Breast Cancer Research and Treatment **32**: 153–164, 1994.
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Report

Expression of insulin-like growth factor binding proteins by T-47D human breast cancer cells: regulation by progestins and antiestrogens

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Key words: antiestrogens, human breast cancer, IGFBP, progestins, T-47D

Summary

We have used ligand blotting and Northern blotting techniques to examine the effects of progestins and antiestrogens on expression of insulin-like growth factor binding proteins (IGFBPs) by T-47D human breast cancer cells under conditions where these agents are growth inhibitory. Under basal conditions, conditioned medium from T-47D cells was found to contain IGFBPs of 39, 33, and 27 kDa. Northern blot and/or Western blot analysis have identified these as IGFBP 2, 5, and 4, respectively. Medroxyprogesterone acetate (MPA) treatment resulted in a time- and dose-dependent decrease in IGFBP 4 and 5 mRNA abundance and secretion of these proteins, while little if any effect was observed on IGFBP 2 expression. A decrease in the steady state mRNA levels for IGFBP 4 and 5 was observed with as little as 0.1 nM MPA. Using 10 nM MPA a maximum decrease in IGFBP 4 and 5 mRNA levels was observed between 12 and 24 hours. While RU 486 alone had little or no effect on IGFBP 4 expression, it inhibited the effect of MPA. However, in the same samples, IGFBP 5 expression was inhibited by RU 486, and RU 486 was unable to reverse the effects of progestins on the expression of IGFBP 5. Furthermore, another synthetic progestin, Org 2058, but not dexamethasone, inhibited IGFBP 4 and IGFBP 5 expression. The antiestrogen ICI 164384 also transiently decreased the steady state mRNA levels of both IGFBP 4 and IGFBP 5. Regulation of expression of the IGFBPs by these agents suggests a potential role for the IGFBPs in the growth response of T-47D cells to these agents.

Introduction

The insulin-like growth factors (IGFs) have been postulated to act in an autocrine/paracrine fashion as well as possibly an endocrine fashion to regulate the growth of human breast cancer [1–5]. Both IGF-I and IGF-II are potent mitogens of breast cancer cells in culture [5–8]. Breast cancer epithelium and breast cancer cells in culture have been shown to produce IGF-II [1, 5, 9], while the stromal elements within the mammary gland produce both IGF-I and IGF-II [1, 3]. IGF-I and IGF-II interact with at least two specific cell surface receptors, referred to as the type I and II IGF receptors and breast cancer cell lines in culture have been shown to express both type I and type II IGF receptors [10, 11]. Interestingly, the mitogenic activity of both IGF-I and IGF-II in human breast cancer cells has been shown to be mediated via the type I IGF-receptor [11].

The actions of the IGFs are modulated by specific high affinity binding proteins. Six distinct types of

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IGF-binding proteins (IGFBPs) have been identified from a variety of cell types including human breast cancer cells [12–20]. Their cDNAs have been cloned and sequenced and these binding proteins show a high degree of homology with one another [15, 21–26]. Despite this homology the binding proteins do exhibit differences in glycosylation, tissue distribution, and hormonal regulation [27, 28]. While the exact biological role for the IGFBPs is unclear, under various *in vitro* conditions IGFBPs have been found to both stimulate and inhibit IGF actions [29–31]. Human breast cancer cells have been shown to express and secrete multiple IGFBPs [13, 19, 32, 33] and in some cases the expression of some of these binding proteins has been found to be regulated by hormones and growth factors [19, 32, 34]. However, the exact role of IGFBPs and other components of the IGF system in human breast cancer cell growth and the response to growth modulating hormones still remains to be elucidated.

Both progestins and antiestrogens are potent growth modulatory agents in T-47D human breast cancer cells [35, 36]. The modulation of autocrine-paracrine growth factor systems is thought to play a role in the growth regulatory effects of these agents. In order to determine a possible involvement of the IGF system in such growth modulation, we have investigated IGFBP expression in T-47D cells and determined the effect of progestins and antiestrogens on their expression.

Materials and methods

Materials

[^{32}P]dCTP and [^{32}P]γ-ATP were purchased from ICN (St-Laurent, Quebec). [^{125}I]IGF-I was purchased from Amersham Canada (Oakville, Ontario). Dulbecco's Minimal Essential Medium (DMEM) powder was purchased from GIBCO/BRL (Burlington, Ontario). Fetal bovine serum was purchased from UBI (Lake Placid, New York) and all other cell culture ingredients were purchased from Flow laboratories (Mississauga, Ontario). Medroxyprogesterone acetate (MPA), progesterone, and dexamethasone were purchased from Sigma (St. Louis, MO). Org 2058 was purchased from Amersham Canada (Oakville, Ontario). RU 486 was a gift from Roussel Uclaf (Romainville, France). R 5020 was purchased from NEN (Lachine, Quebec). ICI 164384 was a gift from ICI (Macclesfield, Cheshire). Antibodies to IGFBP 2 were purchased from UBI (Lake Placid, New York).

Cells and cell culture

The T-47D human breast cancer cells [51] were grown in DMEM supplemented with 5% fetal bovine serum, glucose, glutamine, and penicillin-streptomycin (5% CM) as previously described [51]. Cells were passaged at 70–80% confluency using Earle's EDTA solution.

RNA isolation and Northern blot analysis

Cells were plated at 1×10^6 in 150 mm plastic dishes in 5% CM. Two days later test compounds were added from 1000 × stock solutions to achieve the indicated final concentrations. Control dishes were treated with ethanol vehicle. Cells were harvested at the indicated times by scraping the cells off the monolayer with a rubber policeman. After centrifugation the cell pellet was stored at – 70° C until RNA isolation. RNA was isolated by the guanidium thiocyanate/cesium chloride method [52].

Twenty five μg of total RNA were denatured in 50% (v/v) formamide and 2.2 M formaldehyde and size separated on 1% agarose gels containing 2.2 M formaldehyde. The RNA was transferred to nitrocellulose [53] filters, which were then baked for 2 h at 80° C under vacuum and prehybridized for at least 2 h. The filters were hybridized with cDNAs for IGFBP 1, 3-6 [37] labelled with ^{32}P-dCTP by nick translation, or an end-labelled 23-mer oligonucleotide for IGFBP 2 [23]. Filters were also hybridized with a cDNA for PRA-calcyclin [54] as a control for the differences in RNA loading. Under the conditions of these experiments, steady state levels of PRA/calcyclin mRNA were unaffected by the ste-

roid hormone treatments. Hybridizations, usually for 24 h, were performed at 42° C in the presence of 50% (v/v) deionized formamide, 5 × SSPE (1 × SSPE = 1.15 M NaCl, 0.01 M NaH_2PO_4, 1 mM EDTA), 5 × Denhardt's solution (1 × Denhardt's = 0.02% w/v each of BSA, Ficoll, and polyvinylpyrrolidine), 250 µg/ml denatured salmon sperm DNA, and 0.1% SDS. Blots were washed twice in 2 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS for approx. 20 minutes at room temperature followed by two washes in 0.1 × SSC, 0.1% SDS for approx. 20 minutes at 65° C. Blots hybridized with the IGFBP 2 oligonucleotide were washed three times in 2 × SSC, 0.1% SDS at room temperature for approx. 20 minutes followed by one wash in 2 × SSC, 0.1% SDS at 54° C for approx. 10 minutes. Blots were exposed to Kodak XAR film at – 70° C with or without an intensifying screen.

Quantitation was achieved by densitometric scanning of the autoradiograms using an Apple Color One Scanner and the data were analyzed using the 'Image' program.

Collection of conditioned medium

Cells were plated at 2×10^6 cells per 150 mm dish in 5% CM. Two days later the medium was removed, and cell monolayers were washed twice with 1 × PBS and once with serum free DMEM supplemented with penicillin-streptomycin, glucose and glutamine (SF–CM). SF–CM was then added to the dishes and the cells were subsequently treated with drug or vehicle. Conditioned medium (CM) was collected 24 h later, centrifuged at 4° C, dialyzed overnight at 4° C against distilled, deionized H_2O, lyophilized, and stored at – 70° C until ligand blot analysis.

Ligand and Western blot analysis

Lyophilized samples were reconstituted in water to give a 200-fold concentration. Protein assays were performed on samples according to the method of Lowry [55]. Ligand blotting was performed according to the method of Hossenlopp [56]. Briefly, 50 µg of protein were electrophoresed on 12% SDS-polyacrylamide gels under non-reducing conditions. The size-fractionated proteins were transferred to nitrocellulose filters using a semi-dry transfer cell for 1 h at 12 V, as previously described [37]. The nitrocellulose filter was blocked with 1% BSA in saline (0.15 M NaCl, 0.01 M Tris-HCl, pH 7.4) for 2 h at room temperature, incubated overnight with ^{125}I-IGF-I in saline, 1% BSA, 0.1% Tween-20 at 4° C, washed twice with saline/0.1% Tween-20 and then twice with saline, then exposed to Kodak XAR film at – 70° C with an intensifying screen. Molecular weight estimates were determined from prestained low molecular weight standards (Biorad, Richmond, CA). Bands were quantitated densitometrically using an Abaton flat-bed scanner and the data were analyzed using the 'Image' program.

Western blot analysis was carried out as previously described [57] and the IGFBP 2 protein was visualized using the manufacturer's instructions.

Results

Identification of IGFBP expression by T-47D human breast cancer cells

Ligand blot analysis of conditioned medium from T-47D cells identified three IGFBPs of molecular mass 39, 33, and 27 kDa (Fig. 1). These IGFBPs were found to be of similar size to those present in the conditioned medium of MCF-7 cells, although the relative abundance of each IGFBP differed between the cell lines. These IGFBPs were tentatively identified due to molecular mass as IGFBP 2, IGFBP 5, and IGFBP 4, respectively.

In order to further characterize which IGFBPs were expressed in T-47D cells, RNA was isolated and analyzed by Northern blotting and hybridization with radiolabelled cDNA or oligonucleotide probes to human IGFBP 1 to 6 [37]. Specific hybridization was detected with cDNA probes to human IGFBP 4 and 5 (Fig. 2) but not IGFBP 1, 3, and 6. A major 2.6 kb mRNA was detected using IGFBP 4 cDNA (Fig. 2), while IGFBP 5 cDNA detected a major 6.0 kb transcript (Fig. 2). Multiple bands were found to hybridize to a 23-mer oligonucleo-

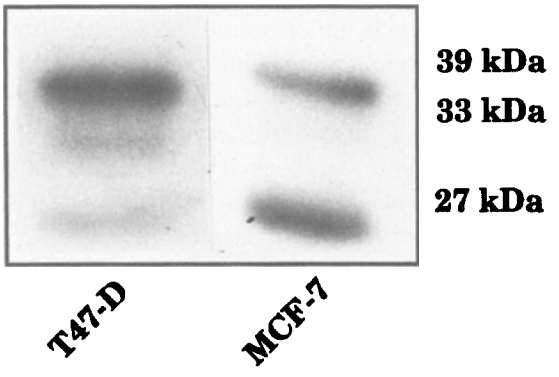

Fig. 1. Detection of IGFBP expression in T-47D human breast cancer cells using ligand blot analysis. Conditioned medium was collected after 24 h as described in Methods. 50 μg of total protein was run on a 12% SDS-PAGE under non-reducing conditions and transferred to nitrocellulose for ligand blotting with ^{125}I-IGF-I. The IGFBPs present in T-47D (left lane) and MCF-7 (right lane) human breast cancer cell CM are shown. A representative ligand blot is shown. The apparent molecular weights were determined by comparison with pre-stained protein molecular weight standards (Biorad).

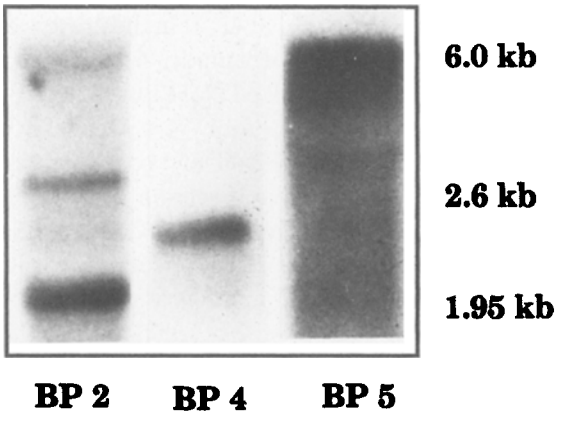

Fig. 2. Northern blot analysis of IGFBP mRNA in T-47D cells. 25 μg of total RNA was run on 1.5% agarose/formaldehyde gel and transferred to nitrocellulose and probed for IGFBP mRNA expression as described in Methods. mRNA transcripts for IGFBP 5 (right lane) and IGFBP 4 (middle lane) were identified using ^{32}P-dCTP labelled cDNA probes as described in Methods. mRNA transcripts for IGFBP 2 (left lane) were identified using a ^{32}P-γATP end-labelled oligonucleotide. Representative autoradiograms of Northern blots are shown. kb, kilobases.

tide with sequence corresponding to nucleotides 433–456 of the IGFBP 2 cDNA sequence [23]. The major transcript detected was 1.95 kb (Fig. 2).

It was concluded from these data, that exponentially growing T-47D human breast cancer cells express and secrete into the medium IGFBP 2, 4, and 5, while IGFBP 1, 3, and 6 were not detected under these conditions with the reagents and techniques used in these experiments.

Effect of progestin treatment on IGFBP expression in T-47D human breast cancer cells

In order to determine the effect of the synthetic progestin MPA on IGFBP expression in T-47D cells, conditioned medium was collected from cells treated for 24 hours with either 10 nM MPA or vehicle alone, and subjected to ligand blot analysis. The results in Fig. 3 indicate that MPA treatment decreased the level of IGFBPs with molecular masses of 33 and 27 kDa, i.e. those binding proteins corresponding to IGFBP 5 and 4, respectively. The 33 kDa protein, IGFBP 5, was reduced to 44 ± 12% (mean ± SEM, n = 3) of vehicle treated control levels, and the 27 kDa protein, IGFBP 4, was reduced to 40 ± 8% (mean ± SEM, n = 3) of vehicle treated control levels. The level of the 39 kDa protein was 87 ± 9% (mean ± SEM, n = 3) of control levels after MPA treatment. This latter IGFBP was tentatively identified as IGFBP 2, and this was confirmed by immunoblotting with a specific IGFBP 2 antibody (data not shown). Therefore, MPA had little if any effect on the level of IGFBP 2 under these conditions.

Northern blot analysis was carried out to determine the effect of MPA on steady state mRNA levels of IGFBPs 2, 4, and 5. Figure 4 shows the results obtained with RNA isolated from T-47D cells after treatment for various periods of time with 10 nM MPA or vehicle alone. The levels of mRNA for IGFBP 4 and IGFBP 5 were significantly reduced after MPA treatment. MPA had little if any consistent effect on the steady state level of IGFBP 2 mRNA. As can be seen in Fig. 4B, there is a marked decrease in the level of IGFBP 4 mRNA after 6 h of MPA treatment, and maximal effects were seen after 24 h of MPA treatment. At 24 h after treatment, a decrease of IGFBP 4 mRNA level to 34 ± 3% (mean ± SEM, n = 3) that of vehicle treated control was achieved.

MPA also decreased the steady state levels of IGFBP 5 mRNA. Maximal inhibition was observed between 12 and 24 h after MPA treatment (Fig. 4c). IGFBP 5 mRNA levels were decreased to 24 ± 14% (mean ± SEM, n = 3) and 26 ± 8% (mean ± SEM, n = 3) of vehicle treated controls, 12 and 24 h, respectively, after MPA treatment.

These data are consistent with the MPA induced decrease in levels of IGFBPs 4 and 5 in the conditioned medium of T-47D cells being due, at least in part, to decreased mRNA levels for these two proteins.

The dose-dependence of the MPA induced decrease in IGFBP 4 and 5 mRNA levels was also examined. Figure 5 shows the effects of 24 h treatment of 0.1–1000 nM MPA on IGFBP 4 and 5 mRNA levels in T-47D cells. As little as 0.1 nM MPA caused a significant decrease in the steady state levels of IGFBP 5 mRNA, while decreases in steady state levels of IGFBP 4 mRNA were only apparent at 1 nM MPA.

The steroid hormone specificity of the decrease in IGFBP 4 and 5 mRNA levels was determined. As can be seen in Fig. 6, Org 2058 (a synthetic progestin) was more effective in reducing the level of mRNA expression for both IGFBP 5 and IGFBP 4 than MPA, a finding which is consistent with the higher affinity of Org 2058 for the progesterone receptor [38]. The glucocorticoid dexamethasone had little or no effect on the level of IGFBP 4 and IGFBP 5 mRNA. The antiglucocorticoid/antiprogestin RU 486 alone had little if any effect on IGFBP 4 mRNA levels and was able to at least partially reverse the effects of MPA. Interestingly, RU 486 alone decreased the level of IGFBP 5 mRNA to 31 ± 9% (mean ± SEM, n = 4) that of vehicle treated controls, and when added together with MPA was unable to reverse the effect of MPA. The antiestrogen ICI 164 384 was also found to decrease the steady state mRNA level of IGFBP 4 but had no effect on the level of IGFBP 5 mRNA.

Effect of antiestrogen treatment on IGFBP expression in T-47D human breast cancer cells

Since the antiestrogen ICI 164 384 appeared to differentially affect IGFBP 4 and 5 (see Fig. 6), the effect of this antiestrogen on the expression of IGFBPs in T-47D cells was further characterized. The effect of ICI 164 384 on IGFBP 4 and IGFBP 5 mRNA steady state levels was determined at various times after treatment (Fig. 7). The results suggest that a decrease in both IGFBP 4 and 5 mRNA levels occurs at 6 h after treatment and is maximal at 12 h after treatment. The decrease at 6 h was not statistically significant but a maximal 39 ± 8% (mean ± SEM, n = 3, p = 0.036) decrease in IGFBP 4 mRNA and a 46 ± 3% (mean ± SEM, n = 3, p = 0.04) decrease in IGFBP 5 mRNA was observed at 12 h after treatment. Thereafter mRNA levels for both IGFBP 4 and 5 begin to increase to control levels. Slight differences in time course between IGFBP 4 and IGFBP 5 which do reach statistical significance in these experiments may explain the differences seen in Fig. 6. Little if any effect was found on IGFBP 2 mRNA level (data not shown).

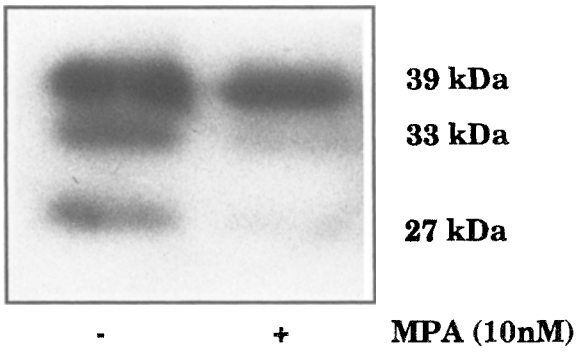

Fig. 3. Ligand blot of CM from T-47D cells after 24 h 10 nM MPA treatment. Conditioned medium was collected after treatment with MPA (+) or ethanol vehicle (–) for 24 h as described in Methods. 50 µg of total protein was run on a 12% SDS-PAGE and transferred to nitrocellulose for ligand blotting with ^{125}I-IGF-I. The apparent molecular weights were determined by comparison with pre-stained protein molecular weight standards (Biorad).

Discussion

Consistent with other reports [13, 19, 33] we have detected three IGFBPs of 27, 33, and 39 kDa in the conditioned medium of T-47D human breast cancer cells. These proteins correspond to IGFBP 4, 5, and

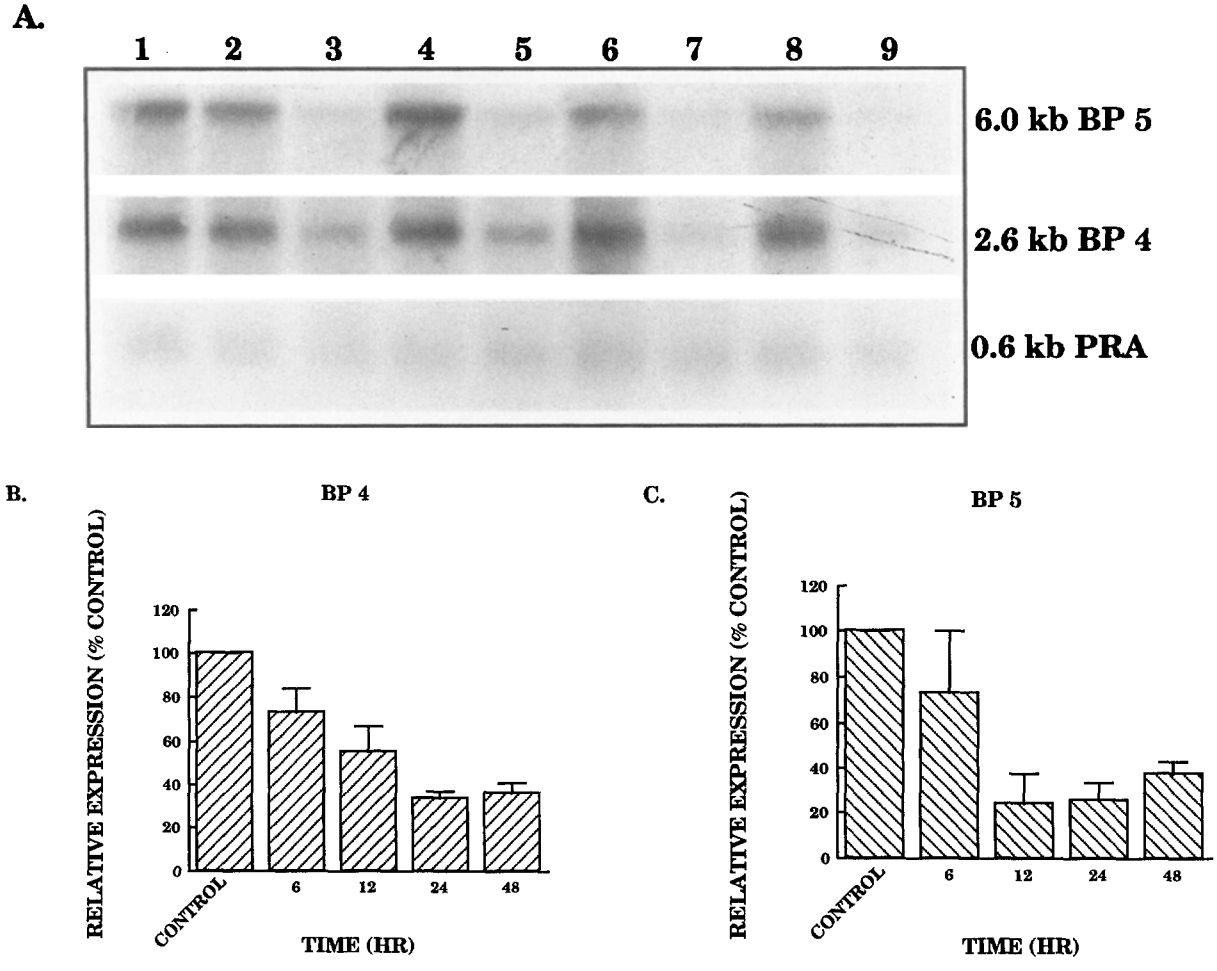

Fig. 4. Time course of MPA treatment on IGFBP mRNA levels in T-47D cells. Cells were treated with 10 nM MPA for various times and 25 μg of total RNA was run on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose and probed as previously described.

A. Representative Northern blots are shown. PRA is used as a loading control. Lane 1, control (ethanol vehicle), lane 2, 6 h control, lane 3, 6 h 10 nM MPA, lane 4, 12 h control, lane 5, 12 h 10 nM MPA, lane 6, 24 h control, lane 7, 24 h 10 nM MPA, lane 8, 48 h control, lane 9, 48 h 10 nM MPA. IGFBP 5 mRNA, top panel; IGFBP 4 mRNA, middle panel; PRA/calcyclin mRNA bottom panel.

B. Histogram of time-course of MPA effects on IGFBP 4 mRNA steady state level. Northern blots are quantitated using scanning densitometry and loading errors were corrected using PRA as a control. The mean ± SEM of 3 separate experiments are shown. In each experiment the MPA treated values are expressed as a percentage of control mRNA isolated at each time point and given an arbitrary value of 100%.

C. Histogram of time-course MPA effects on IGFBP 5 mRNA steady state level. Northern blots were quantitated using scanning densitometry and loading errors were corrected using PRA as a control. The mean ± SEM of 3 separate experiments are shown. In each experiment the MPA treated values are expressed as a percentage of control mRNA isolated at each time point and given an arbitrary value of 100%.

2 respectively, as confirmed either by specific antibody detection and/or Northern blot analysis. The addition of MPA to T-47D cells caused a significant decrease in the expression of both IGFBP 4 and IGFBP 5. This occurred at both the mRNA and protein levels. The antiestrogen ICI 164384 was found to transiently decrease the mRNA level of IGFBP 4 and IGFBP 5. The effect of antiestrogen appeared to be of lesser magnitude than that of MPA. The MPA induced decreased expression occurred at both the mRNA and protein levels. During the preparation of this manuscript another group reported that both estrogen and progestins regulate the expression of IGFBPs in T-47D. They

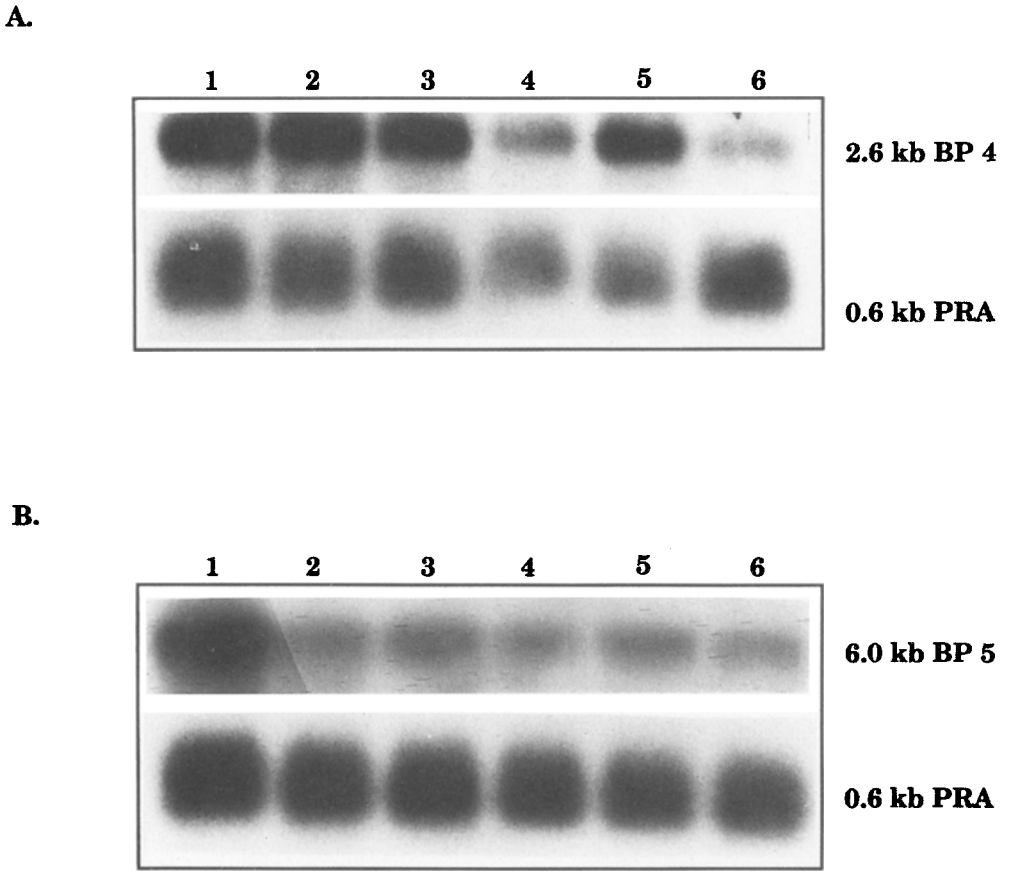

Fig. 5. Dose response of IGFBP 4 and 5 steady state mRNA levels to MPA. Cells were treated with 0.1–1000 nM MPA for 24 h and analyzed by Northern blotting as previously described. Specific hybridization with cDNA probes for IGFBP 4 (A), IGFBP 5 (B), and PRA/calcyclin (bottom panel in both A & B) are shown. Lane 1, control (ethanol vehicle), lane 2, 0.1 nM MPA, lane 3, 1 nM MPA, lane 4, 10 nM MPA, lane 5, 100 nM MPA, lane 6, 1000 nM MPA.

demonstrated that estradiol increased but progesterone decreased the levels of IGFBPs in the conditioned medium of T-47D cells. Our data are in general consistent with their observations [39], since progestins and antiestrogens were found to decrease the levels of IGFBP 4 and 5 expression in T-47D cells. Importantly, our results add significant mechanistic information with regard to the effects of progestins and antiestrogens on IGFBP regulation in T-47D cells. Our data demonstrate that progestins and antiestrogens inhibit the expression of IGFBP 4 and IGFBP 5 at the gene level since mRNA levels for both IGFBP 4 and IGFBP 5 were decreased by these treatments. The effect of progestins, however, was more sustained than that of antiestrogen. Whether the effect on steady state mRNA levels is due to altered gene transcription, some post-transcriptional mechanism, or a combination of both, remains to be established. The effects on IGFBP 2 were small and inconsistent and we conclude that in our system progestins and antiestrogens are not major regulators of IGFBP 2 expression in T-47D cells. Reasons for the discrepancies between our study and a previous study may be due to the different cell culture conditions used. Our study was conducted in fetal calf serum, while the previous study was conducted in the presence of charcoal stripped/phenol red free medium [39]. Charcoal stripping can remove other small molecular weight molecules, e.g. IGF I and II, as well as steroids from fetal calf serum, which may have ef-

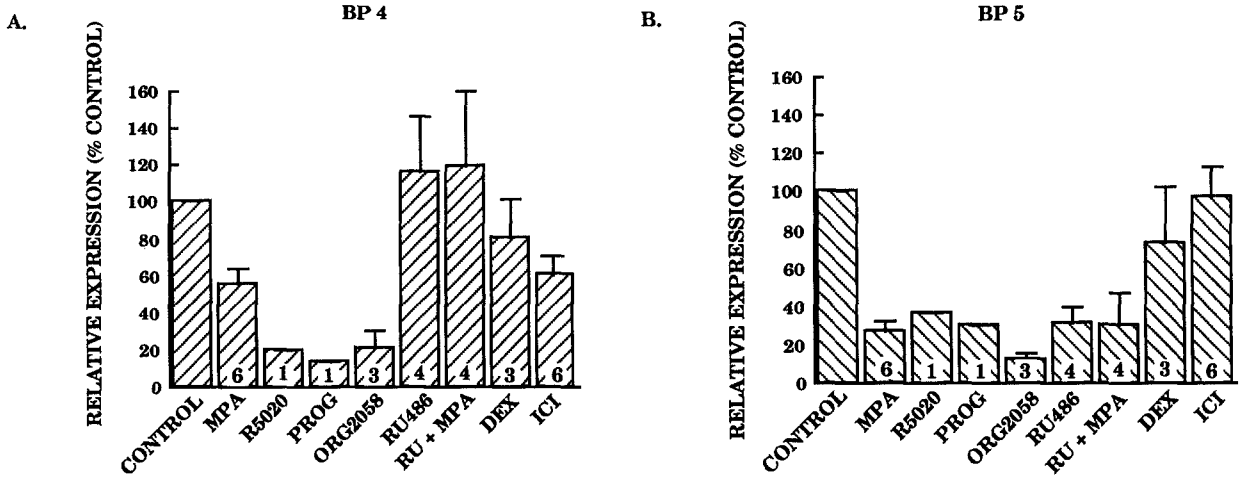

Fig. 6. Specificity experiment for IGFBP 4 (A) and IGFBP 5 (B) mRNA expression. Cells were treated with drug for 24 h and Northern blots were performed as previously described. The blots were hybridized with ^{32}P-dCTP labelled cDNA probes for IGFBP 4 and IGFBP 5 and PRA was used as a loading control. Northern blots were quantitated using scanning densitometry and loading errors were corrected using PRA as a control. The mean ± SEM of 1 to 6 separate experiments are shown. In each experiment the values are expressed as a percentage of vehicle treated control mRNA, which was given an arbitrary value of 100%. Control (ethanol vehicle); 10 nM MPA, R5020, Org 2058, dexamethasone, and ICI 164384; 100 nM RU486 and progesterone.

fects on the regulation of expression of the IGFBPs [28, 32, 40].

Interestingly, Adamo *et al.* [32] have shown that the growth inhibitory effect of retinoic acid in MCF-7 human breast cancer cells was accompanied by increased levels of IGFBP 4 and IGFBP 3 (not normally found in control experiments), while no effect on IGFBP 2 was found. Since retinoic acid inhibits expression of the progesterone receptor [41] the effect of retinoic acid in part may be to remove the inhibitory effect of progestins on the expression of IGFBP. Furthermore, progesterone has been shown to increase total IGFBP activity including probably IGFBP 4 in Hep G2 cells [42] and IGFBP 2 levels in endometrial stromal cells in culture [43]. Such studies, together with the data presented in this manuscript, suggest that progestins are important regulators of IGFBP 4 expression. Our data suggest that such regulation occurs primarily at the mRNA level, and since the rat IGFBP 4 5′-promoter region has been shown to contain potential progesterone response elements [44], we speculate that a direct transcription mechanism is involved, although other post-transcriptional effects cannot be excluded.

Estradiol was shown to increase expression of IGFBP 2, 4, and 5, while decreasing the expression of IGFBP 3 [45, 46] in MCF-7 human breast cancer cells. Although Sheikh *et al.* [19] found that estradiol increased IGFBP 4 mRNA levels in MCF-7 cells as well, this same group found little or no effect on IGFBP 5 mRNA levels. Therefore, our data demonstrating the ability of antiestrogens to decrease IGFBP 4 and IGFBP 5 mRNA levels in T-47D cells are consistent with previous observations that estrogens increase the expression of this gene. Since antiestrogens generally inhibit the effects of estrogens, our data suggest that estrogen-antiestrogen effects on IGFBP 4 and IGFBP 5 expression may occur at least in part by modulation of the mRNA levels.

It is apparent, however, from our study that differences occur between the regulation of IGFBP 4 and IGFBP 5 expression. Firstly, the effect of antiestrogen on IGFBP 5 mRNA may be less sustained than that for IGFBP 4, and inconsistencies with respect to steroid hormone regulation of IGFBP 5 expression have been found previously [19, 45]. Furthermore, while IGFBP 5 expression was clearly regulated at the mRNA level by progestins, a classical mechanism may not be involved since the antiprogestin-antiglucocorticoid RU 486 alone also decreased the expression of IGFBP 5, and RU 486 was unable to reverse the MPA effect on IGFBP 5.

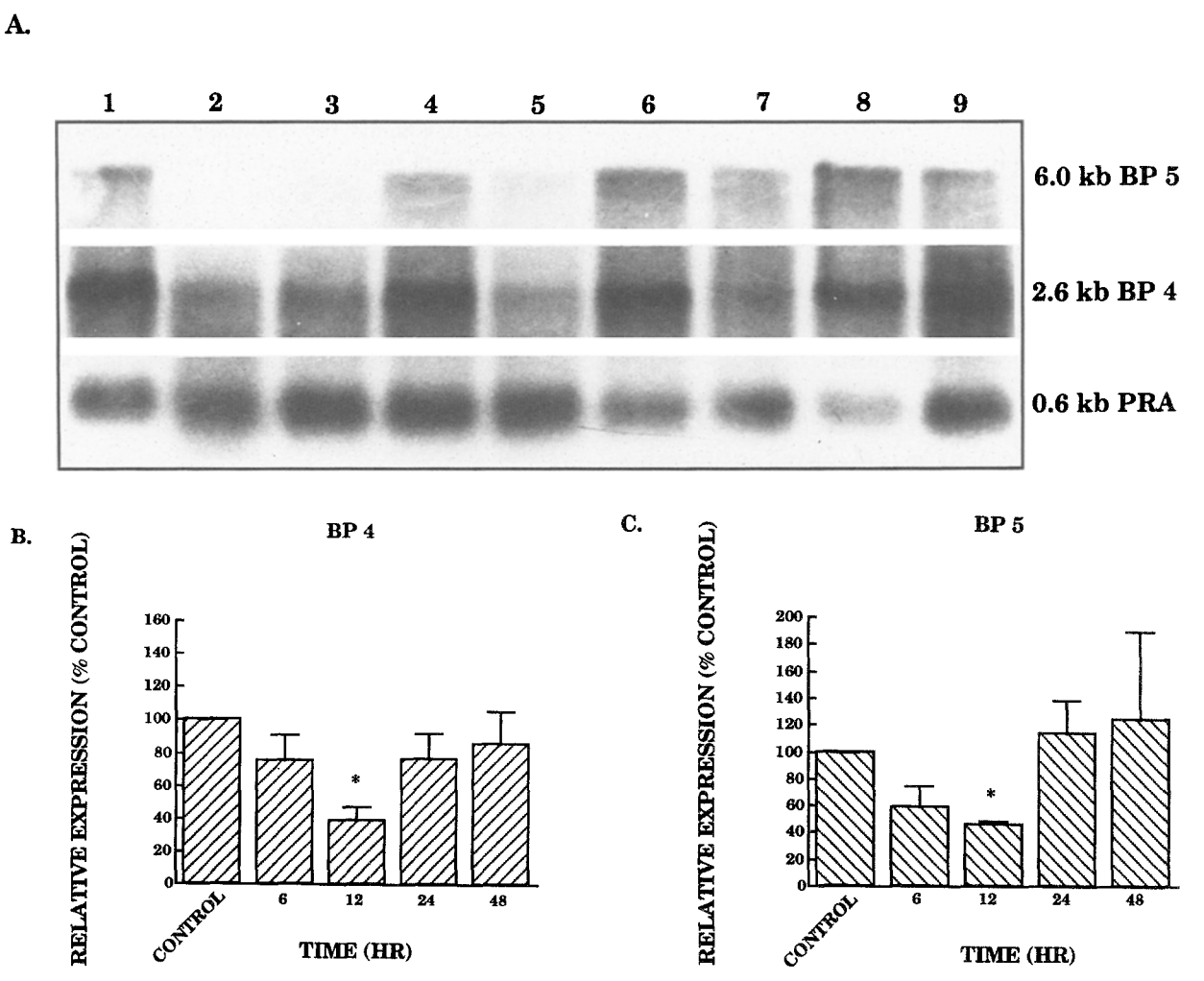

Fig. 7. Time course of ICI 164 384 treatment on IGFBP steady state mRNA levels in T-47D cells. Cells were treated with 10 nM ICI 164 384 for various times and 25 μg of total RNA was run on a 1.5% agarose/formaldehyde gel, transferred to nitrocellulose, and probed as previously described.

A. Representative Northern blots are shown. PRA is used as a loading control. Lane 1, control (ethanol vehicle), lane 2, 6 h control (please note this RNA is partially degraded; the control for quantitation of this experiment was the zero time ethanol treated sample), lane 3, 6 h 10 nM ICI, lane 4, 12 h control, lane 5, 12 h 10 nM ICI, lane 6, 24 h control, lane 7, 24 h 10 nM ICI, lane 8, 48 h control, lane 9, 48 h 10 nM ICI. IGFBP 5 mRNA, top panel; IGFBP 4 mRNA, middle panel; PRA/calcyclin mRNA bottom panel.

B. Histogram of time-course ICI 164 384 effects on IGFBP 4 mRNA steady state level. Northern blots were quantitated using scanning densitometry and loading errors were corrected using PRA as a control. The mean ± SEM of 3 separate experiments are shown. In each experiment the ICI treated values are expressed as a percentage of control mRNA isolated at each time point and given an arbitrary value of 100%. * significantly different from control, $p < 0.05$.

C. Histograms of time-course ICI 164 384 effects on IGFBP 5 mRNA steady state level. Northern blots were quantitated using scanning densitometry and loading errors were corrected using PRA as a control. The mean ± SEM of 3 separate experiments are shown. In each experiment the ICI treated values are expressed as a percentage of control mRNA isolated at each time point and given an arbitrary value of 100%. * significantly different from control, $p < 0.05$.

These effects were not due to some non-specific or toxic action on the cells, since in the same samples RU 486 alone had no effect on IGFBP 4 expression and was able to reverse the progestin induced effect on IGFBP 4 expression. However, the 5′ promoter region of the rat IGFBP 5 gene does contain potential progesterone response elements [47], supporting the observations that this gene is regulated by

progestins, and we speculate that progestins may directly regulate transcription of this gene, although other post-transcriptional mechanisms cannot be excluded.

Our data demonstrate a paradox that has been seen before with respect to many antihormones [48], i.e. that within the one cell type, an antihormone, in this case RU 486, acts as a pure agonist with respect to regulation of one gene (IGFBP 5) and at the same time acts as a pure antagonist with respect to another target gene (IGFBP 4). Since both genes have potential progesterone response elements, it is likely that a direct response to the progesterone receptor may play a role in progestin/antiprogestin regulation of both genes. However, after binding of the complex has occurred, it can be speculated that quite different molecular mechanisms are then involved. RU 486 has been shown previously to have differential agonist/antagonist properties according to promoter context [49], and the characteristics of the IGFBP 4 and 5 promoters, at least with regard to types of cis-acting elements present, appear to be different between the two genes [44, 47]. Therefore, these two genes may provide excellent models with which to dissect the molecular interactions involved in mediating agonistic and antagonistic actions of progestins and antiprogestins. It is noteworthy that progestins, antiprogestins, and antiestrogens all inhibit the expression of IGFBP 5 and all inhibit the growth of T-47D cells.

The fact that progestin, antiprogestin, and antiestrogen, all growth modulatory agents in T-47D cells, can regulate the IGFBPs, suggests a potential role for the IGFBPs in the growth response of T-47D cells to these agents. Moreover, progestins and estrogens have been shown to affect the regulation of several other components of the IGF system in these and other breast cancer cells, e.g. IGF-receptor, and IGF-II and IGF-I-like activities [2, 9, 50]. However, speculation as to the mechanisms by which IGFBPs may be involved in the growth response to these agents remains difficult since the exact physiological functions of the IGFBPs still remain unclear.

Acknowledgements

This work was supported by the Medical Research Council of Canada, the National Cancer Institute of Canada with funds from the Canadian Cancer Society and equipment grants from the H.E. Sellers Foundation and Canadian Breast Cancer Foundation. A.C. is supported by a University of Manitoba Studentship, L.J.M. is the recipient of an Endowed Research Professorship in Metabolic Diseases and an MRC Scientist award and LCM is supported by an NCI (Canada) Scientist award.

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