Insulin-like growth factor-binding protein-3 (IGFBP-3) is inhibitory to the growth of many breast cancer cells in vitro; however, a high level of expression of IGFBP-3 in breast tumors correlates with poor prognosis, suggesting that IGFBP-3 may be associated with growth stimulation in some breast cancers. We have shown previously in MCF-10A breast epithelial cells that chronic activation of Ras-p44/42 mitogen-activated protein (MAP) kinase confers resistance to the growth-inhibitory effects of IGFBP-3 (Martin, J. L., and Baxter, R. C. (1999) J. Biol. Chem. 274, 16407–16411). Here we show that, in the same cell line, IGFBP-3 potentiates DNA synthesis and cell proliferation stimulated by epidermal growth factor (EGF), a potent activator of Ras. A mutant of IGFBP-3, which fails to translocate to the nucleus and has reduced ability to cell-associate, similarly enhanced EGF action in these cells. By contrast, the structurally related IGFBP-5, which shares many functional features with IGFBP-3, was slightly inhibitory to DNA synthesis in the presence of EGF. IGFBP-3 primes MCF-10A cells to respond to EGF because preincubation caused a similar degree of EGF potentiation as co-incubation. In IGFBP-3-primed cells, EGF-stimulated EGF receptor phosphorylation at Tyr-1068 was increased relative to unprimed cells, as was phosphorylation and activity of p44/42 and p38 MAP kinases, but not Akt/PKB. Partial blockade of the p44/42 and p38 MAP kinase pathways abolished the potentiation by IGFBP-3 of EGF-stimulated DNA synthesis. Collectively, these findings indicate that IGFBP-3 enhances EGF signaling and proliferative effects in breast epithelial cells via increased EGF receptor phosphorylation and activation of p44/42 and p38 MAP kinase signaling pathways.

Insulin-like growth factor-binding protein-3 (IGFBP-3), a 45-kDa glycoprotein abundant in the circulation and extracellular environment, is a key regulator of the peptide hormones IGF-I and IGF-II (1). By virtue of its high affinity for these growth factors, IGFBP-3 competes for ligand binding with the receptor primarily responsible for mediating the actions of IGF-I and -II, the type I IGF receptor (IGFR1) (2), and thereby blocks mitogenic and anti-apoptotic signaling initiated by its activation. An important role for IGFBP-3 in modulating the proliferative effects of IGFs in many cell types is well recognized, and both exogenous and endogenous IGFBP-3 have been shown to block IGF action in breast cancer cells in vitro (3–6).

An additional role for IGFBP-3 also exists as a growth modulator with intrinsic bioactivity in breast cancer cells and other cells in vitro. The antiproliferative effects of a number of antitumor agents including transforming growth factor-β (TGF-β), vitamin D, and retinoic acid in breast cancer cells appear to be mediated, at least in part, by IGFBP-3 acting independently of IGF sequestration (7, 8). The IGF independence of IGFBP-3 actions is inferred from an inability of other IGFBPs to mimic the effect, persistence of the growth-inhibitory effect of IGFBP-3 in the presence of insulin or IGF analogs, which activate the IGFR1 but which do not bind IGFBP-3, and no clear evidence of IGFs being present in the system under investigation (7). In addition to its anti-mitogenic effects, IGFBP-3 exhibits pro-apoptotic activity in vitro. IGFBP-3 may sensitize breast cancer cells to apoptotic inducers such as ionizing radiation (9) and ceramide (10), and directly effect apoptosis via induction of pro-apoptotic proteins such as Bax and Bad (9). Collectively, these observations suggest that IGFBP-3 is an important antiproliferative agent in breast cancer cells, acting both through IGF-independent and IGF-modulatory pathways.

By contrast with these findings, however, IGFBP-3 may also be growth-stimulatory in vitro. In MCF-7 breast cancer cells, IGFBP-3 enhanced IGF-stimulated DNA synthesis (11) similar to its effects in fibroblasts reported previously (12). More recent studies have shown that proliferation of airway smooth muscle cells is stimulated by IGFBP-3 in the presence of serum (13), and IGFBP-3 increases proliferation of LNCaP prostate cancer cells in the absence of serum or IGFs (14). In another cell model, T47D breast cancer cells transfected with IGFBP-3 cDNA were initially growth-inhibited by the expressed protein, but with increasing passage number became resistant to its growth-inhibitory effects, and were instead growth-stimulated by the endogenous IGFBP-3 (15).

Such observations of growth-stimulation by IGFBP-3 in breast cancer cells, although difficult to reconcile with the many reports of its antiproliferative actions, are consistent with clinical data, which indicate that IGFBP-3 may be associated with indicators of poor outcome. Thus, IGFBP-3 in breast tumors correlates inversely with estrogen receptor expression, and is positively associated with aneuploidy, S-phase fraction, and tumor size (16–19). Although the significance of these associations is unclear, they imply that there may be...
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changes in sensitivity to the effects of IBGF-3 on breast epithelial cells in vivo, losing growth-inhibitory bioactivity and perhaps even accelerating tumor growth.

While investigating mechanisms that may underlie IBGF-3 insensitivity in breast cancer cells, we found that, whereas IBGF-3 inhibits DNA synthesis in MCF-10A breast epithelial cells, this response is lost when the cells have undergone ma-
lipigenesis via expression of estrogen receptor. Blockade of p44/42 MAP kinase-activation downstream of Ras restored IBGF-3 sensitivity, implicating this pathway in the development of resistance to IBGF-3. The present study was initiated to determine whether activation of Ras and the p44/42 MAP kinase pathway by epidermal growth factor (EGF), a potent mitogen for normal breast epithelial cells and many breast cancer cell lines, could similarly induce resistance to the inhibitory effects of IBGF-3 in MCF-10A cells. We now report that IBGF-3 enhanced the growth stimulatory effects of EGF in this cell line, and that the p44/42 and p38 MAP kinase pathways appear to be involved in this potentiation.

EXPERIMENTAL PROCEDURES

Reagents—Tissue culture reagents and plasticware were from Trace Biosciences (North Ryde, New South Wales, Australia) and Nunc (Roskilde, Denmark). Bovine serum albumin (BSA), bovine insulin, hydrocortisone, and EGF were purchased from Sigma, and cholera enterotoxin was from ICN Biomedicals Australasia (Seven Hills, New South Wales, Australia). Transforming growth factor-α (TGF-α) and heregulin (heregulin-α, EGF domain) were from Upstate Biotechnology, Inc. (Lake Placid, NY). Signaling pathway inhibitors were purchased from Calbiochem-Novabiochem (Alexandria, New South Wales, Australia): MEK inhibitor PD98059, PI 3-kinase inhibitor LY294002, and p38 MAP kinase inhibitor SB203580. Inhibitors were made up as 50 mM stock solutions in dimethyl sulfoxide, and stored at –20 °C. The following antibodies for Western blotting were purchased from Cell Signaling Technology (Beverly, MA): phospho-Thr-202/Tyr-204 and total p44/42 MAP kinase; phospho-Ser-473 and total Akt; phospho-Thr-180/Tyr-182 and total p38 MAP kinase; phospho-Tyr-1068 EGF and total EGF. Natural human IGFBP-3 was purified from Cohn fraction IV of human plasma, as reported previously (21). Recombinant human IGFBP-3 and IGFBP-5 were expressed in human 911 retinoblastoma cells using an adenoviral stock solutions in dimethyl sulfoxide, and stored at –20 °C. SDS-PAGE sample buffer (62.5 mM Tris-HCl, pH 6.8, containing 20 g/liter SDS, 100 ml/liter glycerol, 1 g/liter bromphenol blue, and 50 g/ml dithiothreitol) at 4 °C for 10 min. Lysates were scissored into cold Eppendorf tubes and frozen immediately at –80 °C. Prior to SDS-PAGE analysis, thawed lysates were sonicated for 15 s, heated at 90 °C for 10 min, cooled on ice, and then incubated at 15 °C for 2 min.

SDS-PAGE and Western Analysis—Prepared lysates were subjected to SDS-PAGE (7.5% separating gel for EGFR, 12% separating gel for p44/42, p38, and Akt) and electrophoretic transfer, as described previously (20). After transfer, filters were blocked in either 50 g/liter skim milk powder (for p44/42 and p38 MAPK) or 50 g/liter BSA (for EGFR and Akt) in Tris-buffered saline with Tween 20 (Tris, pH 7.4 containing 1 ml/liter Tween 20). The filters were washed as before, and then 125I-protein A was added to each well.

In Vitro Kinase Assays—Activity of p38 MAPK and p44/42 MAPK in lysates from IBGF-3- and EGF-treated MCF-10A cells was determined by assessing phosphorylation of Elk-1 (for p44/42 MAPK) and Atf-2 (for p38 MAPK) using reagents from Cell Signaling (Beverly, MA). Confluent monolayers in six-well plates were preincubated with or without IBGF-3 for 16 h and stimulated with EGF for 10 min. Cells were harvested with ice-cold phosphate-buffered saline (187.5 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10 g/liter Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μM/ml leupeptin) for 10 min at 4 °C. Lysates were scissored into Eppendorf tubes on ice and sonicated four times for 10 s each, then clarified by centrifugation at 15,000 rpm for 10 min at 4 °C. Supernatants (200 μl containing 200 μg of protein) were transferred to fresh Eppendorf tubes, and phospho-p44/42 MAPK (Thr-202/Tyr-204) and phospho-p38 MAPK (Thr-180/Tyr-182) immunoprecipitated overnight at 4 °C using phosphospecific antibodies immobilized onto agarose hydradze antibodies (Cell Signaling). Beads were washed twice with 500 μl of cell lysis buffer, once with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerol phosphate, 2 mM dithiothreitol, 0.1 mM sodium vanadate, 100 mM MgCl2), and resuspended in 50 μl of kinase buffer. Two micrograms of recombinant Elk-1 or Atf-2 was added to tubes containing the relevant kinase, with ATP to a final concentration of 200 μM. Phosphorylation reactions were carried out over 30 min at 30 °C and terminated by addition of 25 μl of 3X Laemmli sample buffer (187.5 mM Tris-HCl, pH 6.8, 60 g/liter SDS, 30% glycerol, 150 mM dithiobitol) and boiling for 5 min. Samples were vortexed, centrifuged for 2 min, and then loaded onto 10% polyacrylamide gels for SDS-PAGE analysis using antibodies against phosphorylated Elk-1 (Ser-383) or phosphorylated Atf-2 (Thr-71).

Statistical Analyses—All experiments were performed a minimum of two times and are shown as data pooled from the two experiments, unless indicated otherwise. Significance on pooled data was determined by analysis of variance using the Statview package for Macintosh.
RESULTS

We have shown previously that, in the absence of exogenous growth factors, IGFBP-3 is inhibitory to DNA synthesis in MCF-10A breast epithelial cells over 24 h, but that constitutive up-regulation of Ras-MAPK signaling abolishes the growth-inhibitory effect of IGFBP-3 (20). Because EGF stimulates activation of the Ras-MAPK pathway in these cells (data not shown), we therefore investigated whether it similarly affected growth response to IGFBP-3. As shown in Fig. 1A, EGF is a potent stimulator of DNA synthesis in MCF-10A cells over 24 h, with a significant effect apparent with 0.1 ng/ml EGF. In the absence of EGF, natural human IGFBP-3 inhibited DNA synthesis in MCF-10A cells, as reported previously (20) (data not shown). However, IGFBP-3 enhanced the stimulatory effect of low concentrations (0.1 and 1 ng/ml) of EGF (Fig. 1A), with a significant and maximal effect at 10 ng/ml IGFBP-3. The potentiating effect of IGFBP-3 was lost at high (10 ng/ml) EGF concentrations (Fig. 1A).

The effects of IGFBP-3 and EGF on cell proliferation were determined over 7 days in the absence of serum or other exogenous growth factors. EGF alone (1 ng/ml) increased cell numbers determined over 7 days in the absence of serum or other exogenous growth factors. EGF alone (1 ng/ml) increased cell number by 40% relative to control (Fig. 1B), and IGFBP-3 at 10 or 100 ng/ml as indicated, in serum-free medium, and cell numbers were determined 7 days later. Data are expressed as a percentage of the cell number in control (untreated) cultures, and are pooled from two experiments each carried out in triplicate wells. Significant differences shown are $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

IGFBP-3 shares many structural and functional features with another IGF-binding protein, IGFBP-5 (26). Therefore, we investigated whether IGFBP-5 was also able to potentiate EGF action in MCF-10A cells, using purified recombinant human IGFBP-5 expressed in an adenoviral expression system. As shown in Fig. 2A, adenovirus-derived recombinant human IGFBP-5 (adIGFBP-5) enhanced EGF-stimulated DNA synthesis similarly to plasma-derived IGFBP-3, with a significant effect at 1 ng/ml, and maximal enhancement at 10 ng/ml. In the absence of EGF, adIGFBP-5 had no significant effect on DNA synthesis in MCF-10A cells (data not shown). By contrast with adIGFBP-3, adIGFBP-5 did not potentiate EGF-stimulated DNA synthesis, and in fact was slightly inhibitory at low concentrations in the presence of EGF (Fig. 2B).

We then investigated whether a recombinant human IGFBP-3 mutant that exhibits decreased cell binding and nuclear import (27, 28) retains EGF potentiating activity. This mutant, IGFBP-3(mut), has 5 residues in the basic domain in the C-terminal region of IGFBP-3 substituted with the analogous sequence of IGFBP-1, i.e. $^{228}$KGRKR $\rightarrow$ MDGEA. Exogenous IGFBP-3(mut) showed significantly decreased cell association compared with wild type IGFBP-3 in MCF-10A cells (Fig. 2C), similar to that shown previously for endogenous mutant protein in Chinese hamster ovary cells (27). However, in MCF-10A cells some binding was evident at high concentrations of IGFBP-3(mut), with a significant increase in cell-associated IGFBP-3 detected with 1000 ng/ml IGFBP-3(mut), comparable to the binding of 100 ng/ml wild type IGFBP-3 (Fig. 2C). In the presence of EGF, IGFBP-3(mut) enhanced DNA synthesis to a degree similar to that for adIGFBP-3 (Fig. 2D), suggesting that the potentiation effect of IGFBP-3 occurs in the absence of its nuclear localization and under conditions where its cell association is markedly reduced.
The ability of IGFBP-3 to enhance the effects of other growth factors in MCF-10A cells was then investigated. TGF-α, which binds and activates the EGF receptor (also known as ErbB1), stimulated DNA synthesis in MCF-10A cells (Fig. 3A). The stimulation caused by 1 ng/ml TGF-α (−40%) was increased to −120% in the presence 100 ng/ml IGFBP-3 (Fig. 3B). As observed for EGF, the level of potentiation by IGFBP-3 decreased with increasing concentrations of TGF-α, so that with 10 ng/ml TGF-α, DNA synthesis was increased only an additional 20% by IGFBP-3 (Fig. 3C).

To examine whether IGFBP-3 enhanced the effects of hormones that act through other members of the EGF receptor/ErbB family, we tested its effects on DNA synthesis stimulated by heregulin, which binds the EGF receptor family members ErbB3 and ErbB4. In the absence of IGFBP-3, heregulin stimulated DNA synthesis in MCF-10A cells (Fig. 3D), with the maximum dose tested, 25 ng/ml, inducing a 12-fold increase in DNA synthesis. The stimulatory effects of low (0.3 ng/ml, Fig. 3E) or high (10 ng/ml, Fig. 3F) heregulin were not enhanced significantly by IGFBP-3. IGFBP-3 had no effect on DNA synthesis stimulated by platelet-derived growth factor or long-[Arg3]IGF-I, an IGF analog with affinity for IGF receptors but not IGFBPs (data not shown).

To determine whether co-incubation of IGFBP-3 and EGF was necessary for potentiation, MCF-10A cells were preincubated with IGFBP-3 for 24 h prior to its removal, then EGF was added for an additional 24 h. As shown in Fig. 4, pre-exposure to IGFBP-3 resulted in a similar degree of enhancement of EGF-stimulated DNA synthesis to that seen when EGF and IGFBP-3 were added together for 24 h, suggesting that preincubation with IGFBP-3 sensitizes MCF-10A cells to EGF.

We then investigated the effect of IGFBP-3 preincubation on subsequent binding of EGF to cell monolayers. Cells were preincubated with or without IGFBP-3 for 16 h, and then binding of 125I-EGF to cells over 2 h at 4 °C was determined. As shown in Fig. 5A, the amount of 125I-EGF bound to MCF-10A cells was not changed by pre-exposing the cells to IGFBP-3, and displacement curves generated by incubating cells with tracer in the presence of unlabeled EGF were superimposable in IGFBP-3-preincubated and untreated cells. These data suggest that exposure to IGFBP-3 does not alter EGF receptor affinity or number.

The effects of IGFBP-3 on EGFR activation were then examined by assessing Tyr-1068-phosphorylated EGFR in cells pretreated with IGFBP-3 and then exposed to EGF for 5, 15, or 60 min. In the absence of IGFBP-3 pretreatment, EGF-stimulated phosphorylation of EGFR was apparent within 5 min (Fig. 5B); there was no effect of IGFBP-3 on the degree of receptor phosphorylation at this time point. However in cells pretreated for 24 h with either 10 or 100 ng/ml IGFBP-3, EGFR phosphorylation was increased relative to non-preincubated cells within 15 min of exposure to EGF (Fig. 5B). The enhancing effect of IGFBP-3 on receptor phosphorylation was transitory, being
lost within 60 min after addition of EGF. Analysis of data from two similar experiments confirmed that pre-exposure to IGFBP-3 significantly enhanced EGF-stimulated phosphorylation of the EGFR at Tyr-1068 (Fig. 5, panel B). IGFBP-3 tested over similar doses, but without subsequent exposure to EGF, did not stimulate phospho-EGFR (data not shown).

Next, we examined whether IGFBP-3 modulates EGF activation of intracellular signaling, targeting particularly some pathways downstream of the EGFR. MCF-10A cells were preincubated with IGFBP-3 for 24 h and then exposed to EGF for 8 min. Cell lysates were analyzed by Western blotting using phosphospecific antibodies directed against p44/42 MAPK, Akt, and p38 MAPK. In the absence of EGF, there was no detectable phosphorylation of p44/42 or Akt, whereas a low level of phosphorylation of p38 MAPK was apparent (Fig. 6A). Phosphorylation of each of these signaling intermediates was stimulated within 8 min of treatment with 1 ng/ml EGF. Preincubation with IGFBP-3 had no effect on phosphorylation of Akt in response to EGF, but EGF-induced phosphorylation of p44/42 MAPK and p38 MAPK was enhanced by preincubation with IGFBP-3 with a maximal effect apparent between 8 and 15 min of EGF stimulation. Densitometric analysis of these data (Fig. 6B) indicated that IGFBP-3 enhanced EGF-stimulated phosphorylation of p44/42 MAPK and p38 MAPK by -2-fold and 30%, respectively. Analysis of lysates from cells stimulated for 1, 4, 8, 12, 18, and 24 h with EGF indicated that, although IGFBP-3 preincubation increased the magnitude of p44/42 and p38 MAP kinase phosphorylation stimulated by EGF at early time points, this was not sustained. Within 1 h of EGF treatment, phosphorylation of p44/42 and p38 MAP kinases had returned to basal levels, regardless of whether there had been pre-incubation with IGFBP-3 (data not shown). A second smaller peak of phosphorylation of p38 MAP kinase, but not p44/42 MAP kinase, was apparent 18 h after addition of EGF, but this was not enhanced in IGFBP-3-preincubated cells.

The effect of IGFBP-3 preincubation on EGF-stimulated p44/42 and p38 MAP kinase activity was then assessed using in vitro phosphorylation of substrates for these enzymes, Elk-1 and Atf-2, as markers of kinase activity. As shown in Fig. 7, lysates prepared from MCF-10A cells treated for 10 min with EGF showed increased activity of both p44/42 and p38 MAP kinases. The activity of both kinases was enhanced further in lysates from cells incubated with 10 or 100 ng/ml IGFBP-3 prior to exposure to EGF, indicating that IGFBP-3 potentiates EGF-stimulated p44/42 and p38 MAP kinase activity.

To determine the involvement of these pathways in the growth potentiating effects of IGFBP-3 on EGF action, we examined whether attenuation of the p44/42 MAPK or p38 MAPK signaling pathways altered the ability of IGFBP-3 to enhance the effects of EGF. MCF-10A cells were co-incubated for 24 h with IGFBP-3 and EGF in the presence of inhibitors of MEK upstream of p44/42 MAP kinase (PD98059), Akt (LY294002), or p38 MAPK (SB203580). DNA synthesis was determined after 24 h. The dose of inhibitors used in these experiments was intentionally submaximal, because concentrations high enough to block signaling fully through these pathways were cytotoxic over 24 h. As shown in Table 1, the doses used were sufficient to abolish the stimulatory effect of EGF on DNA synthesis, and Western blot analysis confirmed that these concentrations were sufficient to reduce the IGFBP-3-induced enhancement of EGF-stimulated phosphorylation of p44/42 and p38 MAP kinase (data not shown). As shown in Fig. 8, the potentiating effect of IGFBP-3 on EGF action was absent in the presence of the p44/42 MAPK inhibitor PD98059 (20 μM, Fig. 8A); in fact, 100 ng/ml IGFBP-3 caused significant inhibition of
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DNA synthesis in the presence of EGF when p44/42 MAPK pathway was blocked using LY294002 (10 μM) abolished the potentiating effect of IGFBP-3 (lanes 2 and 4) bands have been expressed as a percentage increase above control (no EGF or IGFBP; lane 1) for each protein. *, p < 0.05 compared with EGF alone.

**TABLE I**

| DNA synthesis | Control | 1 ng/ml EGF | % of control |
|---------------|---------|-------------|-------------|
| No inhibitor  | 100     | 441 ± 37    |             |
| PD98059 (20 μM) | 45 ± 8  | 129 ± 13   |             |
| SB203580 (10 μM) | 53 ± 1  | 79 ± 3     |             |
| LY294002 (10 μM) | 24 ± 2  | 106 ± 11   |             |

DNA synthesis in the presence of EGF when p44/42 MAPK activation was blocked (p < 0.05). Similarly, inhibition of the p38 MAPK pathway using SB203580 (10 μM) abolished the potentiating effect of IGFBP-3 on DNA synthesis in MCF-10A cells (Fig. 8F). Consistent with its lack of effect on Akt phosphorylation shown in Fig. 6, IGFBP-3 was still able to potentiate DNA synthesis stimulated by EGF when the PI 3-kinase pathway was blocked using LY294002 (10 μM, Fig. 8C). Collectively, these data suggest that IGFBP-3 potentiates EGF signaling in MCF-10A cells by enhancing its activation of p44/42 and p38 MAPK signaling pathways.

**DISCUSSION**

Numerous studies have shown that IGFBP-3 exerts growth-inhibitory effects in a variety of cell types, either through blockade of IGF-stimulated mitogenesis and cell survival (1) or via antiproliferative activity unrelated to its ability to bind IGFs (7, 9, 10, 20, 29); however, growth-stimulatory effects of IGFBP-3 are less well documented. Early studies in fibroblasts (12, 30) and MCF-7 breast cancer cells (11) suggested that ligand interaction was involved in the stimulatory effect of IGFBP-3 on IGF activity, with its mechanism of action thought to involve either prevention of ligand-induced down-regulation of the IGFR1 (11) or processing of the IGFBP-3 to forms with altered affinity for IGFs (30). A study in airway smooth muscle cells also indicated a growth-stimulatory role for IGFBP-3 in the presence of fetal calf serum (13). In a bovine mammary epithelial cell line transfected to express IGFBP-3, DNA synthesis was increased in response to IGF-I, insulin, and long-[Arg3]IGF-I (31), implying that IGF-IGFBP-3 interaction was not required for a potentiating effect. However, growth-stimulatory interactions between IGFBP-3 and systems other than the IGF axis have not been reported previously.

In this study we have shown that, in MCF-10A breast epithelial cells, IGFBP-3 enhances the potent growth-promoting effects of members of the EGF system, which has been implicated in the development and progression of malignant disease, by priming cells to respond to EGF and TGF-α. Although the underlying mechanism by which IGFBP-3 potentiates EGF action in these cells remains unclear, we made the novel and significant observation that in cells pre-exposed to IGFBP-3, EGF-stimulated phosphorylation of the EGFR at Tyr-1068 was increased. Phosphorylation of this residue is crucial to linking EGFR activation with the Ras-MAPK signaling pathway via Grb2 and Sos (32) and, although we did not explicitly determine whether EGFR kinase activity was increased, our observation of increased phosphorylation and activation of p44/42 and p38 MAPK signaling intermediates downstream of Ras in IGFBP-3-primed cells is consistent with increased Ras activation occurring consequent to increased Tyr-1068 phosphorylation of the EGFR.

At present it is unclear how IGFBP-3 might be bringing about an increase in EGFR phosphorylation. Preincubation with IGFBP-3 did not markedly affect steady-state binding of EGF to cells, suggesting that an overall increase in EGF-EGFR interaction is not involved in the potentiating effect of IGFBP-3, although more dynamic effects on receptor availability arising from changes in internalization or heterodimerization (33) were not investigated. It is possible that IGFBP-3 is modulating the expression or activity of molecules involved in regulating receptor interaction with binding partners such as the Ras exchange factor Sos1 (34), or EGFR dephosphorylation, such as phosphotyrosine phosphatases. IGFBP-3 activation of a phosphotyrosine phosphatase that dephosphorylates the EGFR was recently proposed (35), although the IGF independence of IGFBP-3 action in this model has not been clearly
demonstrated. Increased phosphorylation of the EGFR, as in the present study, would suggest decreased activity of phospho-tyrosine phosphatases rather than increased, and the pos-
sibility that this is involved in IGFBP-3-enhancement of recep-
tor phosphorylation is currently under investigation.

In MCF-10A cells, IGFBP-3 enhanced activation of the p44/42 and p38 MAP kinase signaling pathways, but not the PI 3-kinase pathway, in response to EGF stimulation. Involvement of the p44/42 and p38 MAP kinase pathways in progenestimulating activity of breast cancer cells to respond to EGF has also been demonstrated in T47D breast cancer cells (36), associated with induction and activation of multiple proteins, including EGF receptor family members and Stat (signal transducers and activators of transcription) proteins (36, 37). In experiments not presented in this report, there was no clear change in expression of total Stat1, Stat5a or Stat5b protein in IGFBP-
3-preincubated cells, suggesting that progestin and IGFBP-3
priming of cells to respond to EGF may involve distinct path-
ways and intermediates.

Although the structural elements of IGFBP-3 required for its interaction with EGF signaling have not been elucidated, our experiments suggest that its interaction with major cell-surface moieties is not required. The 228KGRKR motif of IGFBP-3 has been implicated in its cell association (27), and, indeed, we found that the mutant with this region replaced with the corresponding region of IGFBP-1, IGFBP-3(mut), exhibited greatly reduced binding to MCF-10A cells compared with plasma-derived IGFBP-3. Despite this, mutant IGFBP-3 enhanced EGF activity with similar potency to wild type IGFBP-3. Inhibition of IGFBP-3-enhancement of progestin receptor function requires cell-surface moieties, may be sufficient to elicit a response. Although a plasma membrane receptor with IGFBP-3 signal transduction capability, either for growth stimulation or growth inhibition, has not yet been iden-
tified, recombinant IGFBP-3 appeared to show increased po-
cency compared with plasma-derived IGFBP-3, with significant enhancement of EGF activity with 1 ng/ml adenooviral IGF-
BP-3, compared with 10 ng/ml plasma-derived IGFBP-3. Ad-
enooviral IGFBP-3 is essentially unphosphorylated, whereas plasma-derived IGFBP-3 has ~1 mol/mol serine phosphoryla-
tion (data not shown), raising the possibility that differences in the potency of IGFBP-3 from alternative sources may be caused by its differential phosphorylation.

Our observation of activation of the p44/2 and p38 MAPK signaling pathways, but not Akt/PKB, is in contrast with a study that showed, in fibroblasts, the PI 3-kinase pathway appears to be involved in IGFBP-3 potentiation of IGF action, with IGFBP-3 increasing the sensitivity of Akt/PKB to phosphor-
ylation by IGF-1 (47). This implies that IGFBP-3 may impact on numerous growth factor-regulated pathways, per-
haps in a cell- or growth factor-specific manner, to enhance cell proliferation.

Although attenuation of either the p44/42 or p38 MAP kinase pathways was sufficient to block the potentiating effect of IGF-
BP-3, inhibition of the p44/42 MAP kinase pathway alone had the additional effect of reinstating the growth-inhibitory activity of IGFBP-3 in MCF-10A cells. We have shown previously a similar reversal of refractoriness to growth inhibition by IGF-
BP-3 by blocking p44/42 MAP kinase activation in MCF-10A cells, in which chronic activation of this pathway occurs as a result of transfection with oncogenic ras (20). The results of the present study confirm that activation of Ras-MAPK signaling ablates the growth-inhibitory activity of IGFBP-3, and, impor-
tantly, extend these findings to show that interactions between IGFBP-3 and Ras-dependent signaling pathways may result in enhanced growth-stimulatory signaling in breast cells. Inhibi-
tion of this pathway has the potential both to block stimulatory activity arising from the interaction of IGFBP-3 with other induced phosphorylation of TGF-β signaling intermediates similar to plasma-derived IGFBP-3 in a T47D cell line (41), and IGFBP-3(mut) overexpression inhibited growth and induced apoptosis in T47D breast cancer cells (44). In the latter case, endogenous mutant IGFBP-3 may have direct intracellular effects, thereby overcoming the need for secretion and re-up-
take, which might require cell-surface binding. Bioactivity of exogenous mutant IGFBP-3 as in the present study and that of Fanayan et al. (41) is somewhat more difficult to explain, al-
though, as we demonstrated, cell binding of IGFBP-3 was not com-
pletely abolished by substitution of the basic residues, and residual binding of IGFBP-3(mut), perhaps to low abundance cell-surface moieties, may be sufficient to elicit a response. Notably, N-terminal fragments of IGFBP-3 that lack this do-
main are biologically active in a number of systems (6, 45, 46), frequently with increased potency compared with intact wild type IGFBP-3. Understanding the mode of action of IGFBP-3, as either a growth inhibitor or stimulator, and the structural determinants of such action remain important goals.

The concentrations of plasma-derived IGFBP-3 required for stimulation of EGF activity in MCF-10A cells (10–100 ng/ml) are similar to those required for its inhibitory activity in this cell line in the absence of EGF (20), up to an order of magnitude lower than those used in the demonstration of IGFBP-3 bioac-
tivity in some other cell systems (7, 41), and similar to the levels expressed by many cell types (1). In view of the fact that MCF-10A cells secrete ~30 ng/ml IGFBP-3 under the condi-
tions used in these experiments (20), it is somewhat surprising that they remain sensitive to exogenous IGFBP-3 at similar concentrations, and may reflect differences between the cell-
derived IGFBP-3 and exogenous IGFBP-3 used in this study. It is noteworthy that, when compared within the same experi-
ment, recombinant IGFBP-3 appeared to show increased po-
cency compared with plasma-derived IGFBP-3, with significant enhancement of EGF activity with 1 ng/ml adenooviral IGF-
BP-3, compared with 10 ng/ml plasma-derived IGFBP-3. Ad-
enooviral IGFBP-3 is essentially unphosphorylated, whereas plasma-derived IGFBP-3 has ~1 mol/mol serine phosphoryla-
tion (data not shown), raising the possibility that differences in the potency of IGFBP-3 from alternative sources may be caused by its differential phosphorylation.
growth factor systems and to restore its inhibitory activity. Clearly, the identification of the factors involved in the potentiation of IGFBP-3 of EGF action will be the next important step in delineating its growth-stimulatory role in breast cancer cells, and explaining its association with highly malignant cancers with poor prognosis.

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