period (lower panels), and both effects were blocked by AG490. As shown in Fig. 6b, STAT DNA binding activity in nuclear extracts also increased, reaching a peak at 2 h. Notably, the activation of STAT3 in response to serum was also observed in MCF-7 and MDA-MB-468 cells, as shown in Fig. 6c.

Compared with the rapid, acute induction by EGF, the kinetics of STAT activation in response to serum are delayed, suggesting that the up-regulation of STAT DNA binding by serum could involve an autocrine/paracrine mechanism. Therefore, serum-starved BR293 cells were stimulated with 10% FCS, and, after 2 h, half the cells were harvested, while the other cells were washed thoroughly and incubated for a further 4 h in serum-free medium. This medium was then transferred to fresh, serum-starved BR293 cells, which were incubated for a further 15 min. Nuclear extracts were prepared from all of the cells and analyzed for STAT DNA binding. As shown in Fig. 7a, STAT1 and STAT3 DNA binding was stimulated after 2 h by 10% FCS (lanes 3 and 4). In contrast, serum-free conditioned medium from cells incubated previously with 10% FCS for 2 h stimulated the formation of STAT3-DNA complexes after 15 min (lanes 5 and 6). Similarly, conditioned medium from MDA-MB-468 cells cultured for 2 h with 10% FCS was able to stimulate STAT3 DNA binding in BR293 cells within 15 min (result not shown). Treatment of BR293 cells with conditioned medium also induced tyrosine phosphorylation of STAT3 within 15 min, whereas EGF treatment did not (Fig. 7b). We therefore infer that BC cells cultured in 10% FCS release factors that stimulate rapid tyrosine phosphorylation of STAT3, its consequent nuclear translocation, and DNA binding.

Since BR293 cells do not express ErbB1, the involvement of ErbB1 in the serum-dependent activation of STAT3 is unlikely. Consistent with this inference, when FCS was applied to serum-starved MDA-MB-468 cells pretreated with PD153035, the delayed serum stimulation of STAT3 DNA binding was not affected (result not shown). The role of JAKs in the serum-dependent activation of STAT3 was also assessed further. Pretreatment of BR293 cells with 100 μM AG490 for 30 min completely blocked STAT3 activation (Fig. 7c, compare lanes 2 and 3). However, when conditioned medium from serum-stimulated BR293 cells was applied to serum-starved cells treated with AG490, no inhibition was observed (compare lanes 8 and 9). This observation suggests that the primary signal mediating STAT3 activation by serum requires JAK activity, whereas the secondary autocrine signal acts independently of JAKs.

Taken together, the preceding results distinguish two levels of STAT activity in BC cells. In cells expressing ErbB1, STAT-DNA complexes can be induced acutely by EGF, which is dependent upon the kinase activity of both ErbB1 and JAKs. In addition, delayed activation of STAT3 phosphorylation and DNA binding is induced by serum via an autocrine mechanism involving JAKs but not ErbB1.

Inhibition of BC Cell Growth—Given the link between STAT3 activity and cell proliferation observed in a number of different contexts (22–24), the activation of STAT3 in BC cell lines by two distinct mechanisms prompted us to test which, if any, might be important for cell proliferation. Initially, MDA-MB-468 and MCF-7 cells were co-transfected with expression vectors for GFP and STAT3 or one of two trans-dominant negative mutants thereof (24). GFP-positive cells were then selected by fluorescence-activated cell sorting, and their proliferation was subsequently measured by [3H]thymidine incorporation. Expression of the dominant negative STAT3 mutants in MCF-7 cells reduced proliferation by over 50%, while in MDA-MB-468 cells, which gave lower transfection efficiencies, proliferation was reduced by 30–50% (Fig. 8a). These results serve to implicate STAT3 function in the proliferation of these BC cell lines.

We then measured the effects of JAK inhibition on BC cell growth. As shown in Fig. 8b, treatment of MCF-7, MDA-MB-468, and BR293 cells with AG490 for 48 h had a dramatic effect on cell growth, reducing cell proliferation by 75%. AG490 had a similar effect on the other BC cell lines used in this study (results not shown). In direct comparison, however, growth of MCF-10F cells, an immortalized but nontumorigenic breast epithelial cell line, was much less susceptible to AG490. Control experiments confirmed that serum does not stimulate STAT3 phosphorylation or DNA binding in MCF-10F cells (Fig. 8b, right-hand panels), consistent with previous observations of MCF-10A cells (26). Thus, as first seen with lymphoblastic leukemia cells (35), JAK function is important for proliferation of BC tumor cell lines. Since JAKs are involved in both EGF-dependent and -independent STAT3 activity, we also measured the effect on cell growth of two specific ErbB1 inhibitors, PD153035 and PD168393, the latter an irreversible tyrosine kinase inhibitor (36). In this case, treatment of MDA-MB-468 and BR293 cells with either reagent had no effect on their proliferation over 48 h (Fig. 8, c and d). To demonstrate that ErbB1 was indeed inhibited, MDA-MB-468 cells cultured and treated with PD168393 in parallel were stimulated at different time points with EGF for 15 min, and tyrosine phosphorylation of ErbB1 was measured. PD168393 completely inhibited ErbB1 tyrosine kinase activity over 24 h (Fig. 8d, inset). Taken together, these findings suggest that BC cell proliferation correlates with STAT3 activity that is maintained by a serum-dependent autocrine/paracrine pathway.
DISCUSSION

The intended aim of these experiments was to test the notion that STAT3 activity resulting from the overexpression or constitutive activation of ErbB family proteins is a critical determinant of BC cell proliferation. However, we observed a striking variation in ErbB expression levels among the BC cell lines we compared. Moreover, the variations in expression were compounded by differences in tyrosine phosphorylation of ErbB proteins in the various cell lines. Critically, PD153035 and PD168393, which at nanomolar and micromolar concentrations, respectively, inhibit the kinase activity of ErbB1, had no effect on the proliferation of BC cells, whether the cells express (MDA-MB-468) or lack ErbB1 (BR293). Instead, we found that cell growth correlated with an elevated level of STAT3 activity, which was mediated by whole serum in part through an autocrine mechanism involving JAKs.

STAT Activity as an Acute Response to EGF—The degree of STAT activation following stimulation with EGF correlated directly with the level of ErbB1 expressed in the individual BC cell lines. EGF treatment clearly stimulated tyrosine phosphorylation of STAT1 and STAT3 in BT20 and MDA-MB-468 cells within 15 min, whereas in the other cells, no increase in tyrosine phosphorylation could be discerned. In the case of MDA-MB-468 cells, this occurred in the absence of detectable ErbB2 expression, precluding the involvement of ErbB1-ErbB2 heterodimers. However, these cells express high levels of ErbB1, which may obviate a need for ErbB2. In BT20 cells, which express ErbB2 but less ErbB1 than MDA-MB-468 cells, phosphorylation of STAT1 and STAT3 resulted in a lower level of STAT DNA binding. Thus, it appears that ErbB2 expression in BT20 cells cannot compensate for lower ErbB1 expression. STAT activation by EGF was not seen in MDA-MB-231 cells (result not shown) despite detectable ErbB1 expression. BR293 cells, which lack ErbB1 expression, also showed no induction of STAT activity following EGF stimulation. Supershift assays with STAT-specific antibodies enabled the acute DNA-bound complexes in MDA-MB-468 and BT20 cells to be characterized to some extent. In line with previous observations, STAT1 and STAT3 homodimers were prominent EGF-induced complexes on the c-fos promoter element.

As expected, acute STAT activation in response to EGF was dependent on the intrinsic kinase activity of ErbB1, as shown by its complete inhibition by PD153035 and PD168393. After prolonged pretreatment with PD153035 (4–6 h), even the low level of ErbB1 tyrosine phosphorylation corresponding to that seen in unstimulated cells was lost. This may reflect a gradual loss of activity by other ErbB family members as trans-phosphorylation and activation by ErbB1 is curtailed. Acute STAT activation also requires JAK kinase activity, suggesting that JAKs, and possibly STATs, may interact indubitably with ErbB1 via phosphotyrosine residues in the receptor’s cytoplasmic domain. Consistent with this notion, an ErbB1 mutant with an inactive kinase domain (K721A) fails to stimulate STAT DNA binding in response to EGF. However, ErbB1 mutants that retain kinase activity but lack several tyrosine residues still stimulate STATs efficiently, suggesting that ErbB1 phosphorylates a substrate other than itself. This may be JAK2 or a related kinase such as JAK1 or TYK2, which could be associated either with ErbB1 independently of phosphotyrosine residues or with some other vicinal membrane protein. This, in turn, could be another receptor tyrosine kinase serving as a scaffolding protein, as has recently been proposed for the platelet-derived growth factor receptor (37).

Serum-dependent STAT Activity Involving an Autocrine Loop—The STAT3-containing DNA-bound complexes observed in all BC cell lines are dependent on the presence of whole serum in the culture medium. After withdrawing serum for 18 h, its readdition led to a delayed increase in both STAT3 tyrosine phosphorylation and DNA binding. As well as differing kinetically from the acute induction by EGF, the serum-dependent STAT3 activation was unaffected by PD153035, indicating that it occurs independently of ErbB1 kinase function. However, serum activation of STAT3 was blocked by AG490, implicating JAKs in the signal pathway.

Conditioned, serum-free medium from BR293 (Fig. 7) or MDA-MB-468 cells (not shown) stimulated with 10% FCS for 2 h induced STAT3-DNA complexes in BR293 cells within 15 min. This observation provides compelling evidence for the involvement of an autocrine/paracrine loop in the delayed activation of STAT3 DNA binding by serum. Moreover, we found that JAK2 becomes phosphorylated upon serum stimulation and that AG490 inhibited the delayed response to serum but not the rapid response to conditioned medium, suggesting that JAKs mediate the production of autocrine factors by cells but not the cells’ response to them.

Several instances of JAK/STAT activation by autocrine mechanisms have been described to date. For example, in rat cardiomyocytes, angiotensin II has been shown to cause the delayed activation of STAT3 via the secretion of interleukin-6 family cytokines (38, 39). Moreover, the autocrine secretion of prolactin by BC cells has been shown to cause tyrosine phosphorylation and activation of both ErbB2 and JAK2 (40). The autocrine pathway we describe appears not to correspond to either of the above. First, we fail to detect tyrosine phosphorylation of gp130, the signaling chain common to interleukin-6 family receptors, suggesting that interleukin-6-type cytokines are not involved. Second, JAKs are implicated here in the release of autocrine factors rather than in the cells’ subsequent response to them. We are currently characterizing the pathway further with a range of specific molecular inhibitors.

Autocrine Activation of STAT3’s Linked to BC Cell Proliferation—Given that STAT3 activity has been linked to cell proliferation in several contexts (41, 42), the finding that the overexpression of dominant negative STAT3 alleles in BC cell lines reduces proliferation was not unprecedented. In these assays, in which transiently transfected cells were selected on the basis of GFP co-expression, cell proliferation, as measured by [3H]thymidine incorporation, was inhibited by expression of dominant negative STAT3 proteins but not by the wild type protein. The STAT3 Y/F mutant lacks the tyrosine residue involved in dimer formation and may block STAT3 activation by forming nonproductive complexes with activated receptors and kinases. The STAT3 E/V mutant fails to bind DNA (24). The degree of growth inhibition observed was reproducible and statistically significant. Thus, both inhibitory STAT3 mutants suppress the growth of those cells in which they are expressed.

As discussed above, ErbB proteins have also been implicated in BC proliferation, but in contrast to dominant-negative STAT3 proteins, PD153035 and PD168393, which at the concentrations used specifically inhibit ErbB1 kinase activity, failed to suppress BC cell growth. Although we cannot rule out that other ErbB proteins may play a role in driving proliferation, our results do imply that the pronounced expression of ErbB1 in MDA-MB-468 or BT20 cells is unlikely to be a critical factor for their proliferation. This would be in line with the conclusions of others that ErbB proteins influence cell invasion and the metastatic potential of malignant BC cells (14).

Our findings that AG490 strongly inhibits BC cell growth

3 K. Kindle, L. Li, and P. E. Shaw, unpublished data.
4 L. Li and P. E. Shaw, unpublished data.
and serum-dependent, elevated STAT3 activity link the STAT3-dependent BC cell proliferation to an autocrine signaling pathway activated by serum factors. Also consistent with this notion is the observation that MCF-10F nonmammary breast epithelial cells are clearly less susceptible to growth inhibition by AG490. The explanation for this may be the absence of STAT3 phosphorylation and DNA binding activity in these cells, as shown here and reported previously (26). While this manuscript was under revision, another study was published demonstrating the ability of AG490 to block the growth of BC cell lines with constitutive STAT3 activity (43). AG490 was first described as a suppressor of leukemic cell growth via its inhibitory effects on JAKs and has been shown to inhibit STATs and suppress the growth of other cancer cells (41, 42). In our hands, AG490 inhibited the delayed induction of STAT-DNA complexes by serum but failed to have an impact on the rapid response elicited by conditioned medium. This suggests that it is the initial expression of a stimulatory factor or its release from cells that requires JAK function rather than the response to its presence in conditioned medium. The release of autocrine factors may therefore constitute one pathway by which BC cells maintain STAT3 activity and consequently their own proliferation.

Acknowledgments—We thank Drs. Cliff Murray and Sue Watson for provision of BC cell lines and Charles Streuli for the MCF-10F cell line; Axel Ullrich and Lindy Darrant for anti-ErbB1 antibodies; Karin Knolle for the STAT1 and STAT3 antisera; Kurt Horvath and James Darnell for STAT expression vectors; and Colin Stubberfield (Glaxo-Smith-Kline) for PD153055. We are grateful to Heather Judge for cell sorting, to Jackie Bostock for secretarial assistance, and to members of the group for discussions and long term encouragement.

REFERENCES

1. Veu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merline, G. T., Pastan, L., and Lowy, D. R. (1987) Science 238, 1408–1410
2. Lupu, R., Cardillo, M., Harris, L., Hijazi, M., and Rosenberg, K. (1995) Biochim. Biophys. Acta 1279, 365–368
3. Hynes, N. E., and Stern, D. P. (1994) Biochim. Biophys. Acta 1279, 365–368
4. Difore, P. P., Pierce, J. H., Kraus, M. H., Segatto, O., King, C. R., and Aaronson, S. A. (1987) Science 237, 178–182
5. Hoznak, R. M., Schlessinger, J., and Ullrich, A. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7109–7113
6. Stern, D. F., Kamps, M. P., and Cao, H. (1998) Mol. Cell. Biol. 8, 3969–3973
7. Kurnagaran, D., Tzalhas, E., Bernier, R. R., Chen, X., Graus-Porta, D., Ratuskin, B. J., Seger, R., Hynes, N. E., and Yarden, Y. (1996) EMBO J. 15, 254–264
8. Siegel, P. M., Dunkert, D. L., Hardy, W. R., and Muller, W. J. (1994) Mol. Cell. Biol. 14, 7068–7077
9. Siegel, P. M., Ryan, E. D., Cardiff, R. D., and Muller, W. J. (1999) EMBO J. 18, 2149–2164
10. Ottenhoff-Ralf, A. E., Rijken, G., van Beurden, A. E. C. M., Hennipman, A., Michels, A. A., and Staaij, G. E. J. (1992) Cancer Res. 52, 4773–4778
11. Tice, D. A., Biscardi, J. S., Nickels, A. L., and Parsons, S. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 1415–1420
12. Biscardi, J. S., Mao, M.-C., Tice, D. A., Cox, M. E., Leu, T.-H., and Parsons, S. J. (1999) J. Biol. Chem. 274, 8335–8343
13. Cosman, P. J. D., Do, M. T. H., Barth, M., Bowden, E. T., Hayes, A. J., Basyuk, E., Blancto, J. K., Vezza, P. R., MclLeskey, S. W., Mangeat, P. H., and Mueller, S. C. (2000) Nature 406, 742–747
14. Spencer, K. S. B., Gruau-Porter, D., Leng, J., Hynes, N. E., and Klemke, R. L. (1990) J. Cell Biol. 148, 385–397
15. Hackel, P. O., Zwick, E., Preznel, N., and Ullrich, A. (1999) J. Clin. Invest. 114, 184–189
16. Dax, B., Wallasch, C., Lankena, A., Herrlich, A., and Ullrich, A. (1997) EMBO J. 16, 7032–7044
17. Preznel, N., Zwick, E., Daub, H., Tesler, M., Abraham, R., Wallasch, C., and Ullrich, A. (1999) Nature 402, 884–888
18. Luttrell, L. M., Daaka, Y., and Letzkowitz, R. J. (1999) Curr. Opin. Cell Biol. 11, 177–183
19. Leonard, W. J., and Lin, J.-X. (2000) J. Allergy. Clin. Immunol. 105, 877–888
20. Horvath, C. M., and Darnell, J. E. (1997) Curr. Opin. Cell Biol. 9, 233–239
21. Bara, S., Nakajima, K., Fukuda, T., Hibi, M., Nagata, S., Hirano, T., and Fukui, Y. (1997) EMBO J. 16, 3534–35352
22. Matsuda, T., Nakamura, T., Nakao, K., Arai, T., Katsuki, M., Heike, T., and Yokota, T. (1999) EMBO J. 18, 4261–4269
23. Turck, J., Bowman, T., Garcia, R., Caldenhoven, K., De Groot, R. P., and Jove, R. (1998) Mol. Cell. Biol. 18, 2545–2552
24. Ram, P. T., Horvath, C. M., and Iyengar, R. (2000) Science 287, 142–144
25. Sartor, C. I., Deizhimi, M. L., Yu, C. L., Jove, R., and Ethier, S. F. (1997) Cancer Res. 57, 978–987
26. Garcia, R., Yu, C. L., Hudnall, A., Catlett, R., Nelson, K. L., Smithgall, T., Fujita, D. J., Ethier, S. P., and Jove, R. (1997) Cell Growth Diff. 8, 1267–1276
27. Bromberg, J. F., Wrzeszczynska, M. H., Davgan, G., Zhao, Y., Pestel, R. G., Albanese, C., and Darnell, J. E. (1999) Cell 99, 295–303
28. Bowman, T., Garcia, R., Turkson, J., and Jove, R. (2000) Oncogene 19, 2474–2488
29. Sadowski, H. B., and Gilman, M. Z. (1993) Nature 362, 79–83
30. Wagner, B. J., Hayet, E. T., Hoban, C. J., and Cochran, B. H. (1999) EMBO J. 18, 3747–3754
31. Imai, Y., Leung, C. H. K., Friesen, H. G., and Shiu, R. P. C. (1982) Cancer Res. 42, 4384–4389
32. Fry, D. W., Kramer, A. J., McMichael, A., Ambroso, L. A., Nelson, J. M., Leopold, W. R., Conners, R. W., Bridges, A. J. (1994) Science 265, 1095–1099
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J. Biol. Chem. 2002, 277:17397-17405.
doi: 10.1074/jbc.M109962200 originally published online February 21, 2002

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