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Estradiol and Antiestrogens Regulate a Growth Inhibitory Insulin-like Growth Factor Binding Protein 3 Autocrine Loop in Human Breast Cancer Cells*

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MCF-7 human breast cancer cells are commonly used to model tissues responsive to estrogens and antiestrogens. We examined the effects of estradiol and the antiestrogen ICI 182780 on MCF-7 cell proliferation and insulin-like growth factor binding protein 3 (IGFBP-3) gene expression. ICI 182780-induced growth inhibition was associated with increased transcription of the IGFBP-3 gene, increased IGFBP-3 mRNA abundance, and increased IGFBP-3 protein accumulation in the conditioned medium. The growth stimulatory effect of estradiol was associated with opposite effects, and the correlation between cellular proliferation and IGFBP-3 mRNA abundance was strong ($r = -0.91$). Recombinant IGFBP-3 inhibited basal and estradiol-stimulated MCF-7 cell proliferation, and an IGFBP-3 antisense oligodeoxynucleotide abolished antiestrogen-induced growth inhibition. These results provide evidence for an estradiol and antiestrogen-regulated IGFBP-3 growth inhibitory autocrine pathway in MCF-7 cells.

Insulin-like growth factors I and II (IGF-I[1] and IGF-II) are potent mitogens and inhibitors of apoptosis for many normal and neoplastic cell types, including normal and transformed breast epithelial cells (1, 2). Both IGF-I and IGF-II bind with high affinity to specific IGF-binding proteins (IGFBPs), which modulate their bioactivity. At least six IGFBPs have been described (3, 4). IGFBP-3 acts as a growth inhibitor in many (but not all (5)) experimental systems (reviewed in Ref. 6). Examples of data consistent with a growth inhibitory role for IGFBP-3 include the growth inhibition associated with IGFBP-3 gene transfection (6), the increased IGFBP-3 accumulation associated with senescence-related reduction of cellular proliferation (7), the increase in IGFBP-3 production associated with retinoid-induced growth inhibition (8), and the decrease in IGFBP-3 production associated with epidermal growth factor-stimulated proliferation (9). Early studies attributed the growth inhibitory action of IGFBP-3 to the reduction of IGF-I and/or IGF-II bioactivity resulting from competition for somatomedins between IGFBP-3 and the type I IGF receptor (10). However, there is recent evidence that IGFBP-3 also has growth inhibitory activity that is independent of its IGF binding properties (11–14).

Antiestrogens are widely used in breast cancer treatment, and it has been proposed that the inhibitory effect of these compounds on IGF-I expression contributes to their antiproliferative activity (15, 16) (reviewed in Ref. 17). Antiestrogens have significant trophic effects on the uterus (18), and there is a negative correlation between uterine weight and uterine IGFBP-3 expression; the positive uterotrophic actions of both estradiol and the partial estrogen receptor antagonist tamoxifen are associated with suppression of uterine IGFBP-3 expression, while the pure antiestrogen ICI 182,780 (19) causes uterine involution and markedly enhances uterine IGFBP-3 expression (20). We undertook the present study to examine the possibility that the anti-proliferative action of ICI 182780 on breast cancer cells is related to changes in IGFBP-3 gene expression induced by this drug.

EXPERIMENTAL PROCEDURES

The cDNA for human IGFBP-3 (21) and recombinant human IGFBP-3 (22) were provided by Celtrix Pharmaceuticals (Santa Clara, CA), the 18 S cDNA was a gift from Dr. C. Karatzas, and ICI 182,780 (19) was a gift from Dr. A. Wakeling (Zeneka Pharmaceuticals). 17β-Estradiol was purchased from Sigma and 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole from Calbiochem.

Cell Culture and Thymidine Incorporation Assay—Human breast cancer MCF-7 cells were obtained from ATCC and maintained as monolayer cultures in α-modified Eagle's medium (α-MEM) (Life Technologies, Inc.) supplemented with 5 μg/ml bovine insulin (Sigma) and 10% fetal bovine serum (Life Technologies, Inc.). We developed specific culture conditions for assays of DNA synthesis in the absence of serum. Confluent stock cultures were trypsinized and plated at 2.5×10^4 cells/well in 24-well dishes (Becton Dickinson, Lincoln Park, NJ) in α-MEM supplemented with 2.5% fetal bovine serum. After 48 h cell monolayers were rinsed twice with serum-free α-MEM and incubated for a further 24 h in α-MEM supplemented with 2.5% double charcoal stripped serum. Cells were then washed twice with serum-, estrogen-, and phenol red-free (SEPF) α-MEM and then incubated for 48 h in the presence or absence of various concentrations of compounds under study, as indicated in the figure legends. Conditioned media were collected and frozen until assayed for IGFBPs by ligand blotting. The effect of sense and antisense IGFBP-3 phosphorothioate oligodeoxynucleotides on cellular proliferation was studied under the same conditions, except that incubation prior to measurement of thymidine incorporation was for 24 h. The sense oligodeoxynucleotide was a 20-mer corresponding to the 20 amino-terminal nucleotides of the human IGFBP-3 mRNA (23, 24), and the antisense oligodeoxynucleotide was complementary to this sequence. The phosphorothioate oligodeoxynucleotides were prepared by the Biotechnology Research Institute (Montreal, Quebec). The cell number and thymidine incorporation end points have been previously shown to be well correlated in our cell culture system for MCF-7 cells (25). Thymidine incorporation was measured as described previously (25). Experiments were carried out in triplicate.

Northern Analysis—For RNA extraction, cells were treated as for tissue culture, except they were initially plated at 5×10^6 cells in

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[1] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, IGF-binding protein; α-MEM, α-modified Eagle's medium; SEPF, serum-, estrogen-, and phenol red-free; rhIGFBP, recombinant human IGFBP.

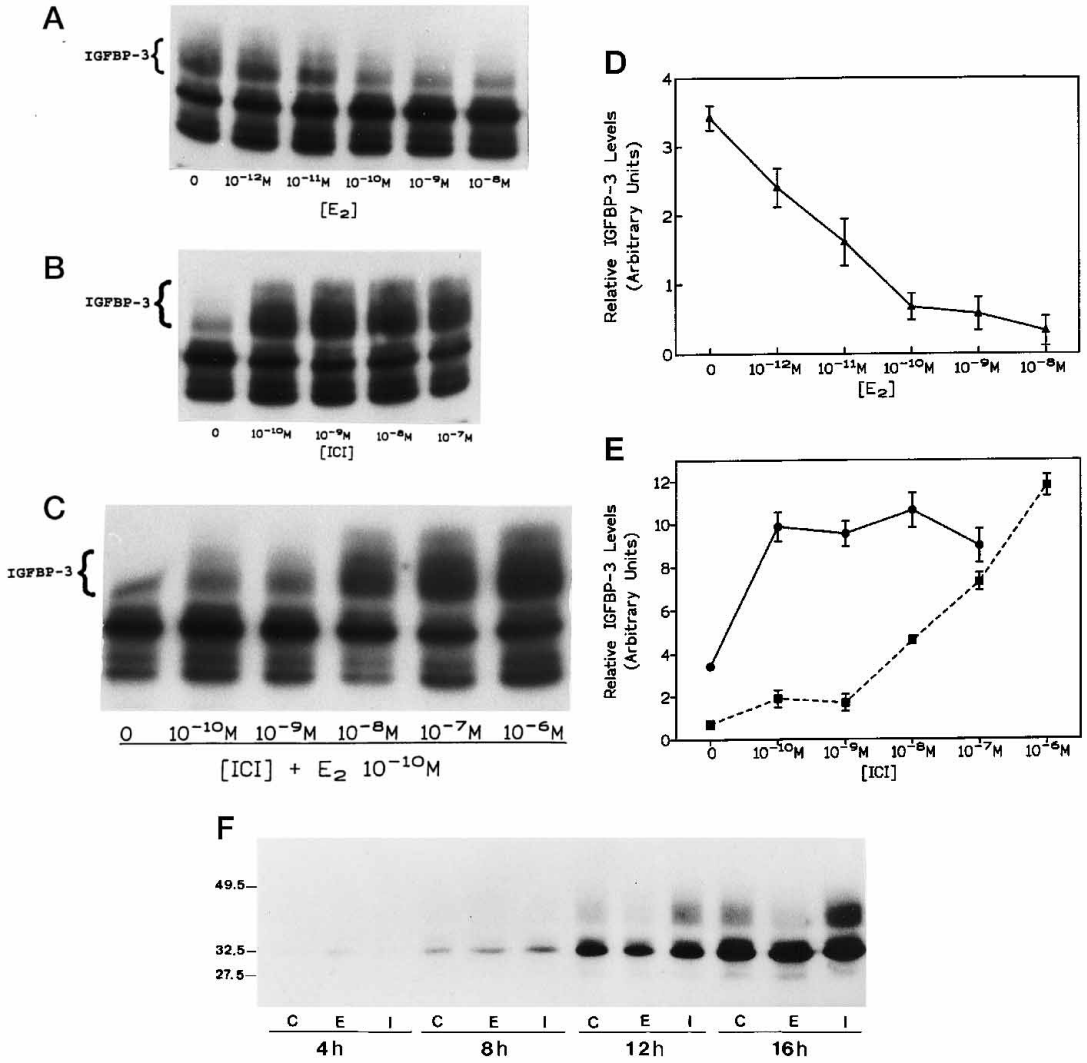

FIG. 1. **IGFBP-3 accumulation in MCF-7 cell-conditioned media during incubation with estradiol, ICI 182780, and ICI 182780 plus estradiol.** MCF-7 cells at 85–90% confluence were grown in SEPF medium for 24 h. Cells were then treated with indicated doses of estradiol (*A*), ICI 182780 (*B*), or 10^{-10} M estradiol plus various doses of ICI 182780 (*C*) for 48 h. Conditioned media were collected and concentrated, and ligand blot analysis was performed as described under "Experimental Procedures." The bands corresponding to IGFBP-3 were quantified densitometrically (*D* and *E*). Means ± S.E. of quadruplicate experiments are plotted. In *E*, the *solid line* joins data points for ICI 182780 alone, and the *dashed line* joins points for various concentrations of the drug in the presence of 10^{-10} M estradiol. The time course of estradiol and ICI 182780 effects on IGFBP-3 accumulation in MCF-7 cell-conditioned media is shown in *F*. MCF-7 cell-conditioned media were harvested at the indicated times after incubation with 10^{-10} M estradiol (*E*) or 10^{-7} M ICI 182780 (*I*), or neither (*C*), concentrated, and analyzed by ligand blotting.

100-mm dishes. Extraction of cytoplasmic RNA using RNAZol premix solution and RNAZol B method (Tel-Test, Friendswood, TX) and Northern blotting of 50-μg samples of total RNA were carried out as described previously (26).

Ligand Blotting—Ligand blotting on proteins present in 1 ml of conditioned medium was performed as described (20).

RESULTS

Effects of Estradiol and ICI 182780 on IGFBP-3 Accumulation in MCF-7 Cell-conditioned Media— Conditioned media from cells grown in the presence of estradiol, ICI 182780, or both, were assayed for IGFBP-3 by ligand blot analysis. MCF-7 cells growing in SEPF medium released four major species of IGFBPs: 24-, 28-, and 32-kDa bands and a complex spanning 38–42 kDa, which was identified as IGFBP-3 by Western blotting (data not shown). A prior report (27) failed to demonstrate the presence of IGFBP-3 in MCF-7-conditioned medium but described assays of media collected shortly after estrogen and/or serum stimulation. Estradiol treatment reduced IGFBP-3 levels compared with controls, while ICI 182780 treatment markedly increased IGFBP-3 accumulation (Fig. 1). The reduction in IGFBP-3 accumulation by estradiol was dose-dependent with 50% inhibition at a dose of 10^{-11} M.

Mechanism of Effects of Estradiol and ICI 182780 on IGFBP-3 Accumulation in MCF-7 Cell-conditioned Media— In keeping with a report showing the absence of IGFBP-3 proteolysis in MCF-7 cell-conditioned medium at physiological pH (28), Western blotting of MCF-7-conditioned media with a polyclonal anti-IGFBP-3 antibody that detects many IGFBP-3 proteolytic fragments showed no evidence of modulation of IGFBP-3 proteolysis by estradiol or ICI 182780 (data not shown). Northern analysis (Fig. 2) using a IGFBP-3 cDNA probe revealed important effects of estradiol and ICI 182780 on IGFBP-3 mRNA abundance. Densitometric scanning of Northern blots revealed that IGFBP-3 mRNA abundance was 3–4-fold higher in ICI 182780-treated cells than in controls and >20-fold higher than in estradiol-treated cells. Estradiol down-regulated IGFBP-3 gene expression in a dose-dependent manner with maximal inhibition at 10^{-9} M. Thus, the direction of change in IGFBP-3 protein accumulation associated with estradiol or ICI 182780 exposure was consistent with the direction of change in IGFBP-3 mRNA levels. Equimolar ICI 182780

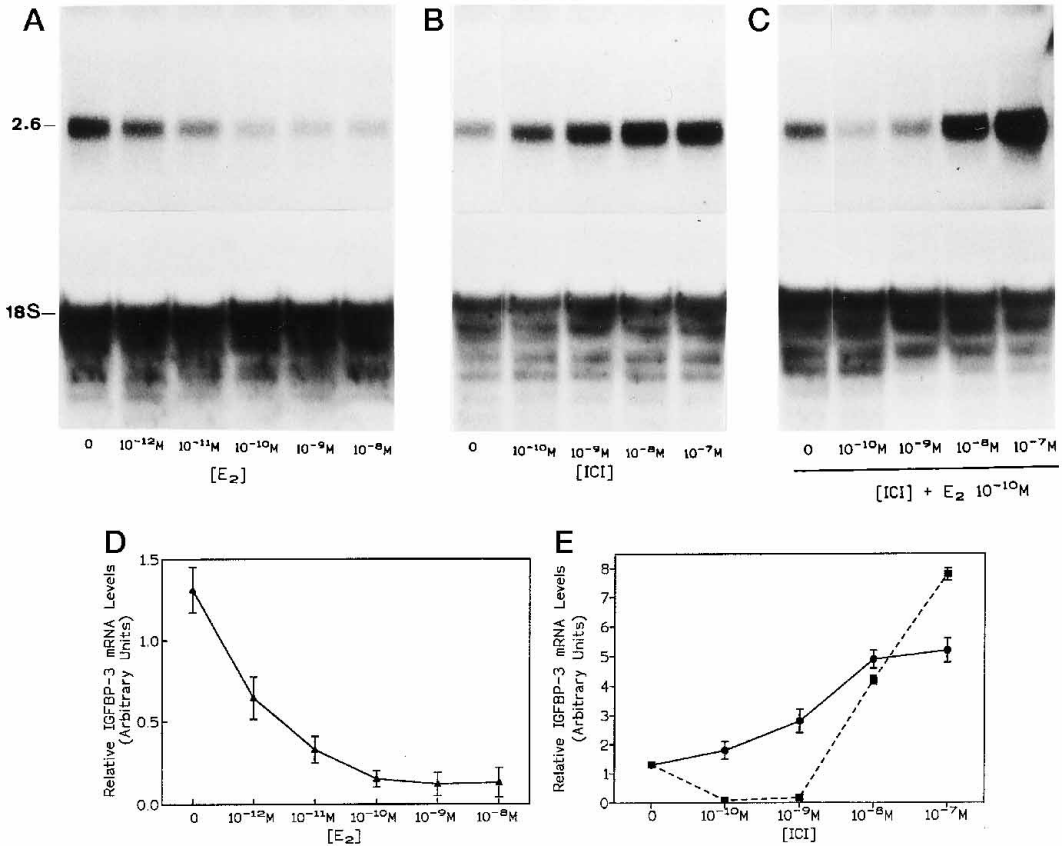

FIG. 2. **Regulation of IGFBP-3 mRNA levels by estradiol and ICI 182780.** MCF-7 cells at 85–90% confluence were grown in SEPF medium for 24 h. Cells were then treated with indicated doses of estradiol (*A*), ICI 182780 (*B*), or 10^{-10} M estradiol plus various doses of ICI 182780 (*C*) for 48 h. Cytoplasmic RNA was isolated and Northern blotting was performed as described under "Experimental Procedures." The 2.6-kilobase IGFBP-3 mRNA (*top panel*) and 18 S ribosomal mRNA (*lower panel*) are shown. The bands corresponding to IGFBP-3 were quantified densitometrically (*D* and *E*). Means ± S.E. of quadruplicate experiments are plotted. In *E*, the *solid line* joins data points for ICI 182780 alone, and the *dashed line* joins points for various concentrations of the drug in the presence of 10^{-10} M estradiol.

did not attenuate the suppression imposed by 10^{-10} M estradiol on IGFBP-3 mRNA abundance, but a 10-fold excess concentration of ICI 182780 abolished the inhibitory effects of estradiol on expression of the gene. A nuclear run-off assay demonstrated that ICI 182780 increased and estradiol decreased the rate of IGFBP-3 gene transcription relative to controls, and studies of IGFBP-3 message stability using the mRNA specific transcription inhibitor 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole demonstrated that the half-life for IGFBP-3 mRNA was approximately 9 h under control conditions and was not altered by estradiol or ICI 182780 treatments (data not shown). Since no estrogen response element has been demonstrated on the promoter region of the human IGFBP-3 gene (23), we speculated that estrogen and ICI 182780 act through other gene(s) whose products regulate the IGFBP-3 gene. To test this hypothesis, cycloheximide was (25 μg/ml) added with estradiol or with ICI 182780, and total RNA was isolated 6 h later for Northern blotting. Inhibition of protein synthesis by cycloheximide up-regulated IGFBP-3 mRNA abundance in estradiol-treated cells but had no effect on ICI 182780-treated cells (data not shown). These results are consistent with the hypothesis that synthesis of a regulatory protein that down-regulates IGFBP-3 gene expression is stimulated by estradiol and inhibited by ICI 182780.

Relationship of Effects of Estradiol and ICI 182780 on IGFBP-3 Gene Expression to Effects on Proliferation—As expected, treatment of MCF-7 cells with estradiol for 48 h resulted in a dose-dependent increase in DNA synthesis (Fig. 3*A*). Importantly, even in the complete absence of estrogenic stimulation (serum-, estradiol-, and phenol red-free conditions), ICI 182780 suppressed MCF-7 cell proliferation, indicating that this compound does not merely block estrogen-stimulated growth (Fig. 3*B*). Evidence for competition between the stimulatory effects of estradiol and the inhibitory effects of ICI 182780 was demonstrated by a series of titration experiments, exemplified by the shift to the right of the ICI 182780 dose-response curve when the experiment was performed in the presence of 0.1 nM estradiol at each ICI 182780 concentration (Fig. 3*B*).

Fig. 3*C* summarizes the relationship between IGFBP-3 gene expression and proliferation in MCF-7 cells by relating the thymidine incorporation data to the mRNA abundance data from the experiment described in Fig. 2. The assays of thymidine incorporation and IGFBP-3 mRNA abundance were performed under identical conditions at the same concentrations of estradiol or ICI 182780. A significant negative correlation ($r = -0.91$) between IGFBP-3 mRNA abundance and proliferation rate was detected, and the relationship fit a negative log function as shown.

In order to determine if IGFBP-3 could mimic the antiproliferative action of ICI 182780 on MCF-7 cells, we examined the effect of recombinant human IGFBP-3 (rhIGFBP-3) on MCF-7 cell proliferation under experimental conditions identical to those used to assay effects of estradiol and ICI 182780. Fig. 3*D* shows ~30% inhibition of both basal and estradiol-stimulated proliferation by 0.9 nM (25 ng/ml) rhIGFBP-3. Concentrations of rhIGFBP-3 up to 250 ng/ml did not further inhibit proliferation. These data raised the possibility that expression of IGFBP-3 is not merely a marker that correlates with actions of estradiol and antiestrogens but that it plays a role in mediating

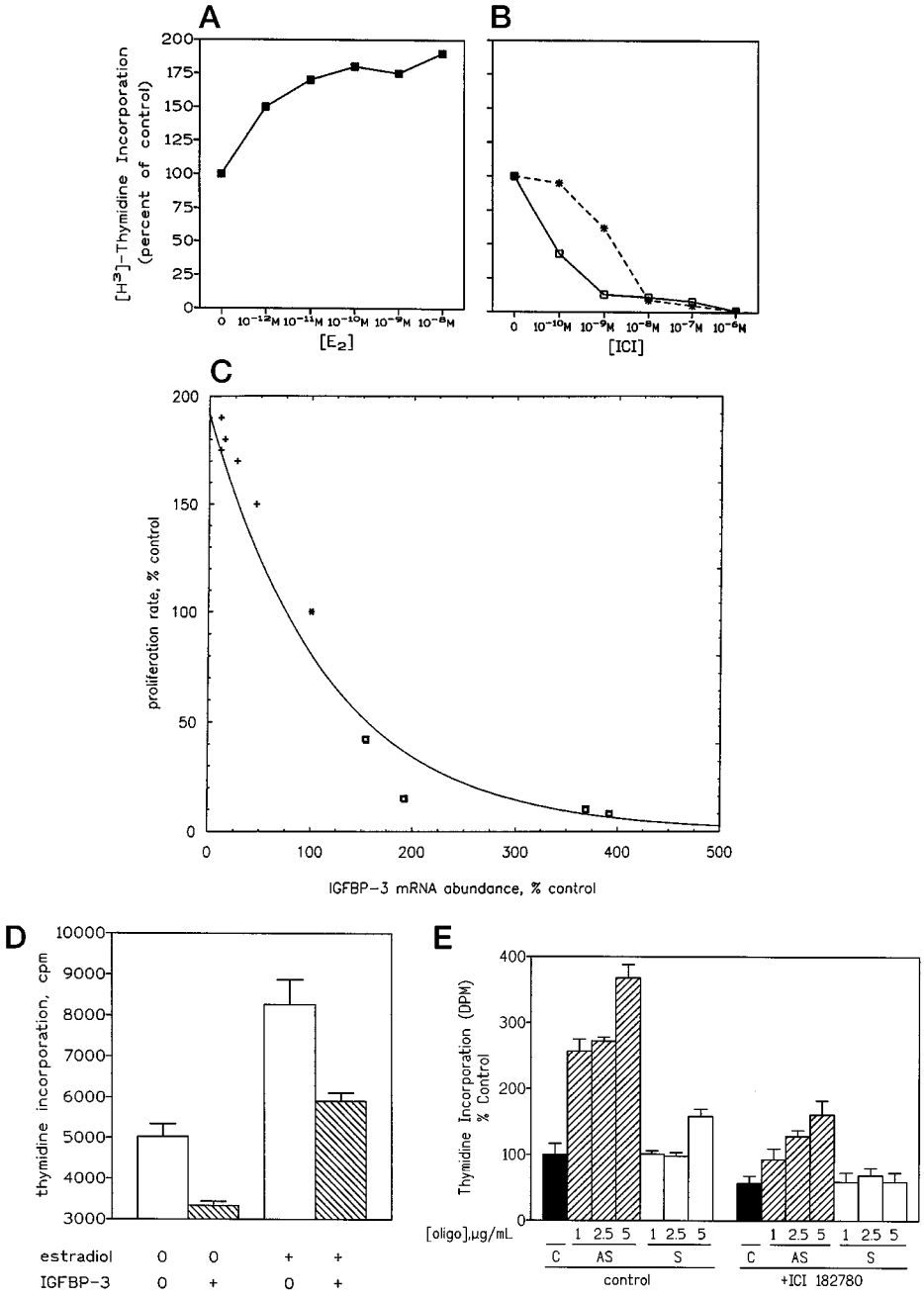

FIG. 3. **Effects of estradiol and ICI 182780 on MCF-7 cell DNA synthesis.** Cells were cultured as described under "Experimental Procedures" and then incubated for 48 h in SEPF medium with indicated doses of estradiol (*A*), ICI 182780 (*B*, *solid line*), or ICI 182780 in combination with 10^{-10} M estradiol (*B*, *dashed line*), prior to measurement of thymidine incorporation. Means of quadruplicate experiments are plotted. S.E. at each point was ≤14%. The relationship between IGFBP-3 mRNA abundance and proliferation rate of MCF-7 cells is shown in *C*. *Points* represent mean values of each variable at the SEPF control condition (*asterisk*) or various concentrations of ICI 182780 (*squares*) or estradiol (*crosses*). The effect of recombinant human IGFBP-3 on basal and estradiol-stimulated MCF-7 cell proliferation is shown in *D*. Cells were cultured as described under "Experimental Procedures" and then incubated for 48 h in SEPF medium in the presence or absence of recombinant human IGFBP-3 (0.9 nM) and of estradiol (10^{-11} M) for 48 h prior to measurement of thymidine incorporation. Means and S.E. of quadruplicate experiments are plotted. In both the presence and absence of estradiol, thymidine incorporation was significantly reduced in the presence of IGFBP-3 ($p < 0.01$, Mann-Whitney *U* test). The effect of sense (*S*) and antisense (*AS*) on MCF-7 cell proliferation is shown in *E*. Results are expressed relative to the control condition in the absence of ICI 182780 and oligodeoxynucleotides. Means and S.E. of quadruplicate experiments are plotted. Basal and ICI 182780-suppressed proliferation rates were significantly increased in the presence of antisense IGFBP-3 oligodeoxynucleotide ($p < 0.001$, Mann-Whitney *U* test).

these actions.

To determine if the antiproliferative action of ICI 182780 is mediated by its effect on IGFBP-3 expression, we used an IGFBP-3 antisense oligodeoxynucleotide (24) to examine the consequences of reducing basal and ICI 182780-stimulated IGFBP-3 secretion. Western blotting confirmed that this oligodeoxynucleotide reduced IGFBP-3 accumulation in MCF-7-conditioned media (data not shown). Basal MCF-7 thymidine incorporation increased with increasing concentration of the antisense IGFBP-3 oligodeoxynucleotide and was 3-fold higher than control at 5 µg/ml. Sense IGFBP-3 oligodeoxynucleotide was without significant effect (Fig. 3*E*). This result was consistent with our prior observation that in the absence of serum and estrogens, MCF-7 cells secrete IGFBP-3 and suggests that the low proliferation rate under these conditions is not entirely attributable to lack of mitogenic stimulation but is in part a consequence of autocrine growth inhibition mediated by IGFBP-3 expression. In the presence of 10^{-10} M ICI 182780, thymidine incorporation by MCF-7 cells was 57 ± 11% of control values, and we observed that this antiestrogen-induced inhibition was completely abolished by 1 µg/ml IGFBP-3 antisense oligodeoxynucleotide, while an equal concentration of the sense oligodeoxynucleotide had no significant effect. While these results imply that in our experimental system the early inhibitory action of ICI 182780 can be entirely attributed to stimulation of IGFBP-3 gene expression, the importance of this mechanism relative to other mechanisms underlying the action of antiestrogens (18, 29) *in vivo* remains to be determined.

DISCUSSION

Our data provide evidence for a previously unrecognized aspect of estrogen and antiestrogen action: estradiol-stimulated proliferation involves relaxation of an autocrine IGFBP-3 inhibitory influence, and the antiproliferative effect of ICI 182780 is related at least in part to up-regulation of IGFBP-3 gene expression (Fig. 4). A precedent for antiestrogen up-regulation of autocrine growth inhibitory pathways comes from the demonstration that tamoxifen up-regulates expression of

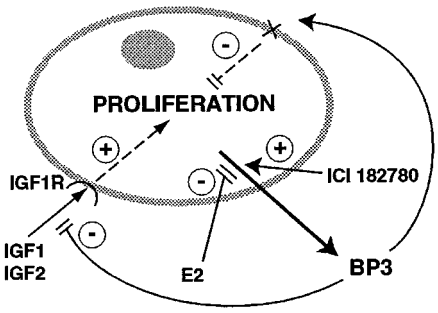

FIG. 4. **Regulation of an autocrine IGFBP-3 inhibitory loop in estrogen receptor positive human breast cancer cells by estradiol and antiestrogens.** Estradiol inhibits and antiestrogens stimulate IGFBP-3 production. Maximal stimulation of IGFBP-3 gene expression by the pure antiestrogen ICI 182780 is approximately 8-fold higher than achievable with tamoxifen, an antiestrogen commonly used in breast cancer treatment. IGFBP-3, in turn, may inhibit proliferation by a direct pathway (11–14) and/or by competing with IGF-I receptors for IGF-I or IGF-II.

TGFβ (reviewed in Ref. 30). As TGFβ increases IGFBP-3 accumulation in media conditioned by fibroblasts (31, 32) and recent data suggest that IGFBP-3 plays a role in mediating TGFβ-induced growth inhibition in Hs578T cells (24), we considered the possibility that ICI 182780 stimulation of IGFBP-3 expression might be mediated via changes in TGFβ expression but observed no significant changes in TGFβ expression in MCF-7 cells by ICI 182780 (data not shown). In our experimental system, exogenous IGFs are absent, and autocrine expression of IGF-I and IGF-II could not be detected (data not shown). Therefore, the observed inhibitory action of IGFBP-3 may involve a direct growth inhibitory signal transduction pathway (11, 12) rather than reduction of bioavailability of IGFs for cell surface receptor binding. However, these mechanisms are not mutually exclusive, and both may be relevant to the *in vivo* actions of estrogens and antiestrogens.

A recent clinical trial (33) demonstrates that ICI 182780 can induce clinical responses even in breast cancers resistant to tamoxifen, an antiestrogen widely used clinically. In addition, a recent *in vivo* study demonstrated that tamoxifen is a less potent inhibitor of *in vivo* MCF-7 cell proliferation than ICI 182780 (34). Tamoxifen is a partial antagonist to the estrogen receptor and exhibits estrogenic or antiestrogenic activities in a species-specific and tissue-specific manner (18). For example, in the oophorectomized rat model (as well as in clinical use) tamoxifen has an estrogen-like stimulatory effect on uterine growth, while ICI 182780 causes uterine involution. It has been demonstrated that these uterine actions of antiestrogens are well correlated with effects on IGFBP-3 expression, assuming a growth inhibitory action of this protein; the positive uterotrophic effect of tamoxifen is associated with down-regulation of uterine IGFBP-3 expression, while ICI 182780-induced uterine involution is associated with up-regulation of uterine IGFBP-3 expression (20). In view of these results, we determined the effect of tamoxifen on MCF-7 cell proliferation and IGFBP-3 gene expression under our *in vitro* conditions. We observed that tamoxifen was 2 orders of magnitude less potent than ICI 182780 in up-regulating IGFBP-3 mRNA abundance and that maximum stimulation was only to ~125% of control values, as compared with the 8-fold stimulation seen with ICI 182780 (data not shown). Taken together, these data demonstrate a consistent relationship between the effects of antiestrogens on proliferation and on IGFBP-3 expression, with respect to both the degree of growth inhibition in the case of MCF-7 cells and to the direction of proliferative change in the case of the uterus.

We have noted (data not shown) that estrogen receptor negative breast cancer cell lines exhibit high rates of base-line proliferation relative to estrogen receptor positive cells despite the fact that they constitutively express IGFBP-3. In view of the inhibitory effect of estradiol on IGFBP-3 expression in estrogen receptor positive cells, the constitutive expression of IGFBP-3 in estrogen receptor negative breast cancer cell lines is not unexpected and may be a consequence of the absence of function of the signal transduction pathway that links the estrogen receptor to the expression of the IGFBP-3 gene. While the high proliferation rate of estrogen receptor negative breast cancer cells in the presence of abundant autocrine IGFBP-3 appears paradoxical in the context of the action of IGFBP-3 on MCF-7 cells reported here, it is possible that this is a consequence of defects in the signal transduction pathways that mediate the "direct" growth inhibitory actions of IGFBP-3 (11, 12) and/or of unrelated aspects of neoplastic progression that bypass the inhibitory influence of IGFBP-3. The influence of agonist and antagonist estrogen receptor ligands on IGFBP-3 expression in MCF-7 cells appears greater in magnitude than the effect of TGFβ on IGFBP-3 expression by an estrogen receptor negative breast cancer cell line, which constitutively secretes this protein (24).

In summary, expression of the IGFBP-3 gene by MCF-7 cells is detectable under serum-free conditions, enhanced by ICI 182780 in the presence or absence of estrogens, suppressed by estradiol, and negatively correlated with proliferation. This demonstrates that ICI 182780 does not merely act as a competitive antagonist to block estrogen action but rather actively regulates IGFBP-3 gene expression and cellular proliferation in a direction opposite to that of estradiol. Furthermore, estrogen-stimulated MCF-7 cell proliferation is attenuated by IGFBP-3, and the antiproliferative action of the antiestrogen ICI 182780 is reduced by blocking IGFBP-3 translation. While antiestrogens have previously been noted to have effects on IGF-I expression (15–17), these data provide evidence for a novel mechanism by which estrogens and antiestrogens influence IGF physiology and cellular proliferation, suggest novel mechanisms that may be involved in the important clinical problem of antiestrogen resistance, and raise the possibility that in general steriod hormone action involves modulation of expression of IGF binding proteins.

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