Estrogen and Antiestrogen Modulation of MCF7 Human Breast Cancer Cell Proliferation Is Associated with Specific Alterations in Accumulation of Insulin-like Growth Factor-binding Proteins in Conditioned Media[1]

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ABSTRACT

Many neoplastic cell lines secrete insulin-like growth factor binding proteins (IGFBPs). The physiological role of these proteins is incompletely characterized; under various conditions IGFBPs have been observed to either enhance or inhibit the biological activity of insulin-like growth factors. MCF7 human breast cancer cells are known to be mitogenically responsive to insulin-like growth factors and estrogens, to secrete several IGFBPs, including BP-2, BP-4 and BP-5, and to be growth inhibited by antiestrogens. We report here that the pure antiestrogen ICI 182,780 and, to a lesser extent, the commonly used drug tamoxifen significantly increase levels of a M_r 43,000-46,000 IGFBP (BP-3) and significantly reduce levels of a M_r 24,000 IGFBP (BP-4) in the conditioned medium of MCF7 cells. Effects of estradiol and antiestrogens on M_r 30,000 and M_r 36,000 IGFBPs are also described. The effects of ICI 182,780 on IGFBPs in the conditioned medium of MCF7 cells may contribute to the remarkable ability of this compound to attenuate insulin-like growth factor I stimulated MCF7 cell proliferation.

INTRODUCTION

There is ample evidence that breast cancer cells are mitogenically responsive to IGFs[3] (1, 2). Type I IGF receptors are present in both breast cancer biopsy specimens (3, 4) and breast cancer cell lines (5, 6). Furthermore, the mitogenic response of breast cancer cells to IGFs can be attenuated by a blocking antibody against the type I IGF receptor, both *in vitro* (7, 8) and *in vivo* (9). A positive correlation between estrogen receptor level and type I IGF receptor expression has been observed in primary breast cancers (10, 11). In ER+ breast cancer cell lines, the mitogenic effects of IGFs (12, 13) or insulin at concentrations sufficient to interact with the type I IGF receptor (14) are enhanced in the presence of estrogens. Antiestrogens reduce proliferation of estrogen dependent tumors *in vivo* (15, 16) and of ER+ cell lines cultured not only in the presence but also in the absence of estrogens (17–20). Furthermore, in the absence of estrogenic stimulation, antiestrogens have been shown to attenuate the actions of insulin, IGF-I, and other growth factors (18, 21). Both estrogens and antiestrogens have effects on IGF-I binding (12, 22) and type I IGF receptor gene expression (12) in ER+ cell lines, indicating that IGF responsiveness may be regulated in part at the receptor level.

Six IGFBPs have been characterized and have been shown to modulate IGF action (23, 24). Patterns of IGFBP expression have been examined in a number of breast cancer cell lines (25–27). Estradiol has been shown to influence IGFBP production and gene expression in the ER+ MCF-7 cell line (26–28); however, effects of the antiestrogen tamoxifen on IGFBP expression have only been observed at the RNA level (27).

The ability of "antiestrogens" to attenuate IGF-I stimulated proliferation of ER+ breast cancer cells, even in the absence of estrogens, suggests that these agents can actively induce growth inhibitory signals, rather than merely block estrogen stimulated proliferation. To date, the only candidate antiestrogen activated growth inhibitor described is TGFβ (29). Because IGFBPs may also function as growth inhibitors (24, 30), we undertook to more completely describe estrogen and antiestrogen regulation of IGFBPs in MCF7 cells.

MATERIALS AND METHODS

Cell Culture. MCF-7 cells (obtained from American Type Culture Collection) were maintained in log-phase culture in α-modified Eagle's medium (GIBCO) supplemented with 10% FCS (GIBCO), 5 μg/ml bovine insulin (Sigma), and garamycin at 37°C and a 5%CO_2 environment. Experimental conditions involved plating at 25,000–50,000 cells/cm^2 in phenol red-free Dulbecco's modified Eagle's medium (4 mg/ml glucose; GIBCO) supplemented with DCC-FCS to remove steroids. The cells were incubated in this medium for 3 days prior to treatment in order to eliminate endogenous steroid hormones. The medium was changed to 5%DCC-FCS/phenol red-free DMEM including tamoxifen (Sigma), ICI 182,780 (a generous gift from A. Wakeling; ICI Pharmaceuticals), and/or 17β-estradiol (Sigma) which were prepared as stock solutions in ethanol. Control conditions included the appropriate concentration of ethanol, which was $\leq$0.1%.

[^{3}H]Thymidine Incorporation. After 2 days of treatment, [^{3}H]thymidine was added to medium to achieve a final concentration of 0.1 μCi/ml. Incorporation was allowed to proceed for 2 h at 37°C, after which, monolayers were precipitated with 10% trichloroacetic acid for 1 h at 4°C. The acid insoluble precipitate was solubilized in 1 N NaOH and incorporated radioactivity was evaluated by a liquid scintillation counter. Under these experimental conditions, [^{3}H]thymidine incorporation data correlated with results obtained through cell growth studies (data not shown).

IGFBP Evaluation. After 2 days of treatment in 5%DCC-FCS containing medium, the treatment of subconfluent monolayers was continued in serum free phenol-free DMEM for 16 h. One milliliter of conditioned medium from each experimental condition was centrifuged at 2000 rpm for 10 min prior to concentration (10-fold) with a Centricon 10 microconcentrator (Amicon). Concentrated conditioned medium proteins were separated on a 12% sodium dodecyl sulfate-polyacrilamide gel under nonreducing conditions and transferred to nitrocellulose paper (Bio-Rad). IGFBPs were detected by the Western ligand blotting method of Hossenlopp *et al.* (31) using [^{125}I]-IGF-I which had been iodinated to a specific activity of 150–200 μCi/μg by the chloramine T method. Using methods similar to those reported by Giudice *et al.* (32), the IGFBP bands were quantitated by an LKB densitometric scanner (Pharmacia) interfaced with Gelscan software (Pharmacia). The IGFBP band densities are expressed in arbitrary units which denote the integrated area under the absorbance peak.

At the time of conditioned medium collection, replicate monolayers were analyzed for protein content. The protein content of the 0.1 nM 17β-estradiol, 1 μM tamoxifen, and 1 μM ICI 182,780 treated monolayers were 131 ± 7%, 95 ± 9%, and 85 ± 9%, respectively, of the control condition. These values were used in the correction of raw data for the statistical analysis in Fig. 7.

Western Immunoblot Analysis. The identities of the M_r 43,000–46,000 and M_r 24,000 binding proteins were confirmed through Western immunoblot-

ting of ligand blots. Briefly, blocked ligand blots were exposed to a rabbit polyclonal antibody directed towards rBP-3 (1:1000 dilution; Celltrix Pharmaceuticals) or to a rabbit antibody preparation directed towards rBP-4 (1:100 dilution; Austral Biomedical). Binding was detected after incubation with an alkaline phosphatase conjugated goat anti-rabbit serum (Bio-Rad) and NBT/BCIP substrate solutions (Bio-Rad).

RESULTS

The effects of 17β-estradiol, tamoxifen, and the pure antiestrogen, ICI 182,780 on MCF-7 cell proliferation were compared under estrogen-free, serum containing conditions (5%DCC-FCS in phenol red-free DMEM) (Fig. 1). Under these conditions, 0.1 nM 17β-estradiol elicited a significant 1.7-fold increase in the amount of [^{3}H]thymidine incorporation after 2 days of treatment, and tamoxifen and ICI 182,780 at concentrations of 1 μM depressed MCF-7 cell proliferation by 73 and 90%, respectively. IGF-I (1.3 nM) stimulated [^{3}H]thymidine incorporation 1.6-fold under these same conditions, and this stimulation was significantly inhibited when cells were coincubated with IGF-I and 1 μM ICI 182,780 (Fig. 2).

Under the same conditions used to evaluate MCF-7 cell proliferation in Fig. 1, the effects of 17β-estradiol, tamoxifen, and ICI 182,780 on IGFBPs were evaluated by performing Western ligand blots on equivalent volumes of cell conditioned medium after 2 days of treatment. Fig. 3 depicts a typical Western ligand blot. Control cells (Fig. 3, *Lane 1*) displayed three predominant IGF-I binding proteins with molecular weights of M_r 24,000 30,000, and 36,000. The M_r 24,000 binding protein corresponds with the molecular weight observed for BP4 (33), and the identity of this band as BP-4 was confirmed through immunoblotting (data not shown). The M_r 36,000 binding protein corresponds to the binding protein which has been designated as BP2 (34), and the M_r 30,000 protein, which occurs as a broad band, is most likely BP5 (35) in accordance with the findings of McGuire *et al.* (28).

The effects of 17β-estradiol on IGFBP expression are illustrated in Fig. 2, *Lanes 2–4*, and quantitative evaluation through scanning densitometry reveals a dose dependent increase in IGF-I binding to BP-4, as well as to the M_r 30,000 and M_r 36,000 IGFBPs (Fig. 4). These findings agree with previous reports showing that estradiol treatment of MCF-7 cells results in increased levels of BP2 and BP4 (26) and elevated mRNA levels of BP2, BP4, and BP5 (27, 28).

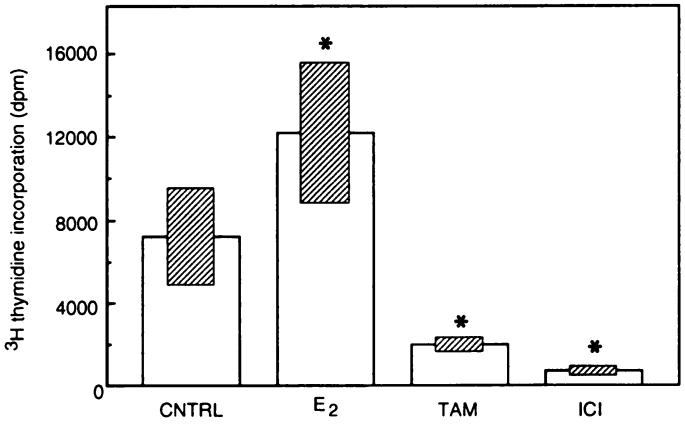

Fig. 1. Effects of estradiol, tamoxifen, and ICI 182,780 on MCF7 cell proliferation. MCF7 cells were cultured for 3 days in phenol-red free DMEM containing 5% DCC-FCS to remove endogenous steroids. Cell proliferation was assessed by [^{3}H]thymidine incorporation after 2 days of treatment in the continuing presence of 5% DCC-FCS, as described in "Materials and Methods." CNTRL, control (estrogen free) condition; E_2, 0.1 nM 17β-estradiol; TAM, 1 μM tamoxifen; ICI, 1 μM ICI 182,780. Data are expressed as the mean ± SEM and were tested for statistical significance relative to the control by a paired *t* test (n = 7). *, $P < 0.05$.

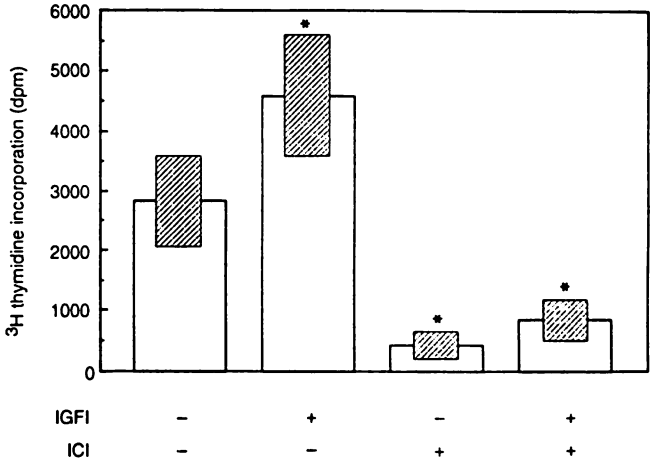

Fig. 2. Effects of ICI 182,780 on IGFI stimulation of MCF7 cell proliferation. Cell culture conditions were as described in Fig. 1. MCF7 cell proliferation was assessed by [^{3}H]thymidine incorporation following 2 days of in the absence (-) or presence (+) of 1 μM ICI 182,780 and in the presence (+) and absence (-) of 1.3 nM IGF-I. Data are expressed as the mean ± SEM and were tested for statistical significance relative to the control by a paired *t* test (n = 4). *, $P < 0.05$.

Antiestrogen treatment with tamoxifen or ICI 182,780, in the absence of estrogen (Fig. 3, *Lanes 5* and *9*, respectively) resulted in decreased levels of BP-4 when compared to the control condition, and increased detection a M_r 43,000–46,000 binding protein which was confirmed as BP3 (36) by immunoblotting (data not shown). Under culture conditions which included estradiol during the course of treatment, we observed that a reversal of the antiestrogen effect on IGFBPs occurs, particularly with respect to BP-3 and BP-4 (Fig. 3).

The concentration effects of ICI 182,780 on IGFBP accumulation in conditioned medium were examined, and quantitative evaluation revealed that regulation of IGFBP levels occurs in a dose dependent manner (Fig. 5). ICI 182,780 treatment, in the absence of estrogens, most dramatically influences the levels of BP-4 and BP-3 detected in the conditioned medium. Fig. 6*A* demonstrates that as proliferation declines, a reciprocal regulation of BP-3 and BP-4 occurs under conditions of increasing concentrations of ICI 182,780 (Fig. 6*B*). Both BP-4 and BP-3 band densities demonstrated a high correlation with cellular proliferation (r = 0.98 and -0.83, respectively) in this data set.

Fig. 7 presents statistical analysis of IGFBP levels under the four conditions presented in Fig. 1 (*i.e.*, estrogen free control *versus* cells treated with 0.1 nM estradiol, 1 μM tamoxifen, or 1 μM ICI 182,780). Prior to analysis, densitometric values for each condition were corrected for monolayer protein content at the time of conditioned medium collection. To normalize the data from each autoradiograph for statistical analysis, a standard for each ligand blot was created by averaging the corrected band densities across the four conditions under study and assigning the average density a value of 100 arbitrary units. The data from each experimental band were then expressed relative to the standard value for the IGFBP band on that autoradiograph. The normalized data from five independent experiments were then evaluated for statistical differences relative to the control condition. The data in Fig. 7 present the mean (± SEM) of these measurements. Asterisks indicate statistically significant ($P < 0.05$) differences as determined by the Mann-Whitney U test.

In the experiments evaluated in Fig. 7, we were able to demonstrate the presence of BP-3 at low but detectable levels in the control condition. Estradiol treatment resulted in significant increases in BP-4 and the M_r 30,000 and M_r 36,000 binding proteins and decreased levels of BP-3. Neither tamoxifen nor ICI 182,780 affected the M_r 36,000 protein, while both antiestrogens caused a significant decrease

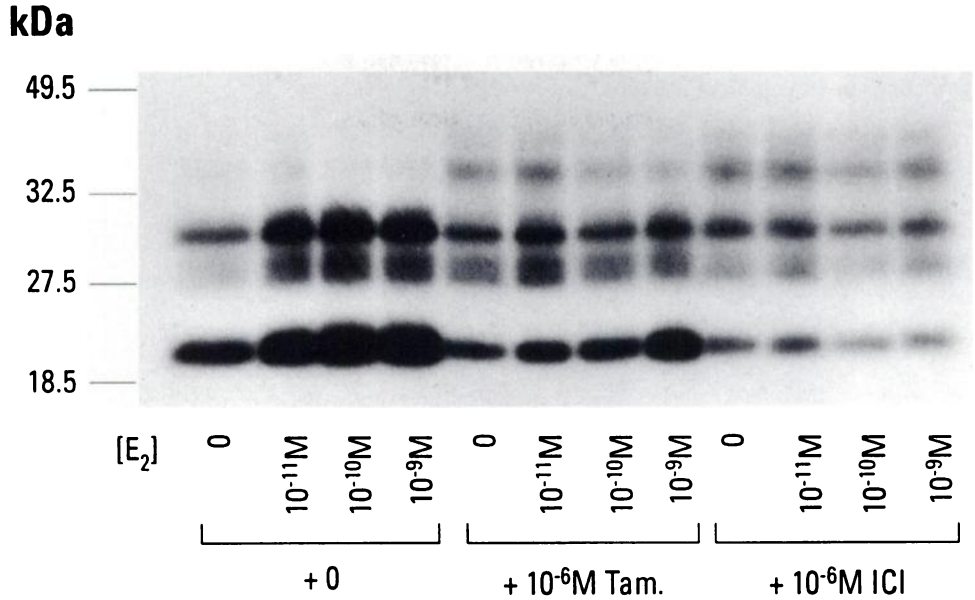

Fig. 3. Western ligand blot of [^{125}I]-IGF-I detected IGFBPs. Cells were cultured and treated for 2 days as described in Fig. 1. IGFBPs were then collected in serum free treatment containing medium as described in "Materials and Methods." Each lane contains the equivalent of 1 ml of cell conditioned medium. In this representative ligand blot, cells were treated with increasing concentrations of 17β-estradiol (10^{-11} M – 10^{-9} M) alone or in combination with 1 μM tamoxifen or 1 μM ICI 182,780.

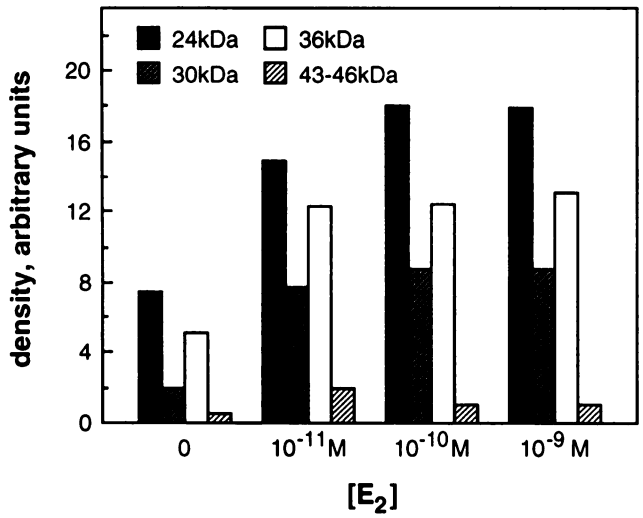

Fig. 4. Effects of 17β-estradiol on IGFBP levels. Estrogen withdrawn MCF7 cells were treated with increasing concentrations of 17β-estradiol, and equivalent volumes of conditioned medium were evaluated by Western ligand blot. IGFBP bands were quantitated by densitometric scanning. *Bars* correspond to the relative abundance of each of the four IGFBP species at each estradiol concentration. Data presented are from a representative experiment.

in BP-4. Increases in BP-3 occurred with both antiestrogens, but were greater with ICI 182,780 treatment. The M_r 30,000 binding protein was differentially regulated by the antiestrogens, being significantly increased by tamoxifen, and virtually unaltered by treatment with ICI 182,780.

Table 1 quantitates the total IGF-I binding capacity of the IGFBPs in the conditioned medium under the control, 0.1 nM 17β-estradiol, 1 μM tamoxifen, and 1 μM ICI 182,780 treatment conditions and presents the relative contributions of each of the IGFBP species to the total. Only estradiol treatment resulted in an appreciable change (1. 5-fold increase) in the total IGFBP binding capacity of the conditioned medium. However, the relative contribution of each of the IBFBPs to the total following estradiol treatment remains nearly identical to that observed in the control condition. The M_r 36,000 protein is clearly a dominant IGFBP in MCF7 cells, and the contribution of this IGFBP to the total (35–39%) remained largely unaffected across the four conditions. A modest increase in the M_r 30,000 IGFBP occurred in all the treatment conditions when compared to the control, yet this protein remains a minor contributor (10–18%) to the total IGFBP. The IGFBPs most affected by the treatments in this analysis are BP-3 and BP-4. BP-4 is the predominant IGFBP in the conditioned medium of control and estradiol treated cells. Although BP-4 levels are significantly elevated with estradiol treatment (Fig. 7), its contribution to the total (44%) remains the same as that observed in the control cells (43%). With antiestrogen treatment, however, the contribution of BP-4 to the total declines (31% with tamoxifen treatment and 24% with ICI 182,780). BP-3, on the other hand, is a minor band in the control condition (9%) and is reduced to only 2% of the total IGFBP production by estradiol treated cells. With antiestrogen treatment, BP-3 con-

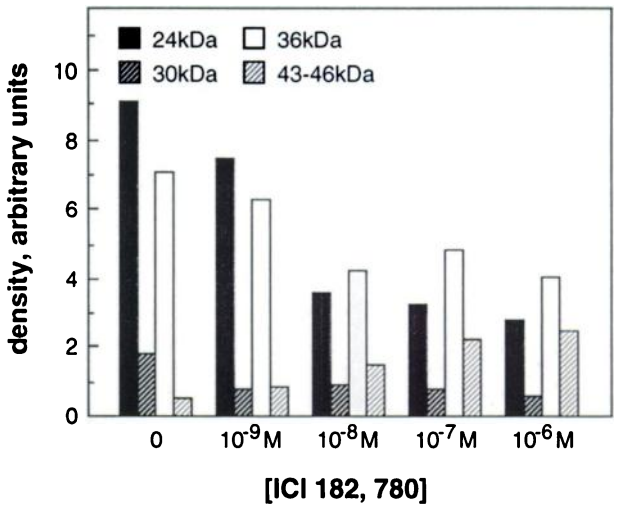

Fig. 5. Effects of ICI 182,780 on IGFBP levels. Estrogen withdrawn MCF7 cells were treated with increasing concentrations of ICI 182,780, and equivalent volumes of conditioned medium were evaluated by Western ligand blot. IGFBP bands were quantitated by densitometric scanning. *Bars* correspond to the relative abundance of each of the four IGFBP species at each concentration of ICI 182,780. Data presented are from a representative experiment.

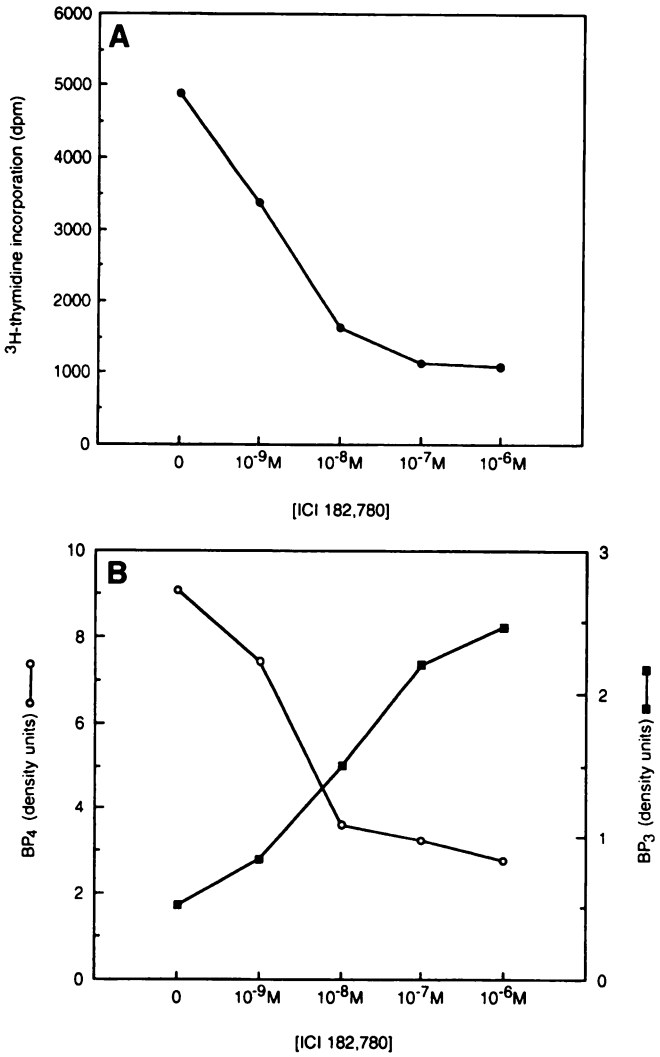

Fig. 6. Effects of ICI 182,780 on MCF7 proliferation and on BP3 and BP4 levels. *A*, effects on increasing concentrations of ICI 182,780 on [^{3}H]thymidine incorporation in estrogen withdrawn MCF7 cells. *B*, effects of increasing concentrations of ICI 182,780 on band densities of BP3 (M_r 43,000–46,000) and BP4 (M_r 24,000) proteins. Values in *A* and *B* were derived from the same experiment.

tribution increases dramatically and represents 22% of the total IGFBP presence in the ICI 182,780 treated condition.

DISCUSSION

We demonstrate that ICI 182,780 inhibits IGF-I stimulated MCF7 cell proliferation and describe for the first time increased levels of a M_r 43,000–46,000 IGFBP (BP-3) in the conditioned medium of MCF7 cells following treatment with tamoxifen or ICI 182,780. In the absence of antiestrogens and under conditions optimized for detection of the predominant BP species secreted by MCF7 cells, other investigators have failed to detect a band corresponding to BP-3 (26–28), although BP-3 mRNA has been detected using RNase protection assay (27). In fact, the BP-3 level in the conditioned medium of control MCF-7 cells is low but detectable and is suppressed almost completely by estradiol. It is noteworthy that tamoxifen and ICI 182,780 do not merely attenuate the estradiol-induced suppression of BP-3, but even under estrogen-free conditions they increase BP-3 to well above control levels. BP-3 has been shown to be an inhibitor of proliferation in some (30, 37) but not all (38, 39) experimental systems, and we observed a strong negative correlation between BP-3 levels and proliferation in our experiments. While several mechanisms by which antiestrogens reduce cellular proliferation have already been described (40), it is possible that increased levels of BP-3 in response to these drugs contribute to growth inhibition, assuming that BP-3 acts as an inhibitor of IGF-I action in the microenvironment of MCF7 cells.

It has recently been demonstrated that retinoids inhibit proliferation of breast cancer cells under certain conditions (41, 42) and that increased levels of BP-3 in the conditioned medium occur in reponse to retinoids (41, 42). Our results show that antiestrogens have a similar effect, and these findings may be clinically relevant in view of the recent proposal to coadminister retinoids and antiestrogens in the treatment of breast cancer (reviewed in Ref. 43). Our observations may also be relevant to recent results concerning induction of TGFβ by antiestrogens (29), since TGFβ has been shown to increase BP-3 in certain experimental systems (44).

Our data demonstrate that a M_r 24,000 IGFBP (BP-4) is also regulated by estradiol, tamoxifen, and ICI 182,780. BP-4 is the major IGFBP species in MCF7 conditioned medium under control conditions, and its accumulation is enhanced by estrogens, reduced by antiestrogens, and positively correlated with cellular proliferation. While it has been demonstrated that BP-4 attenuates the mitogenic action of free IGF-I *in vitro* (33), estrogen has been shown to enhance responsivity to IGF-I (12) and to act synergistically with IGF-I in promoting MCF7 cell proliferation (12, 13). Therefore, if BP-4 were acting as an inhibitor of IGF-I action in our experimental system, one would have to conclude that estrogen stimulates proliferation despite a concomitant inhibition of IGF-I action, which seems unlikely. Thus, our observations do not suggest a growth inhibitory role for BP-4 in our experimental system. In other systems, certain IGFBPs have been shown to enhance proliferation under specific conditions (45), and a growth stimulatory role for estrogen induced IGFBPs is possible, particularly in the context of the proposal that *in vivo*, low molecular weight IGFBPs enhance extravascular diffusion of IGFs (24).

The ability of ICI 182,780 to inhibit IGF-I stimulated proliferation is in keeping with accumulating evidence suggesting that there are multiple levels at which estrogens and antiestrogens interact with the IGF system. Antiestrogens have been shown not only to modulate IGFBP levels in IGF-responsive cells as reported here, but also to modulate the expression of type I IGF receptors (12), the expression of IGF-I by liver and other tissues (46), and the circulating IGF-I concentration (47, 48). Furthermore, the view that ICI 182,780 merely acts to block estrogen stimulated proliferation appears incomplete, given the fact that it inhibits MCF7 proliferation even under estrogen-free conditions (20). Our data suggest that one mechanism by which ICI 182,780 is able to block IGF-I stimulated MCF7 cell proliferation is to increase BP-3 to levels higher than those seen in the presence or the absence of estradiol. Regulation of BP-3 levels in the conditioned medium may occur at the transcriptional or translational levels of protein processing, or BP-3 levels may be controlled at the posttranslational level. BP-3 specific proteases have been shown to regulate IGFBP levels in serum (49, 50), tissue extracts (49), and in other biological fluids (50). The possibility exists that estrogens and antiestrogens modulate BP-3 levels, and therefore IGF-I bioactivity, through the regulation of BP-3 proteolytic enzyme activity and/or alteration of BP-3 secretion.

We cannot rule out the possibility that the inhibition of MCF7 proliferation by antiestrogens is unrelated to the specific changes in IGFBP levels. However, our observations are consistent with the view that antiestrogens influence breast cancer proliferation not only by previously described mechanisms (40, 48) but also by modulating various IGFBP species that influence IGF-I bioactivity. More specifically, our results are consistent with a growth inhibitory role of BP-3

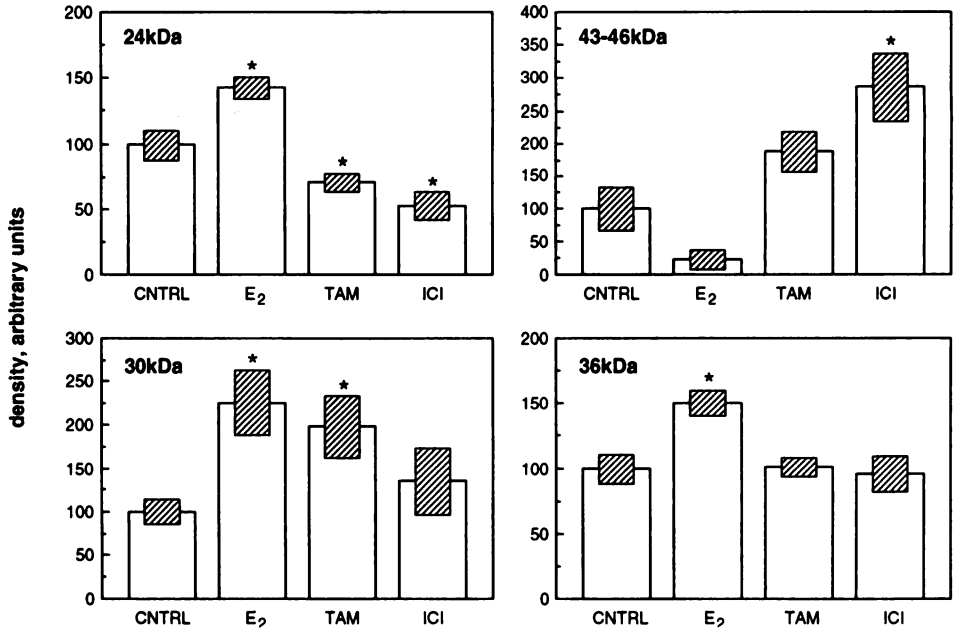

Fig. 7. Effects of estradiol, tamoxifen, and ICI 182,780 on MCF7 IGFBP levels. Western ligand blots of IGFBPs from estrogen withdrawn MCF7 cells (CNTRL) and cells treated with 0.1 nM 17β-estradiol (E_2), 1 μM tamoxifen (TAM), or 1 μM ICI 182,780 (ICI) were quantitated by scanning densitometry. Data were normalized as described in "Results" and analyzed for statistical significance by the Mann Whitney U test ($n = 5$). Data are expressed as the mean ± SEM; *, < 0.05.

Table 1 *IGFBP binding capacity of MCF-7 cell conditioned medium*[a]

	Control	E_2	TAM	ICI
Total IGFBP binding,[b] Arbitrary units	15.65 ± 2.28	23.39 ± 4.13	16.40 ± 2.53	14.17 ± 3.11
IGFBP contribution, % of total[c]				
M_r 43,000–46,000	9.0 ± 4.1	2.4 ± 1.8	14.5 ± 3.1	22.5 ± 1.7
M_r 36,000	36.4 ± 2.5	38.3 ± 3.9	35.4 ± 2.1	38.7 ± 3.5
M_r 30,000	10.6 ± 1.4	16.2 ± 2.6	18.8 ± 0.9	14.6 ± 2.8
M_r 24,000	44.0 ± 4.8	43.1 ± 2.2	31.3 ± 5.1	24.2 ± 2.9

[a] Cell monolayers were treated for 2 days in phenol red-free DMEM containing 5% DCC-FCS prior to collection of serum-free treatment containing conditioned medium. IGFBPs in conditioned medium from control cells and cells treated with E_2 (0.1 nm 17β-estradiol), TAM (1 μM tamoxifen), or ICI (1 μM ICI 182,780) were evaluated by Western ligand blotting with [^{125}I]-IGF-I. Data are expressed as the mean ± SEM of five independent experiments.

[b] Total IGFBP represents the sum of all IGFBP bands as quantitated by scanning densitometry and was normalized to the protein content of the monolayers under each condition.

[c] Represents the densitometric contribution of each IGFBP band to the total.

and a growth enhancing role for BP-4 on the proliferation of MCF7 breast cancer cells.

ACKNOWLEDGMENTS

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Note Added in Proof

Our observation concerning the positive correlation between IGF-BP4 accumulation and proliferation is of interest in the context of the recent finding that the human gene encoding IGF-BP4 has been mapped to a region of chromosome 17 defined by markers THRA1 and D17S579, which is known to contain the gene for hereditary breast-ovarian cancer (P. Tonin, E. Ehrenborg, G. Lenoir, J. Feunteun, H. Lynch, K. Morgan, H. Zazzi, A. Vivier, M. Pollak, H. Huynh, H. Luthman, C. Larsson, and S. Narod. The human *IGF-BP4* gene maps to chromosome region 17q12-21.1 and is close to the gene for hereditary breast-ovarian cancer. *Genomics,* in press, 1993).

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