Regulation of IGF binding protein synthesis by a bovine mammary epithelial cell line

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Abstract

IGF-I has been proposed as a key regulator of mammary epithelial cell (MEC) growth and differentiation. As IGF-I bioactivity is modulated by specific, high-affinity binding proteins (IGFBP), the forms of IGFBP that are secreted by the bovine MEC line, MAC-T, were identified. Media conditioned by MAC-T cells contained four forms of IGFBP that were identified, by western blotting with specific antibodies, as IGFBP-2, -3, -4 and -6. The amounts of IGFBP-3 in conditioned media were relatively low under basal conditions when analyzed by ligand blotting with ¹²⁵I-IGF-II, but were increased dramatically relative to serum-free controls by exposure to IGF-I (100 ng/ml) or IGF-II (100 ng/ml) for 24 h. These increases in IGFBP-3 protein corresponded with dose-dependent increases in IGFBP-3 mRNA, with IGF-II eliciting a smaller response than was elicited by IGF-I at each concentration. Leu-IGF-I, which has reduced affinity for the IGF-I receptor but normal affinity for IGFBPs, failed to increase IGFBP-3 protein and mRNA levels, whereas B-chain IGF-I (normal affinity for the receptor but reduced affinity for IGFBPs) elicited the response, thus implying an IGF-I receptor-mediated event. Time-course studies indicated that IGFBP-3 mRNA was increased fourfold by 3 h of IGF-I treatment, with maximal increases of eightfold above serum-free controls observed between 8 and 13 h of treatment. By 24 h of treatment, IGFBP-3 mRNA levels had declined and were approximately threefold above controls in cells exposed to IGF-I. Amounts of messenger RNA of IGFBP-6 and IGFBP-2

were not increased by IGF treatment. However, retinoic acid (10^{-6} M) stimulated both IGFBP-2 and IGFBP-6 protein and mRNA levels, but it decreased IGFBP-3 mRNA levels relative to controls. The combination of retinoic acid plus IGF-I had no additional effect on IGFBP-6 or -2 above that observed with retinoic acid alone, whereas IGF-I together with retinoic acid attenuated the decrease in IGFBP-3 observed with retinoic acid alone.

Protein kinase A-mediated pathways were also shown to alter IGFBP synthesis. Forskolin, which increases cAMP, increased IGFBP-3 protein and mRNA levels. The combination of IGF-I plus forskolin resulted in greater increases in both protein and mRNA than were observed with either treatment alone. In contrast, forskolin decreased IGFBP-6 mRNA relative to controls, but had no effect on IGFBP-2. The decrease in IGFBP-6 was less marked when cells were treated with a combination of IGF-I and forskolin. Forskolin had no effect on IGFBP-2 mRNA levels.

In summary, the ability of IGF-I specifically to regulate IGFBP-3 synthesis represents a mechanism whereby IGF-I may regulate its own bioactivity. In addition, the differential regulation of IGFBP-2, -3 and -6 by retinoic acid (which inhibits proliferation) and IGF-I (which stimulates proliferation) suggests that these forms of IGFBP have different roles in regulating mammary epithelial cell physiology.

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Introduction

Insulin-like growth factor-I (IGF-I) is a potent mitogen for the majority of cell types in the body, including mammary epithelial cells (MEC) (Winder *et al.* 1989, McGrath *et al.* 1991, Zhao *et al.* 1992, Chen *et al.* 1994). This ability to stimulate cell division requires a complex system to regulate its activity. The mechanisms by which IGF-I regulates mammary gland physiology are not well under-

stood. While circulating IGF-I may influence the mammary gland in an endocrine manner, there is also evidence for local production of IGF-I within the gland itself. IGF-I mRNA has been detected in mammary tissue from several species (Yee *et al.* 1989, Hauser *et al.* 1990, Murphy *et al.* 1987) and has been shown to be localized in the stroma of the bovine mammary gland and in both normal and neoplastic human mammary tissue (Yee *et al.* 1989, Hauser *et al.* 1990). In the bovine,

immunofluorescent staining has identified IGF-I in the cytoplasm of the mammary secretory epithelia (Glimm *et al.* 1988). These data support a paracrine role for IGF-I and suggest that interactions between distinct cell types such as stromal fibroblasts and epithelial cells may be important.

The IGFs are found in association with specific, high-affinity binding proteins (IGFBPs) that modulate their bioactivity. Six unique forms of IGFBP have been identified (Cohick & Clemmons 1993, Jones & Clemmons 1995). Recently, a protein that may represent a seventh member of this family has been characterized (Oh *et al.* 1996). Multiple functions for the IGFBPs have been proposed, including prolonging the half-life of the IGFs in the circulation, transporting the IGFs out of the circulation into the extracellular space, and targeting IGF-I and -II to specific tissue sites. At the cellular level, IGFBPs have been shown to both potentiate and inhibit IGF-I action (Cohick & Clemmons 1993, Jones & Clemmons 1995). As IGFBPs alter the bioactivity of IGF-I, understanding mechanisms that regulate their availability in the extracellular environment is critical to delineating IGF action in the mammary gland.

The MAC-T cell line established by Huynh *et al.* (1991) is an immortalized bovine MEC line that retains morphological and biochemical characteristics of differentiated mammary cells *in vitro*. These cells possess IGF-I receptors and respond to IGF-I in terms of increased DNA synthesis (Zhao *et al.* 1992, Woodward *et al.* 1996). Furthermore, MAC-T cells secrete several forms of IGFBP (Romagnolo *et al.* 1994, Woodward *et al.* 1996). Therefore they represent an appropriate model for studying mammary epithelial cell growth at the cellular and molecular levels. The objective of the present study was to determine mechanisms that regulate the availability of these IGFBPs in the extracellular environment of the mammary gland.

Materials and Methods

Materials

Phenol-red-free Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin and gentamicin were purchased from Gibco-BRL (Grand Island, NY, USA). Trypsin was obtained from Boehringer Mannheim (Indianapolis, IN, USA). Tissue culture plasticware was supplied by Becton-Dickinson (Lincoln Park, NJ, USA). Recombinant bovine IGF-II and recombinant human IGF-I were gifts from Monsanto Co. (St Louis, MO, USA) and Genentech (San Francisco, CA, USA), respectively. [Leu²⁴]-IGF-I(1-62) (Leu-IGF-I) and B-chain IGF-I mutant were provided by Dr Margaret Cascieri (Merck, Sharp and Dohme, Rahway, NJ, USA). Forskolin, 4 β -phorbol 12-myristate 13-acetate (PMA), and all *trans*-retinoic acid were pur-

chased from Sigma Chemical Co. (St Louis, MO, USA). Restriction enzymes were obtained from New England Biolabs (Beverly, MA, USA). Recombinant human IGFBP-6 and antisera against human IGFBP-6 were obtained from Austral Biologicals (San Ramon, CA, USA). Antisera against bovine IGFBP-2, -3 and human IGFBP-4 were provided by Dr David Clemmons (University of North Carolina, Chapel Hill, NC, USA).

Reverse transcriptase-polymerase chain reaction (RT-PCR)

A partial bovine IGFBP-6 cDNA was obtained by RT-PCR. Oligonucleotide primers were used to amplify a region of 309 base pairs in the coding region of the bovine IGFBP-6 gene (Moser *et al.* 1992). The sense primer was 5'-GAGAAAGAGGATTTGCCTTTG-3' and the antisense primer was 5'-ATGGTCACAATTA GGCACGTA-3'. Two micrograms total RNA isolated from MAC-T cells was used for cDNA synthesis. The reaction was carried out in a final volume of 20 μ l 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM each deoxy-NTP, 5 U RNasin (Promega), 0.5 μ g random hexamer primer (Boehringer Mannheim) and 1 U AMV reverse transcriptase (Promega). PCR was performed in a DNA Thermal Cycler (Perkin-Elmer Cetus, Norwalk, CT, USA) for 35 cycles with each cycle consisting of 1 min of denaturation at 94 °C, 1 min of annealing at 50 °C, and 1 min of extension at 72 °C. The amplified PCR product was fractionated on a 3% low-melting-point agarose gel, isolated and subsequently ligated into pCR II vector (Invitrogen Co., San Diego, CA, USA) according to the manufacturer's recommendations. Double-stranded DNA sequencing performed by the Sanger dideoxy chain termination method (Sanger *et al.* 1977) using the Sequenase Version 2.0 kit (United States Biochemical, Cleveland, OH, USA) confirmed that the amplified 309 base pair product was identical to the sequence published for bovine IGFBP-6 (Moser *et al.* 1992).

Cell culture

The MAC-T epithelial cell line was established from primary bovine MEC by immortalization with SV40 large-T antigen by Huynh *et al.* (1991). The cells were grown on 100 mm dishes in phenol-red-free DMEM supplemented with 4.5 g/l glucose, 10% FBS, 4 mM glutamine, gentamicin (50 μ g/ml), penicillin (100 U/ml) and streptomycin (100 g/ml). The cells were passaged by exposure to 0.14% trypsin-0.3% EDTA and replated at 1.0×10^4 cells/cm².

Secretion experiments

Cells were plated at 1.0×10^4 cells/cm², grown to confluence, rinsed twice with serum-free DMEM, then

incubated for 24 h at 37 °C. After the wash-out period, media were aspirated and treatments were added in a final volume of 3 ml serum-free DMEM. Cells were then incubated for 1–24 h, depending on the experiment. Conditioned media were collected and analyzed for IGFBP content by ligand and immunoblotting techniques.

Northern blotting

Cells were lysed in Tri reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and RNA was isolated according to the manufacturer's recommendations. Samples were denatured at 55 °C for 1 h with 1.0 M glyoxal and 50% DMSO in 10 mM sodium phosphate buffer, pH 6.8. Total RNA was separated by electrophoresis on 1% agarose gels prepared in 10 mM sodium phosphate buffer, pH 6.8 and transferred to nylon membranes (Biotrans, ICN Biomedical Inc., Irving, CA, USA). Membranes were prehybridized for 2 h at 65 °C in 0.25 M sodium phosphate, 7% SDS, and 1 mM EDTA, pH 7.2, then hybridized overnight with [³²P]CTP-labeled cDNA probes. The bovine IGFBP-2 and IGFBP-3 cDNA probes have been described previously (Cohick & Clemmons 1991). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and 18S ribosomal RNA cDNA probes were obtained from Ambion (Austin, TX, USA). Blots were washed twice in 2 × SSC plus 0.1% SDS and twice in 0.1 × SSC containing 0.1% SDS at 65 °C for 30 min per wash and then exposed to Kodak AR film with intensifying screens at −70 °C. The relative band intensities were quantified by PhosphorImager analysis (Molecular Dynamics, Sunnyvale, CA, USA).

Ligand and immunoblot analysis

Conditioned media were separated by SDS-PAGE using 12.5% polyacrylamide gels under non-reducing conditions as previously described (Hossenlopp *et al.* 1986). The proteins were transferred to polyvinylidene difluoride filters (0.45 µm pore size; Millipore Co., Bedford, MA, USA), incubated overnight with [¹²⁵I]-IGF-I or [¹²⁵I]-IGF-II, washed, and autoradiographed at −70 °C for 1–3 days with intensifying screens. To identify the specific forms of IGFBP present in MAC-T cell-conditioned media, 2 ml 72 h-conditioned media was concentrated with a centricon-10 microconcentrator (Amicon, Inc., Beverly, MA, USA) and separated by SDS-PAGE. After transfer, the filter was cut into strips and either incubated with [¹²⁵I]-IGF-II overnight or immunoblotted with antisera against bovine IGFBP-2 (1:6000), bovine IGFBP-3 (1:2500), human IGFBP-4 (1:1000), or human IGFBP-6 (1:1000). Filters were incubated with primary antisera against IGFBPs for 2 h at room temperature, washed as previously described (Cohick *et al.* 1993), and incubated with 1:20 000 anti-rabbit IgG conjugated to horseradish peroxidase (Sigma). After washing, peroxi-

Figure 1 Identification of the forms of IGFBP secreted by MAC-T cells. Media (2 ml) conditioned by insulin (5 µg/ml)-stimulated MAC-T cells for 72 h was concentrated with a centricon-10 microconcentrator and separated by SDS-PAGE. After transfer, the filter was cut into strips and either ligand blotted with [¹²⁵I]-IGF-II, or immunoblotted with antisera to IGFBPs. Primary antibody dilutions were 1:6000 for bovine IGFBP-2, 1:2500 for bovine IGFBP-3, and 1:1000 for human IGFBP-4 and human IGFBP-6.

dase activity was detected using the enhanced chemiluminescence detection system (ECL, Amersham) according to the manufacturer's recommendations.

Statistical analysis

Differences between serum-free controls and treated groups were compared by Student's *t*-test. *P* < 0.05 was considered significant.

Results

The forms of IGF binding proteins (IGFBP) secreted by MAC-T cells were identified by western blotting techniques (Fig. 1). Ligand blotting with [¹²⁵I]-IGF-II indicated that numerous bands were present in conditioned media of MAC-T cells that had been exposed to insulin (5 µg/ml) for 72 h. Immunoblotting with specific antibodies identified these bands as IGFBP-3 (49 000 to 43 000 *M_r*), IGFBP-2 (34 000 *M_r*), IGFBP-6 (30 000 to 25 000 *M_r*), and IGFBP-4 (24 000 *M_r*). In contrast to IGFBP-2, -3, and -4, IGFBP-6 was not detected by ligand blotting with [¹²⁵I]-IGF-I (data not shown).

The growth of MAC-T cells and other MECs is stimulated by the IGFs and inhibited by retinoic acid (Fontana *et al.* 1991, McGrath *et al.* 1991, Zhao *et al.* 1992, Woodward *et al.* 1996). In addition, retinoic acid inhibits the mitogenic effects of IGF-I on MECs (Fontana *et al.* 1991, Woodward *et al.* 1996). To determine if these factors regulate IGFBP synthesis by MAC-T cells, confluent cultures were incubated in serum-free media in

Figure 2 Representative autoradiogram of the effect of IGF-I, retinoic acid, forskolin and PMA on IGFBP levels in conditioned media of MAC-T cells. After a 24 h exposure to serum-free media, confluent cultures of MAC-T cells were exposed to serum-free media with or without treatments for 24 h. Treatments were: 5 μ M forskolin (lane 1), 100 ng/ml IGF-I (lane 2), 5 μ M forskolin plus 100 ng/ml IGF-I (lane 3), serum-free control (lane 4), 100 ng/ml PMA (lane 5), 100 ng/ml PMA plus 100 ng/ml IGF-I (lane 6), 1 μ M retinoic acid (lane 7), serum-free control (lane 8), 100 ng/ml IGF-I (lane 9), and 1 μ M retinoic acid plus 100 ng/ml IGF-I (lane 10). Lanes 1–6 were run on one gel and lanes 7–10 on a second gel. Membranes were hybridized with [125 I]-IGF-II as described in Materials and Methods. Similar results were obtained in at least two additional experiments.

the presence of each factor for 24 h. As shown in Fig. 2, cultures incubated in serum-free media without additives secreted predominately IGFBP-2, -4, and -6. The amount of IGFBP-3 detectable by ligand blotting was very low under these conditions (lanes 4 and 8). However, exposure to 100 ng/ml IGF-I dramatically increased IGFBP-3 protein levels relative to serum-free controls. This effect was specific for IGFBP-3, as IGF treatment did not result in consistent increases in the other forms of IGFBP. Treatment with retinoic acid consistently increased protein levels of both IGFBP-2 and IGFBP-6. In contrast, IGFBP-3 levels were decreased by retinoic acid (detectable with longer autoradiographic exposures). The combination of IGF-I and retinoic acid was not able to overcome completely the decrease in IGFBP-3 observed with retinoic acid alone, although the decrease was attenuated.

To determine if agents that activate either the protein kinase A or protein kinase C (PKC) intracellular signaling pathways affect IGFBP protein levels, confluent cultures were exposed to either forskolin or PMA, respectively. As shown in Fig. 2, both forskolin and PMA increased IGFBP-3 levels in conditioned media. However, the combination of forskolin and IGF-I resulted in dramatically greater increases in IGFBP-3 compared with either treatment alone. The amounts of IGFBP-3 protein in conditioned media of cells treated with PMA plus IGF-I were not different from those observed with PMA alone. Forskolin consistently decreased IGFBP-6, while having no effect on IGFBP-2. PMA had no effect on protein levels of IGFBP-2, -4 or -6.

Northern analysis was performed to determine if alterations in IGFBP protein levels corresponded with changes in the abundance of IGFBP mRNA (Fig. 3). Exposure to 100 ng/ml IGF-I increased IGFBP-3 mRNA levels 2.5-, 3- and 10-fold above those of serum-free controls in

each of three experiments. In these experiments, cells were exposed to treatments for 24 h. This duration of exposure underestimated the maximal IGFBP-3 responses, which were obtained between 8 and 13 h of treatment (see Fig. 6 below). IGFBP-3 mRNA was also increased between two- and sixfold after exposure to IGF-II for 24 h. Messenger RNA levels of IGFBP-2 or -6 were not altered by IGF treatment.

In contrast to the IGFs, retinoic acid suppressed IGFBP-3 mRNA levels relative to those of serum-free controls, whereas it increased IGFBP-2 and -6 mRNA. The addition of retinoic acid plus IGF-I attenuated the decrease in IGFBP-3 mRNA, while having no additional effect on message levels of IGFBP-2 or -6 above that observed with retinoic acid alone.

Exposure to forskolin also increased IGFBP-3 mRNA levels. The combination of forskolin and IGF-I resulted in a dramatic increase in IGFBP-3 mRNA that was greater than the additive effects of either agent alone (5-, 19- and 23-fold in three experiments after 24 h of treatment). In contrast to its effects on IGFBP-3, forskolin treatment markedly suppressed the levels of IGFBP-6 mRNA compared with serum-free controls. The inhibition was less marked when cells were exposed to IGF-I in combination with retinoic acid. Forskolin had no effect on IGFBP-2 mRNA levels. In contrast to forskolin, PMA had no effect on IGFBP-3 mRNA levels, even though the amounts of protein in conditioned media were increased. However, the combination of PMA plus IGF-I increased IGFBP-3 mRNA levels to a greater extent than did IGF-I alone in two of three experiments. PMA either alone or in combination with IGF-I had no effect on IGFBP-2 or -6 mRNA levels.

To determine if the ability of IGF-I to increase IGFBP-3 synthesis was mediated by the IGF-I receptor, IGF-I analogs with varying affinities for the IGF-I receptor and IGF binding proteins were tested (Fig. 4). Exposure to 50 ng/ml B-chain IGF-I (reduced affinity for the IGF binding proteins but normal affinity for the IGF-I receptor) also increased IGFBP-3 protein and mRNA levels. When quantitated by phosphorimager analysis, the IGFBP-3 mRNA response to 50 ng/ml B-chain IGF-I was found to be greater than the response to a similar concentration of native IGF-I in each of three individual experiments. Increasing concentrations of native IGF-I up to 200 ng/ml further increased the IGFBP-3 response (lane 9). IGF-II also increased IGFBP-3 mRNA levels, although the response was less than that observed with a similar concentration of IGF-I. Treatment with 5 μ g/ml insulin also resulted in large increases in IGFBP-3 mRNA. In contrast, 50 ng/ml Leu-IGF-I (normal affinity for IGF binding proteins but reduced affinity for the IGF-I receptor) failed to stimulate IGFBP-3 mRNA levels. These results indicate that IGF-I increases the levels of IGFBP-3 mRNA and protein by an IGF-I receptor-mediated event.

Figure 3 Regulation of IGFBP mRNA levels by the IGFs, retinoic acid, forskolin and PMA. After a 24 h exposure to serum-free media, confluent cultures of MAC-T cells were exposed to serum-free media with or without treatments for 24 h. Treatments were: serum-free control (lane 1), 100 ng/ml IGF-I (lane 2), 100 ng/ml IGF-II (lane 3), 1 µM retinoic acid (lane 4), 1 µM retinoic acid plus 100 ng/ml IGF-I (lane 5), 5 µM forskolin (lane 6), 5 µM forskolin plus 100 ng/ml IGF-I (lane 7), 100 ng/ml PMA (lane 8), 100 ng/ml PMA plus 100 ng/ml IGF-I (lane 9). (A) Representative autoradiogram of total RNA (15 µg) sequentially hybridized with cDNAs for bovine IGFBP-3, IGFBP-6, and IGFBP-2. (B) Quantitation by phosphorimager analysis of three individual experiments. Bars represent means $\pm$ S.E. of three separate experiments expressed as a percentage of mRNA levels in the untreated cells. Open bars represent the treatment alone; hatched bars represent the treatment plus 100 ng/ml IGF-I. *Significant difference between the treatment and serum-free control ($P < 0.05$, Student's *t*-test).

Figure 4 Regulation of IGFBP mRNA levels by insulin, IGF-I and IGF-I analogs with varying affinities for the IGF-I receptor. After a 24 h exposure to serum-free media, confluent cultures of MAC-T cells were incubated in serum-free media for 16 h with or without treatments: 50 ng/ml B-chain IGF-I (lane 1), 50 ng/ml Leu-IGF-I (lane 2), 5 µg/ml insulin (lane 3), 50 ng/ml IGF-II (lane 4), serum-free control (lane 5), 50 ng/ml IGF-I (lane 6), 25 ng/ml IGF-I (lane 7), 10 ng/ml IGF-I (lane 8), and 200 ng/ml IGF-I (lane 9). (A) Autoradiogram of total RNA (15 µg) hybridized with cDNAs for bovine IGFBP-3, IGFBP-6, IGFBP-2 and GAPDH. (B) Quantitation by phosphorimager analysis of three individual experiments. Bars represent means $\pm$ S.E. of three separate experiments expressed as a percentage of mRNA levels in the untreated cells.

A full dose-response curve was performed to compare further the ability of IGF-I and IGF-II to increase IGFBP-3. As shown in Figs 4 and 5, IGFBP-3 mRNA was increased in a dose-dependent manner by between 10 and 200 ng/ml IGF-I. In contrast, IGFBP-3 mRNA

Figure 5 Dose-dependent effects of IGF-I and IGF-II on IGFBP mRNA levels. After a 24 h exposure to serum-free media, confluent cultures of MAC-T cells were incubated in serum-free media for 16 h with or without 0, 10, 25, 50 or 200 ng/ml IGF-I (continuous lines) or IGF-II (dashed line). Total RNA (15 µg) was hybridized with cDNAs for bovine IGFBP-3 (●), IGFBP-6 (◆), IGFBP-2 (▼) and GAPDH (▲). Membranes were analyzed by phosphorimager analysis. Values are expressed as a percentage of mRNA levels in the untreated cells. A representative experiment is shown; similar results were obtained in two other experiments.

levels were not affected by 10 or 25 ng/ml IGF-II, but increased with 50 or 200 ng/ml IGF-II. The specificity of the IGFBP-3 response was determined by sequentially hybridizing each membrane with cDNA probes for IGFBP-2 or -6 or GAPDH. Concentrations up to 200 ng/ml IGF-I or -II failed to affect IGFBP-2 or -6 mRNA levels. These data indicate that the large increases in IGFBP-3 mRNA obtained in these experiments were not due to differences in sample loading. In contrast, IGF-I consistently increased GAPDH levels as much as threefold. Therefore, while this mRNA is not an appropriate indicator of sample loading, the much greater increases in IGFBP-3 mRNA levels observed in response to the IGFs relative to GAPDH provide additional evidence of the specificity of this response.

The time-course for IGFBP-3 mRNA induction is presented in Fig. 6. IGFBP-3 mRNA levels were not increased following 1 h of exposure to IGF-I (100 ng/ml). However, by 3 h, IGFBP-3 mRNA was increased fourfold above that in serum-free controls and reached maximal values of six- to eightfold between 8 and 13 h. By 24 h of exposure to IGF-I, the IGFBP-3 mRNA levels had declined and were only threefold above serum-free controls. Hence, the much larger increases

Figure 6 Time-course of IGF-I induction of IGFBP-3 mRNA levels in MAC-T cells. After a 24 h exposure to serum-free media, confluent cultures of MAC-T cells were exposed to serum-free media or 100 ng/ml IGF-I for increasing periods of time between 1 and 24 h. Total RNA (15 µg) was sequentially hybridized with cDNAs for bovine IGFBP-3, bovine IGFBP-6 and 18S ribosomal RNA. Values (obtained by phosphorimager analysis) represent means ± S.E. of three separate experiments expressed as a percentage of mRNA levels in the untreated cells. *Significant difference between the treatment and serum-free control ($P < 0.05$, Student's *t*-test).

obtained with IGF-I and -II treatment shown in Figs 4 and 5 relative to Fig. 3 are due to the difference in the duration of exposure to these factors (16 compared with 24 h). In contrast to IGFBP-3, IGFBP-6 mRNA was not increased above serum-free controls at any time point.

Discussion

Understanding the regulation of mammary growth and development is important for both normal lactation physiology and the transition to disease states (i.e. transformation and neoplasia). IGF-I has consistently been shown to be a potent mitogen for both primary MECs (Winder *et al.* 1989, McGrath *et al.* 1991) and MEC lines, including the MAC-T cell line (Zhao *et al.* 1992, Chen *et al.* 1994, Hadsell *et al.* 1994). The MAC-T cell line represents the only available MEC line of bovine origin, therefore it may serve as a useful model to study IGF-IGFBP interactions in the bovine MEC. Media conditioned by confluent cultures of MAC-T cells contained four distinct forms of IGFBP, which were identified by immunoblotting as IGFBP-2, -3, -4 and -6. The MAC-T cell line has previously been shown to secrete IGFBP-2, -3 and a 24 kDa IGFBP (Romagnolo

et al. 1994) that we have now identified as IGFBP-4. In addition, the present study demonstrated that this cell line also synthesizes IGFBP-6, which is detectable by ligand blot analysis when [125 I]-IGF-II is utilized as the ligand. The inability to detect IGFBP-6 by ligand blotting using [125 I]-IGF-I as the ligand was expected, and is consistent with the lower affinity of IGFBP-6 for IGF-I than for IGF-II (Martin *et al.* 1990). The greater molecular weight observed for IGFBP-6 in bovine-cell-conditioned media relative to that of the recombinant protein expressed in yeast has been reported by others (Bach *et al.* 1993, Martin *et al.* 1994) and shown to be due to O-linked glycosylation (Bach *et al.* 1992).

Primary bovine MECs have also been shown to secrete IGFBP-2 and -3, in addition to unidentified 28 and 21 kDa bands, by ligand blotting with [125 I]-IGF-I in the presence of serum, IGF-I and epidermal growth factor (McGrath *et al.* 1991). In addition, numerous breast cancer cell lines and rodent MEC lines have been shown to secrete multiple forms of IGFBP, depending on estrogen receptor status and hormonal stimulation (Adamo *et al.* 1992, Sheikh *et al.* 1992, Skaar & Baumrucker 1993). Synthesis of IGFBP-5 (M_r 31 000–32 000) has been reported for several breast cancer cell lines (Sheikh *et al.* 1992). However, no bands migrated between IGFBP-2 (M_r 34 000) and IGFBP-4 (M_r 24 000) by ligand blot analysis using [125 I]-IGF-I as the ligand, and IGFBP-5 mRNA was not detected by northern blot analysis. Therefore it appears that IGFBP-5 is not synthesized by confluent cultures of MAC-T cells.

The amounts of IGFBP-3 in conditioned media of MAC-T cells were increased dramatically by exposure to IGF-I, confirming previous reports (Woodward *et al.* 1996). IGF-I also increased IGFBP-3 levels in conditioned media of primary bovine MECs (McGrath *et al.* 1991), murine MECs (COMMA-D; Skaar & Baumrucker 1993) and human breast cancer cells (Camacho-Hubner *et al.* 1991, Sheikh *et al.* 1993). The IGFBP-3 response to IGF-I has also been observed in cells of non-mammary origin, including human retinal pigment cells (Feldman & Randolph 1994), Sertoli cells (Smith *et al.* 1992), ovarian granulosa cells (Grimes & Hammond 1992), bovine and human fibroblasts (Conover 1990, 1991), and bovine endothelial cells (Erondur *et al.* 1996). However, the mechanism by which IGF-I increases IGFBP-3 availability appears to vary depending on the cell system.

In the present study, levels of IGFBP-3 mRNA were increased by exposure of MAC-T cells to IGF-I, suggesting that IGF-I affected IGFBP-3 protein synthesis at a pretranslational level. Similar findings have been reported for bovine fibroblasts (Bale & Conover 1992), porcine granulosa cells (Grimes & Hammond 1992) and bovine endothelial cells (Erondur *et al.* 1996). Recently, Erondur *et al.* (1996, 1997) have shown that

IGF-I treatment does not alter IGFBP-3 mRNA stability, and that the transcriptional activity of IGFBP-3 is increased by IGF-I in bovine endothelial cells. Further studies will determine if similar mechanisms are operative in MAC-T cells.

In the present study, both B-chain IGF-I and insulin at 5 µg/ml increased IGFBP-3 protein and mRNA levels. These factors activate the IGF-I receptor, but bind with little or no affinity to IGFBP-3. In contrast, Leu-IGF-I, which has low affinity for the IGF-I receptor but normal affinity for bovine IGFBP-3, was ineffective at increasing IGFBP-3 protein or message levels. These data indicate that the ability of IGF-I to increase IGFBP-3 levels in conditioned media of MAC-T cells does not require a physical interaction between IGF and the IGFBPs, but is an IGF-I receptor-mediated event. Similar findings have been reported for MAC-T cells transfected with IGF-I (MD-IGF-cells) (Romagnolo *et al.* 1994). The lower potency of IGF-II relative to IGF-I in stimulating this response may reflect the lower affinity of IGF-II for the IGF-I receptor. Alternatively, IGFBP-6 present in the conditioned media may sequester the IGF-II and decrease its availability to the receptor.

In contrast to our findings, studies with various cell types of non-bovine origin have found that IGF-I treatment increased IGFBP-3 in conditioned media without concomitant increases in IGFBP-3 mRNA (Conover 1991, Martin *et al.* 1992, Smith *et al.* 1992, Sheikh *et al.* 1993, Feldman & Randolph 1994). Thus it is possible that the mechanism by which IGF-I increases IGFBP-3 availability is species-dependent. In these studies high concentrations of insulin or IGF analogs that did not bind IGFBPs failed to increase IGFBP-3 protein levels, suggesting that IGF-I increased IGFBP-3 protein levels through a direct interaction between the two proteins that was independent of the IGF-I receptor. In human fibroblasts, IGF-I mediated this effect by binding to cell-surface-associated IGFBP-3, resulting in the release of IGFBP-3 into the conditioned media (Martin *et al.* 1992).

Whereas IGF-I had dramatic effects on IGFBP-3 synthesis, levels of IGFBP-2 and IGFBP-6 mRNA were not affected. There have been several reports that IGF-I increases the concentrations of IGFBP-2 in conditioned media of MECs (Adamo *et al.* 1992, Sheikh *et al.* 1993, Woodward *et al.* 1996). However, corresponding increases in IGFBP-2 message have not been reported (Adamo *et al.* 1992, Sheikh *et al.* 1993), suggesting that this is a post-translational effect. We also observed an increase in IGFBP-2 protein in response to IGF-I in some experiments. However, even in experiments in which the amounts of IGFBP-2 in conditioned media were increased, IGFBP-2 mRNA levels were not altered.

The intracellular signaling agents, cyclic AMP and PKC, have both been shown to affect cellular growth and differentiation (Yang *et al.* 1980, Ethier *et al.* 1989, Asaoka

et al. 1992) and to regulate IGFBP concentrations (Bachrach *et al.* 1991, Gong *et al.* 1992). In MAC-T cells, exposure to forskolin (which increases cAMP) increased IGFBP-3 expression, but the tumor promoter, PMA (which activates PKC), had no effect. However, the combination of either forskolin or PMA with IGF-I induced greater increases than were observed with either agent alone. A similar effect on IGFBP-3 synthesis was observed with forskolin and the combination of forskolin plus IGF-I in a bovine kidney epithelial cell line (Cohick & Clemmons 1991). Forskolin has also been shown to increase IGFBP-3 mRNA levels in human fibroblasts (Camacho-Hubner *et al.* 1992) and in human breast tumor MCF-7 cells (Martin *et al.* 1995). The mechanism by which forskolin (i.e. cAMP) increases IGFBP-3 mRNA levels is not known. The promoter region of the bovine IGFBP-3 gene has recently been cloned and found to contain a GC-rich region containing two overlapping putative AP-2 binding sites (Erondur *et al.* 1997). Both the cAMP-dependent protein kinase A and the phorbol ester-activated PKC pathways induce AP-2 mediated transcriptional activation (Imagawa *et al.* 1987, Hyman *et al.* 1989). Deletion of this region in the IGFBP-3 promoter abolished the ability of IGF-I to stimulate IGFBP-3 promoter activity (Erondur *et al.* 1997). This raises the possibility that AP-2 could be involved in the synergism between IGF-I and forskolin, PMA, or both, in inducing IGFBP-3 synthesis.

Retinoids are important regulators of normal epithelial cell growth and differentiation (Sporn & Roberts 1991, Love & Gudas 1994). Retinoic acid has been shown to inhibit IGF-I-stimulated thymidine incorporation in bovine MAC-T cells (Woodward *et al.* 1996) and in breast epithelial cells (MCF-7; Fontana *et al.* 1991). In the present study, exposure to retinoic acid inhibited both basal and IGF-I-stimulated IGFBP-3 expression. In contrast, retinoic acid increased the synthesis of both IGFBP-2 and -6, and the addition of IGF-I had no additional effect. Thus the relative distribution of the different forms of IGFBPs was very different after exposure to retinoic acid, with IGFBP-2 and -6 predominating relative to IGFBP-3. Woodward *et al.* (1996) reported that IGF-I-stimulated DNA synthesis in MAC-T cells was inhibited to a lesser degree by retinoic acid when IGF-I analogs with reduced affinities for the IGFBPs were used compared with native IGF-I. These data, together with the present finding that retinoic acid increases IGFBP-2 and -6, suggest a possible inhibitory role for these forms of IGFBP in bovine MEC growth. In contrast, the finding that IGF-I specifically increases the synthesis of IGFBP-3, whereas retinoic acid inhibits it, raises the possibility of a stimulatory role for IGFBP-3. The differential regulation of IGFBP-3, -2 and -6 expression and their varying affinities for the IGFs offers a sophisticated mechanism for regulation of mammary epithelial cell growth.

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