IGF-I differentially regulates IGF-binding protein expression in primary mammary fibroblasts and epithelial cells

J M Fleming, B J Leibowitz, D E Kerr¹ and W S Cohick

Department of Animal Sciences, Rutgers The State University of New Jersey, 108 Foran Hall, 59 Dudley Road, New Brunswick, New Jersey 08901-8520, USA

¹Department of Animal Science, College of Agriculture and Life Sciences, University of Vermont, 570 Main Street, Burlington, Vermont 05405, USA

(Requests for offprints should be addressed to J M Fleming; Email: jfleming@AESOP.Rutgers.edu)

Abstract

Elucidating how mitogens facilitate epithelial/stromal interactions is critical given that mitogens regulate mammary gland development and function. IGF-I is a potent mammary cell mitogen that is locally produced in the mammary gland. Since IGF-binding proteins (IGFBPs) regulate IGF-I bioavailability, we characterized the cell-type-specific production of IGFBP in primary bovine mammary epithelial (BME) and fibroblast (BMF) cells. Cells were treated with IGF-I and mRNA levels were analyzed via quantitative real-time (qRT)-PCR and Northern blot analysis. Media conditioned by cells treated with IGF-I for 48 h were analyzed via ligand blotting with ¹²⁵I-labeled IGF-I and -II and immunoblotting with specific IGFBP antibodies. A reciprocal regulation of IGFBP-3 and -5 by IGF-I was observed between the two cell types. IGF-I induced large dose-dependent increases in IGFBP-3 mRNA and protein levels in BME cells, while IGFBP-5 protein was barely detectable and mRNA levels were detectable only by qRT-PCR. In BMFs, IGF-I induced large increases in IGFBP-5 mRNA and protein while IGFBP-3 mRNA was only slightly increased by IGF-I treatment and the protein was difficult to detect. IGFBP-6 mRNA was detected by Northern blot analysis in both cell types but was not regulated by IGF-I. In BME cells, IGFBP-6 protein levels were readily detectable under basal conditions and were increased by IGF-I. Interestingly, IGFBP-6 protein could not be detected in media conditioned by BMFs. IGFBP-4

mRNA was readily seen by Northern blot analysis in BMFs, however qRT-PCR was required to detect IGFBP-4 mRNA in BME cells. IGF-I increased IGFBP-4 mRNA levels by 2-fold in both cell types. IGFBP-4 protein was only detectable in media conditioned by BME cells when stimulated by IGF-I. In contrast, IGFBP-4 was present in media conditioned by untreated BMFs but was not consistently increased by IGF-I treatment. This was explained by the finding that IGF-I stimulated proteolysis of IGFBP-4, as evidenced by the appearance of two immuno-responsive fragments of 18 and 14 kDa. This proteolysis was specific to IGFBP-4, and was not observed in BME cells. We confirmed the protease to be pregnancy-associated plasma protein A (PAPP-A) by immunoblotting with an antibody against human PAPP-A/proMBP (pro form of eosinophil major basic protein) complex. *In vitro* immuno-neutralization experiments showed that blocking PAPP-A prevented the ability of IGF-I to stimulate IGFBP-4 proteolysis. IGFBP-2 mRNA and protein levels were observed under basal conditions in both cell types, with no significant regulation by IGF-I. The analysis of cell-type-specific regulation of the IGF system in both primary mammary epithelial cells and stromal cells will assist in the characterization of the mechanisms behind the role of the IGF system in normal mammary physiology and ultimately breast cancer.

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Introduction

The insulin-like growth factor (IGF) system regulates the growth, development and function of the mammary gland through complex interactions with other growth factors and hormones. Both *in vivo* and *in vitro* studies have established a vital role for IGF-I in mammary gland development. For example, studies with IGF-I knockout mice have demonstrated that a mammary gland devoid of IGF-I fails to develop (Ruan & Kleinberg 1999). Additionally, studies

with transgenic mice have shown that mammary-specific expression of IGF-I during early puberty initiates premature ductal branching and alveolar development (Weber *et al.* 1998). *In vitro* studies have established that IGF-I possesses the ability to stimulate proliferation and inhibit apoptosis of normal mammary epithelial cells (MECs) (Cohick 1998). Even though IGF-I has unequivocally been shown to play a critical role in regulating mammary gland physiology, the precise molecular mechanisms by which this regulation occurs are poorly understood.

The IGF system is composed of two polypeptide growth factors (IGF-I and -II), two receptors and six high-affinity binding proteins (IGFBP-1 to -6). The temporal expression of ligands, receptors and each of the IGFBPs has been reported for mammary tissue (Allar & Wood 2004). The IGFBPs function to transport, prolong the half-life and regulate the bioavailability of the IGFs (Mohan & Baylink 2002). However, studies are continually emerging that suggest that both IGF-dependent as well as IGF-independent roles for the IGFBPs at the cellular level are important in mammary gland physiology (Marshman & Streuli 2002).

It is well established that epithelial/mesenchymal interactions are important for postnatal development, differentiation and hormonal responsiveness in mammary cells (Parmar & Cunha 2004). *In vivo* reconstitution studies have demonstrated that specific types of mammary stromal cells can dictate and even alter the developmental fate of normal and tumorigenic mammary epithelial cells (Haslam & Woodward 2003). Furthermore, epithelial cells cultured on a reconstituted basement membrane undergo differentiation and secrete milk proteins, whereas cells cultured on a non-specific attachment factor remain undifferentiated (Howlett & Bissell 1993). The mesenchyme is composed of multiple cell types that secrete different factors – including extracellular matrix components and growth regulatory factors – which mediate cell–cell interactions through paracrine and/or autocrine mechanisms. These secreted components provide the necessary environment for the different phases of mammary gland development and are often involved in the progression of breast cancer (Bissell *et al.* 2003, Haslam & Woodward 2003).

The goal of this study was to determine the forms of IGFBP that are synthesized by primary mammary epithelial cells compared with fibroblasts, and to determine how IGF-I regulates their production and/or availability.

Materials and Methods

Reagents

Recombinant human IGF-I was obtained from GroPep (North Adelaide, Australia). Cell culture reagents were from Invitrogen, with the exception of fetal bovine serum (Gemini Bio-Products, Woodland, CA, USA) and phenol-red-free Dulbecco's modified Eagle's medium (Sigma). Tissue culture plasticware was from Becton-Dickinson (Franklin Lakes, NJ, USA). Bovine insulin and D-glucose were from Sigma. Antisera against bovine IGFBP-2, -4 and -5 were generous gifts of Dr David Clemmons (University of North Carolina, Chapel Hill, NC, USA). Human IGFBP-3 and IGFBP-6 antisera were obtained from Diagnostic Systems Laboratories Inc. (Webster, TX, USA) and Austral Biologicals (San Ramone, CA, USA) respectively. Pregnancy-associated plasma protein A (PAPP-A)/proMBP (pro form of eosino-

phil major basic protein) antibody was kindly provided by Dr Claus Oxvig (University of Aarhus, Denmark).

Cell culture experiments

Primary bovine mammary fibroblasts (BMFs) isolated from a lactating heifer were kindly provided by Dr Anthoula Lazaris (Nexia Biotechnologies, Montreal, Canada). Primary MECs were isolated from a mastitis-free, culture-negative, lactating cow selected from the Department of Animal and Food Science, University of Vermont herd. The cow was euthanized 6 h after morning milking at a commercial, USDA-inspected abattoir by penetrating captive bolt. Mammary tissue samples were recovered within 30 min of death. Primary MECs were isolated as previously described (Wellnitz & Kerr 2004). The bovine MEC line MAC-T was established from primary bovine MECs by immortalization with the SV40 large-T antigen (Huynh *et al.* 1991). Stock plates of primary cells were maintained in phenol-red-free DMEM supplemented with 4.5 g/l D-glucose, 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 50 µg/ml gentamicin (complete media), at 37 °C in a humidified atmosphere with 5% CO₂. Stock plates of MAC-T cells were maintained in the same media supplemented with phenol red and 5 µg/ml bovine insulin.

For all experiments, cells were plated in complete media without insulin or phenol red. Cells were grown to confluence, washed twice with phenol-red-free, serum-free (PRFSF) DMEM, and incubated in PRFSF DMEM with 0.2% BSA and 30 nM sodium selenite. Following a 24-h wash-out period, spent media were aspirated and replaced with PRFSF DMEM without additives ± treatments. For RNA analysis, cells were lysed in Trizol (Invitrogen) and stored at –80 °C until analysis. For analysis of secreted IGFBP and PAPP-A protease experiments, conditioned media were collected, cleared by centrifugation and stored at –20 °C until analysis.

Western ligand blotting

Total protein content of the conditioned media was determined using the Bradford protein assay kit (BioRad). Conditioned media containing equal amounts of protein were separated by electrophoresis through 12.5% resolving SDS-polyacrylamide gels under non-reducing conditions, as previously described (Cohick & Turner 1998). Briefly, the proteins were transferred to nitrocellulose membranes, incubated overnight with ¹²⁵I-labeled IGF-I or -II (Amersham), washed, and then autoradiographed for 1–3 days with intensifying screens.

Western immunoblotting

Total protein content of the conditioned media was determined and equal amounts of protein were separated

by electrophoresis as described above. Membranes were blocked in 5% non-fat dried milk for 1 h at room temperature, incubated with primary antisera overnight at 4 °C (incubation with human IGFBP-3 was for 2 h at room temperature), washed and incubated with the appropriate secondary antibody conjugated to horseradish peroxidase (Amersham) as previously described (Cohick & Turner 1998). After washing, peroxidase activity was detected using the enhanced chemiluminescence detection system (ECL Plus, Amersham) according to the manufacturer's recommendations. For IGFBP-6 and PAPP-A protein detection, 15 ml of conditioned media were concentrated using Amicon Ultra centrifugal filters (Millipore, Bedford, MA, USA) with molecular weight cut-offs of 10 000 and 100 000 kDa respectively, prior to electrophoresis.

Immuno-neutralization experiments

Serum-free conditioned media were collected from BMFs after 48 h as described above. The media (50 µl) were preincubated with vehicle (PBS), 10 µg antihuman PAPP-A/proMBP polyclonal antibody, or non-immune immunoglobulin (Ig)G for 1 h at 37 °C in a humidified atmosphere with 5% CO₂. After preincubation, the reaction mixture was incubated with or without IGF-I under the same conditions overnight. Following termination of the incubation, the samples were analyzed by Western ligand blotting.

Northern blotting

Total RNA was isolated and analyzed by Northern blotting as described previously (Cohick *et al.* 2000). Briefly, denatured RNA (10 µg/lane) was separated by electrophoresis, transferred to nylon membranes (Biotrans; ICN, Irvine, CA, USA), then hybridized overnight with [³²P]-dCTP-labeled cDNA probes for: bovine IGFBP-2, -3, and -6; human IGFBP-4 and -5; or for 18S ribosomal RNA. Membranes were washed and then exposed to Kodak AR film with intensifying screens at -80 °C. Differences in relative band intensities were determined by PhosphorImager analysis (Molecular Dynamics, Inc., Sunnyvale, CA, USA).

Reverse transcription, PCR and quantitative real-time (qRT)-PCR

Unless otherwise stated, all reagents were purchased from Invitrogen. Total RNA was treated with DNase (Ambion, Austin, TX, USA) according to manufacturer's instructions. RNA quality following DNase digestion was verified by inspection of the 18S and 28S rRNA bands after agarose gel electrophoresis. DNase-treated RNA (2 µg) was reverse transcribed using oligo-dT primers, SuperScript II Reverse Transcriptase and First Strand

Synthesis buffer amended with dithiothreitol (DTT). For standard PCR, reactions contained PCR buffer mix, 1 unit of Taq DNA polymerase, 0.5 mM of each dNTP, 0.75 mM MgCl₂, 0.1 µM of each primer, 2 µl template cDNA and distilled water to a final volume of 50 µl. The primers encoding IGF-I, IGF-II and IGF-I receptor (IGF-IR) sequences were as follows. IGF-I: forward, 5'-GCCT GCGCAATGGAATAAAGTCCT-3'; reverse, 5'-TGG GCATCTTCACCTGCTTCAAGA-3'. IGF-II: forward 5'-TGGACACCCTCCAGTTTGTCTGT-3'; reverse, R 5'-TCGGAAGCAACACTCTTCCACGAT-3'. IGF-IR: forward, 5'-ATCCACAGCTGTAACCATGAG GCT-3'; reverse, 5'-TGGGATTCTCAGGTTCTGGC CATT-3'. Amplification conditions were a single denaturation step at 94 °C for 2.5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min followed by a final extension for 5 min at 72 °C.

For quantitative PCR, individual standard curves were established for each mRNA analyzed using specific primer sets and serial dilutions (1:2, 1:20, 1:200 and 1:2000) of a common pool of cDNA. The IGFBP-3 primer set was obtained from Voge *et al.* (2004). Primer sets for cyclophilin (forward, 5'-GTTCCAAAGACAGCAGA-3'; reverse, 5'-CCTAAATAGACGGCTCC-3'), IGFBP-5 (forward, 5'-AGTCGTGCGGCGTCTACACTGAG-3'; reverse, 5'-AAGATCTTGGGCGAGTAGGTCTCC-3') and IGFBP-4 (forward, 5'-TGTGTGCGTGTGTGTT AATGAGCC-3'; reverse, 5'-TTGAAACATACCAG GGCTCTCCT-3') were developed using PrimerQuest (IDT, Coralville, IA, USA). To determine gene expression profiles, individual samples were diluted 1:4 and 5 µl were amplified in a 27.5 µl reaction mix containing 12.5 µl Platinum Quantitative PCR Supermix-UDG, 6.5 µl distilled H₂O, 0.5 µl (~200 µM) of each forward and reverse gene-specific primer and 2.5 µl of a 1:10⁵ dilution of SYBR Green fluorescent dye (Molecular Probes, Eugene, OR, USA). Quantitative PCR was performed in single wells on 96-well plates (TKR Biotech, PA, USA) and fluorescent PCR products were detected using a BioRad iCycler iQ. Cycle threshold (Ct) values were determined with iCycler iQ Real-time Detection System Software, version 3.1 (BioRad). Data were normalized using cyclophilin and relative expression of treated samples to untreated samples was determined. Products were verified by agarose gel electrophoresis and melting curves were generated for each sample.

Statistical analysis

Data from experiments were analyzed by ANOVA with differences considered significant where $P < 0.05$. For time course data, individual *t*-tests were run as *post-hoc* tests with a Dunn-Sidak correction for overall protection at $P < 0.013$. For dose-response data, REGWQ were run as *post-hoc* tests. Analyses were performed in both the

Figure 1 IGF-I differentially regulates IGFBP in primary BMFs and BME cells. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then treated with serum-free media ± IGF-I (100 ng/ml) for 48 h. Conditioned media containing equal amounts of protein (BME, 6 µg; MAC-T, 5 µg; BMF, 2 µg) for each pair of samples were separated by SDS-PAGE. Following transfer, the membranes were ligand blotted with ¹²⁵I-labeled IGF-I (A) or ¹²⁵I-labeled IGF-II (B). Results are representative of a minimum of three experiments.

SigmaStat (2.03) program for Windows (SPSS Inc., Chicago, IL, USA) and the SAS program 9.1 for Windows (SAS Institute Inc., Cary, NC, USA).

Results

Regulation of IGFBP secretion by IGF-I is similar for primary bovine mammary epithelial (BME) and MAC-T cells

We have previously shown that under basal, serum-free conditions the BME cell line, MAC-T, secretes IGFBP-2, -4 and -6 and that exposure to IGF-I results in a large induction of IGFBP-3 protein secretion (Cohick & Turner 1998). To determine how the IGFBP secretion profile in this non-transformed, immortalized cell line compares with primary mammary epithelial cells, we examined the IGFBP profile secreted by primary BME cells from a similar physiological state. As shown in Fig. 1, the IGFBP profiles present in conditioned media of cells treated with or without IGF-I were very similar for primary BME and MAC-T cells. Under basal, serum-free conditions, IGFBP-2 and -6 proteins were readily detected in media conditioned by BME cells for 48 h. IGFBP-2 was detected by ligand blotting with either ¹²⁵I-labeled IGF-I or IGF-II. However, IGFBP-6 was only detected when IGF-II was used as the radiolabel, as previously reported (Cohick & Turner 1998). In contrast, IGFBP-3 and -4 were minimally detected in media conditioned by both MAC-T and primary BME cells under basal conditions. Treatment with 100 ng/ml IGF-I for 48 h dramatically increased IGFBP-3 and -6 protein levels as detected by

ligand blotting in both MAC-T and primary BME cells (Fig. 1). Smaller increases were also observed in IGFBP-2 and -4 protein secretion in response to IGF-I.

IGF-I differentially regulates IGFBP secretion in primary BMFs and BME cells

Given the importance of stromal–epithelial interactions in the mammary gland, we next examined the IGFBP profile secreted by BMFs isolated from a similar physiological state. Under basal, serum-free conditions the major IGFBPs detected by ligand blotting using ¹²⁵I-labeled IGF-I as the ligand were IGFBP-2, -4 and -5. When IGF-II was used as the radioligand, small amounts of IGFBP-3 were also observed under basal conditions. IGFBP-6 protein was undetectable in media conditioned by BMFs. Following treatment with IGF-I, a large increase in IGFBP-5 protein levels was observed. Smaller increases in IGFBP-2 and -4 protein secretion were observed, similar to that seen in BME cells. The increases in IGFBP-4 were variable and not always observed. In addition, a band of 18 kDa was present following exposure to IGF-I when IGF-II was used as the ligand. In contrast to BME cells, secretion of IGFBP-3 protein was not altered by exposure to IGF-I.

The identity of the individual forms of IGFBP as well as the ability of IGF-I to regulate their secretion was confirmed by Western immunoblotting with antisera to the specific IGFBP (Fig. 2). Similar to results obtained with ligand blotting, treatment with IGF-I increased IGFBP-2, -3, -4 and -6 levels in BME cells, and increased IGFBP-2

Figure 2 Identification of IGFBP secreted by primary BMFs and BME cells. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then treated with serum-free media $\pm$ IGF-I (100 ng/ml) for 48 h. Representative immunoblots of media conditioned by IGF-I-stimulated primary epithelial cells and fibroblasts for 48 h are shown. Media were concentrated and separated by SDS-PAGE. After transfer, the membranes were immunoblotted with specific IGFBP antisera. Results are representative of a minimum of three experiments.

and -5 in BMFs. The 18 kDa fragments as well as a 14 kDa fragment were detected with IGFBP-4 antisera. While ligand blotting analysis indicated no detectable effect on IGFBP-3 protein levels in BMFs, small increases were detected by immunoblotting. However, this increase

was markedly less than the increase in IGFBP-3 observed when BME cells were treated with IGF-I.

IGF-I regulates IGFBP-3 mRNA levels in primary BME cells in a time- and dose- dependent manner

We next determined whether changes in protein secretion corresponded with changes in mRNA levels in each cell type. Similar to our previous findings in the MAC-T cell line (Cohick & Turner 1998), IGFBP-2 and -6 mRNA levels were readily detected in BME cells under basal, serum-free conditions but were not increased in response to IGF-I (Fig. 3). Exposure to IGF-I significantly increased IGFBP-3 mRNA levels above those of untreated serum-free controls in a dose-responsive manner ($P < 0.001$) (Fig. 3). Time-course analysis indicated that an approximately 10-fold increase above serum-free levels was observed by 8 h of treatment, with elevated levels sustained through 24 h ($P < 0.05$) (Fig. 4A and B). While mRNA levels of IGFBP-2 were not significantly altered by IGF treatment, they did significantly decrease between 4 and 24 h (Fig. 4C) ($P < 0.01$). Since IGFBP-4 and -5 mRNA levels were not detectable by Northern blot analysis, quantitative real-time (qRT)-PCR was used to determine if IGF-I treatment increased mRNA levels of

Figure 3 IGF-I increases IGFBP-3 mRNA levels in a dose-responsive manner in primary BME cells. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then exposed to serum-free media $\pm$ increasing doses of IGF-I for 8 h. Total RNA was isolated and analyzed by Northern blotting via hybridization with 32 P-labeled bovine IGFBP-2, -3, -6 and 18S cDNA. (A) Representative Northern blot. (B) Relative intensity by PhosphorImager analysis (corrected for 18S): data represent means $\pm$ S.E.M. of at least three separate experiments. IGF treatment was determined as significant, $P < 0.001$, (ANOVA) with a *post-hoc* REGWQ multiple-range test indicating groups b and c are significant compared with group a, and group c is significant compared with group b.

Figure 4 Temporal induction of IGFBP mRNA levels by IGF-I in primary BME cells. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then exposed to serum-free media $\pm$ IGF-I (100 ng/ml) for indicated times. Total RNA was isolated and analyzed by Northern blotting by hybridizing with 32 P-labeled bovine IGFBP-2, -3, -6 or 18S cDNA. (A) Representative Northern blot. (B and C) Relative intensity by PhosphorImager analysis for IGFBP-3 and -2 respectively; data represent means $\pm$ S.E.M. of at least three separate experiments. *Significance of $P < 0.05$ for overall protection (ANOVA).

these IGFBPs in BME cells. IGFBP-4 mRNA levels were increased by IGF-I between 8 and 12 h of treatment, but had returned to basal levels by 24 h (Fig. 5). In contrast, IGFBP-5 mRNA levels, while detectable by qRT-PCR, were not consistently increased in response to IGF-I (Fig. 8).

IGF-I differentially regulates IGFBP mRNA levels in primary BMFs compared with BME cells

As shown in Fig. 6, mRNA for IGFBP-2, -4 and -6 were expressed by BMFs under basal, serum-free conditions. In

contrast, IGFBP-3 and -5 were difficult to detect by Northern blotting without overexposing the autoradiogram. Following exposure to IGF-I, dose-responsive increases in IGFBP-3, -4 and -5 were observed (Fig. 6). Relative to the other IGFBPs, IGFBP-5 mRNA levels were regulated to the greatest degree by IGF treatment, with significant increases of 6-fold observed between 8 and 12 h ($P < 0.008$) (Figs 6B and 7B). IGFBP-4 mRNA levels were increased 2-fold by IGF-I treatment, with significant increases observed at 8 and 12 h ($P < 0.02$) (Fig. 7C). Time-course analysis revealed that IGFBP-3 mRNA levels also significantly increased with IGF-I

Figure 5 IGF-I regulates IGFBP-4 mRNA levels in primary BME cells. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then exposed to serum-free media $\pm$ IGF-I (100 ng/ml) for indicated times. Total RNA was isolated, DNase treated and analyzed by qRT-PCR as described in the Materials and Methods. Data represent means $\pm$ S.E.M. of two separate experiments.

treatment over time ($P < 0.003$). As for BME cells, mRNA levels of IGFBP-2 and -6 were not significantly altered by IGF treatment.

Northern blot analysis indicated that IGF-I induced a greater increase in IGFBP-5 mRNA levels in BMFs compared with changes in IGFBP-3 mRNA. In contrast, IGF-I had a greater effect on IGFBP-3 mRNA levels in BME cells. To evaluate this difference in response between the two cell types more quantitatively, qRT-PCR was employed. Treatment of BMFs with IGF-I induced a 10-fold increase in IGFBP-5 mRNA levels and only a 2-fold increase in IGFBP-3. The converse was observed in BME cells, i.e. IGF-I treatment induced a 40-fold increase in IGFBP-3 mRNA while having little to no effect on mRNA levels of IGFBP-5 (Fig. 8).

IGF-I stimulates IGFBP-4 proteolysis in primary BMFs but not in BME cells

As previously mentioned, exposure of BMFs to IGF-I resulted in the appearance of two small immunoresponsive fragments of approximately 18 and 14 kDa (Fig. 2). IGFBP-4 fragments were not detected in BME cells treated with IGF-I. IGF-I-dependent proteolysis of IGFBP-4 by the protease PAPP-A has been previously reported in human skin fibroblasts as well as preovulatory follicles from multiple species including bovine (Lawrence *et al.* 1999, Mazerbourg *et al.* 2001). Therefore, we immunoblotted BMF-conditioned media with an antibody against human PAPP-A/proMPB complex and found that PAPP-A is also secreted by BMFs (Fig. 9A). To determine if an antibody against PAPP-A could inhibit the IGFBP-4 proteolysis induced by IGF-I, cell-free, immuno-neutralization experiments were performed. Anti-PAPP-A/proMBP polyclonal IgG, but not non-specific rabbit IgG, inhibited IGF-dependent IGFBP-4 protease activity present in conditioned media (Fig. 9B and C). Ligand blot analysis of the immuno-neutralization experiments indicated that IGF-II appeared to have a

higher affinity for the IGFBP-4 fragments compared with IGF-I (Fig. 9B and C). Since slight proteolysis of IGFBP-4 was evident in media conditioned by BMFs without the addition of exogenous IGF-I (Fig. 9B), we hypothesized that these cells produce endogenous IGFs that could induce constitutive proteolysis of IGFBP-4 by proteases such as PAPP-A. Therefore, IGF-I and -II mRNA expression was analyzed using qRT-PCR in both BME cells and BMFs. IGF-IR expression was used as a control and was detected in both cell types (Fig. 10). Both IGF-I and -II mRNA levels were readily detectable in BMFs, however mRNA for IGF-I and -II was undetectable in BME cells.

Discussion

The biological action of IGF in the mammary gland is regulated by a complex system comprised of multiple IGFBPs. Our findings indicate that stromal and epithelial components of the gland differentially express multiple forms of IGFBP that are regulated by IGF-I in a cell-type-specific manner. This supports the hypothesis that the local production and regulation of the IGF axis is fundamental to physiological events within the gland. Recent studies investigating the expression of the IGF axis throughout the murine mammary gland have shown that each IGFBP has a distinct spatial and temporal expression during postnatal phases of mammary gland development (Richert & Wood 1999, Allar & Wood 2004, Boutinaud *et al.* 2004). IGFBP-3 mRNA exhibited the highest level of expression of all IGFBPs across developmental stages, when analyzed via qRT-PCR, with the exception of early involution – where IGFBP-5 mRNA levels were an order of magnitude higher than IGFBP-3 (Boutinaud *et al.* 2004). This supports the hypothesis that although IGFBP-3 is the major IGFBP in serum, there is also a requirement for significant local production of IGFBP-3. *In situ* hybridization analysis indicated that IGFBP-2 and -4 mRNA were

Figure 6 IGF-I increases IGFBP-5, -4 and -3 mRNA levels in a dose-responsive manner in primary BMFs. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then exposed to serum-free media $\pm$ increasing doses of IGF-I for 8 h. Total RNA was isolated and analyzed by Northern blotting via hybridization with 32 P-labeled bovine IGFBP-2, -3, -6 or human IGFBP-4 and -5 cDNA. (A) Representative Northern blot. (B and C) Relative intensity by PhosphorImager analysis of IGFBP-5 and -4 respectively: data represent means $\pm$ S.E.M. of at least three separate experiments. IGF-treatment was determined significant at $P < 0.02$ (ANOVA) with a *post-hoc* REGWQ multiple-range test indicating that group b is significantly different compared with group a.

predominately found in the stroma while IGFBP-3 and -5 were found in both stromal and epithelial compartments. In addition, within a given compartment each IGFBP had

a distinct mRNA expression pattern (Allar & Wood 2004). Messenger RNA levels of IGF-I, -II, IGFR, and all six IGFBP transcripts have been analyzed across physiological stages in the bovine mammary gland (Plath-Gabler *et al.* 2001). Similar to the murine data, RT-PCR of whole mammary extracts identified IGFBP-3 and -5 as the predominant IGFBPs. Surprisingly, in contrast to the numerous reports describing the involvement of IGFBP-5 during rodent involution, IGFBP-5 mRNA levels were increased during lactation and decreased upon the onset of involution in the bovine (Plath-Gabler *et al.* 2001). Despite the apparent species differences, few studies have investigated the specific cellular expression and regulation of the IGF axis in the bovine mammary gland.

The MAC-T cell line has been used as a model to study the IGF axis in the bovine mammary gland (Cohick & Turner 1998, Romagnolo *et al.* 1994, Woodward *et al.* 1996). One goal of the present study was to determine whether IGF regulates IGFBP expression in primary BME cells in the same way as it does in this immortalized, non-transformed cell line. The basal and IGF-stimulated secretion patterns of IGFBP were essentially analogous in the primary BME cells compared with MAC-T cells when analyzed by ligand blotting with either 125 I-labeled IGF-I or -II, although a few subtle differences were observed. While media conditioned by either MAC-T cells or BME cells contained primarily IGFBP-2 and -6 under basal, serum-free conditions, IGFBP-3 levels were slightly higher in conditioned media of MAC-T cells when 125 I-labeled IGF-II was used as the ligand. However, a difference was not detected at the mRNA level. A second subtle difference in the IGFBP secretion pattern between primary BME and MAC-T cells was the detection of low levels of IGFBP-5 protein in the MAC-T but not the BME cells.

IGF-I induced a similar stimulation of IGFBP-2, -3, -4, and -6 protein in both MAC-T and BME cells. These results are consistent with the only other study investigating IGF regulation of IGFBP in primary BME cells. In that report, IGFBP-2 and -3, and two unidentified IGFBPs, were secreted under basal conditions and IGF-I was shown to regulate IGFBP-2 and -3 protein secretion (McGrath *et al.* 1991). This study did not examine whether changes occurred at the mRNA or post-transcriptional levels.

IGFBP-2, -3 and -6 were all detectable by Northern blot analysis under basal conditions in BME cells, similar to previous reports in MAC-T cells. However, IGFBP-4 and -5 mRNA levels were not previously detected by Northern blot analysis in MAC-T cells due to their

Figure 7 Temporal induction of IGFBP mRNA levels by IGF-I in primary BMFs. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then exposed to serum-free media $\pm$ IGF-I (100 ng/ml) for indicated times. Total RNA was isolated and analyzed by Northern blotting via hybridization with 32 P-labeled bovine IGFBP-2, -3, -6 or human IGFBP-4 and -5 cDNA. (A) Representative Northern blot. (B, C and D) Relative intensity by PhosphorImager analysis of IGFBP-5, -4 and -3 respectively: data represent means $\pm$ S.E.M. of at least three separate experiments. *Significance of $P < 0.05$ for overall protection (ANOVA); Dunn-Sidak correction for individual test, $P < 0.01$.

Figure 8 IGF-I differentially regulates IGFBP mRNA levels in primary BME cells compared with BMFs. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation, then exposed to serum-free media ± IGF-I (100 ng/ml) for indicated times. Total RNA was isolated, DNase treated and analyzed by qRT-PCR as described in the Materials and Methods. The graph represents fold increase over serum-free controls (shown in parentheses), data represent means ± S.E.M. of at least two separate experiments.

relatively low abundance. Allar and Wood (2004) demonstrated in the murine mammary gland that IGFBP-5 mRNA levels were lowest during lactation and that IGFBP-4 mRNA was primarily restricted to stroma. This may account for the need of a more sensitive detection assay to detect mRNA levels of these IGFBPs in cells isolated during lactation. Similar to the murine data, IGFBP-4 mRNA was barely detectable during lactation in bovine mammary tissue extracts (Plath-Gabler *et al.* 2001).

In the present study, IGFBP-3 and -4 mRNA levels were both increased by IGF-I treatment. In contrast, IGFBP-2, -5 and -6 levels were not affected by IGF-I even though IGF-I increased protein levels of IGFBP-2 and -6. The discrepancies in the changes in IGFBP-2 and -6 mRNA and protein levels agrees with results in MAC-T cells (Cohick & Turner 1998). Interestingly, in the murine MEC line HC11, IGFBP-4 and -6 mRNA levels were undetectable even by qRT-PCR (Boutinaud *et al.* 2004), yet in the bovine these IGFBP are detectable. Additionally, IGFBP-3 and -5 mRNA levels were the most highly expressed IGFBP in HC11 cells (Boutinaud *et al.* 2004), whereas in BME cells, IGFBP-5 expression was much lower compared with IGFBP-3. This differential expression may be due to species variation, or the stage of differentiation during cell isolation: HC-11 cells were derived from a mid-pregnant mouse mammary gland while the BME cells were isolated from a lactating cow. The regulation of IGFBP by IGF-I has not been previously investigated in mammary fibroblasts. Interestingly, there appears to be a reciprocal relationship between the mRNA levels of IGFBP-5 and -3 and their regulation by IGF-I in BMFs compared with BME cells. While IGFBP-3 protein was appreciably increased by IGF treatment in BME cells, it was difficult to detect in BMFs and the response was variable. This contrasts with studies in

Figure 9 Primary BMFs secrete PAPP-A, an IGF-dependent protease of IGFBP-4. Confluent cells were rinsed, serum-starved for 20 h, then exposed to serum-free media for 48 h. (A) Lane 1, media (15 ml) were concentrated, separated by SDS-PAGE and immunoblotted with an antibody to the PAPP-A/proMBP complex; lane 2, 100 ng of purified human PAPP-A/proMBP protein positive control. (B and C) Representative autoradiogram of ligand blots from an immuno-neutralization study of IGFBP-4 protease activity: 50 µl of media were incubated ± IGF-I (100 ng/ml) and ± an antibody to PAPP-A/proMBP complex at 37 °C for 20 h, then separated by SDS-PAGE. After transfer, the membrane was ligand blotted with ¹²⁵I-labeled IGF-II (B) and ¹²⁵I-labeled IGF-I (C). Results are representative of a minimum of three experiments.

Figure 10 IGF-I and -II mRNA is present in primary BMFs but not BME cells. Confluent cells were rinsed twice in serum-free DMEM followed by 20 h serum starvation and exposed to serum-free media for 8 h. Total RNA was isolated, DNase treated then analyzed by RT-PCR with primers designed against bovine IGF-I, -II and the IGF-IR. Figure shows representative ethidium bromide stain of PCR products separated by agarose gel electrophoresis. Results are representative of three experiments.

bovine and human skin fibroblasts where IGF-I increased levels of IGFBP-3 in conditioned media (Conover *et al.* 1995a). In contrast to IGFBP-3, the increase in IGFBP-5 mRNA and protein levels observed in the present study has also been reported in human skin fibroblasts (Camacho-Hubner *et al.* 1992). It is noteworthy that both IGFBP-3 and -5 have been reported to enhance as well as inhibit IGF action on mammary cell growth, and both IGFBP have been associated with breast cancer (Schedlich & Graham 2002, Butt *et al.* 2003, Hao *et al.* 2004). For example, equimolar concentrations of IGFBP-3 inhibited IGF-stimulated DNA synthesis in bovine mammary extracts cultured in three-dimensional collagen gels (Weber *et al.* 1999). In contrast, IGFBP-3 was shown to potentiate IGF-I action via a mechanism that was independent of IGF binding when over-expressed in MAC-T cells (Grill & Cohick 2000). Interestingly, addition of exogenous IGFBP-3 could not mimic the effects of endogenous IGFBP-3 and actually inhibited IGF-I action in this study. Other studies have found that IGFBP-3 can affect cell behavior, independent of IGF-I, depending on environmental conditions. For example, IGFBP-3 has been shown to differentially affect cell attachment depending on the extracellular matrix composition (McCaig *et al.* 2002). Neuenschwander *et al.* (1996) investigated the role of IGFBP-3 in the mammary gland *in vivo* using a transgenic mouse model in which the whey acidic protein promoter directed expression of IGFBP-3 to mammary tissue during late pregnancy and throughout lactation. This study illustrated that mammary-specific up-regulation of IGFBP-3 decreased the amount of apoptotic cells and delayed involution, suggesting that IGFBP-3 enhanced cell survival.

There are conflicting reports on the physiological role of IGFBP-3 in fibroblasts. Conover *et al.* (2000) demonstrated that the addition of exogenous IGFBP-3 increased

cell sensitivity to IGF stimulation in bovine skin fibroblasts; however, it was recently reported that in primary human mammary fibroblasts exogenous IGFBP-3 had no effect on basal and IGF-stimulated cell growth (Strange *et al.* 2004).

IGFBP-5 protein, while difficult to detect in media conditioned by BME cells, was comparatively the most regulated IGFBP by IGF-I stimulation in BMFs. IGFBP-5 has typically been associated with involution of the mammary gland. IGFBP-5 production was shown to increase during rat and murine involution (Tonner *et al.* 1997, Plath-Gabler *et al.* 2001, Boutinaud *et al.* 2004). A delay in involution that corresponded with reduced IGFBP-5 expression was observed in STAT3 knockout mice (Chapman *et al.* 2000b). Moreover, both IGFBP-5 expression and involution were accelerated in interferon regulatory factor-1 knockout mice (Chapman *et al.* 2000a,b). However, in contrast to these data, there are numerous reports supporting other roles for IGFBP-5 that include enhancing IGF action and cellular differentiation in the mammary gland and other tissues (Jones *et al.* 1993, Phillips *et al.* 2003, Allar & Wood 2004). It has been hypothesized that the ratio of IGF:IGFBP-5 may determine the overall effect of IGF-I on cell fate (Allan *et al.* 2004). In the present study, IGFBP-5 was up-regulated by IGF, a survival factor, suggesting that IGFBP-5 secreted by BMFs is involved in growth-promoting effects of IGF. However IGF-independent roles for IGFBP-5 cannot be ruled out. Further studies will be undertaken to determine the role of stromal-derived IGFBP-5 in the mammary gland.

While IGFBP-6 protein was undetectable in BMFs, it was readily detectable and regulated by IGF-I in BME cells. This suggests that mammary cells require a localized mechanism to regulate the effects of IGF-II, for IGFBP-6 has a 20- to 100-fold higher binding affinity for IGF-II over IGF-I (Bach 1999). Interestingly, IGFBP-6 was shown to inhibit the proteolysis of IGFBP-4 when incubated in media conditioned by murine osteoblasts (Fowlkes *et al.* 1997). This inhibition was reversed by co-incubation with a peptide based on the heparin-binding domain of IGFBP-6. In the present study, IGFBP-6 and intact IGFBP-4 are present in media conditioned by IGF-stimulated BME cells, while IGFBP-6 is not detectable and IGFBP-4 is proteolyzed in media conditioned by IGF-stimulated BMFs. The biological action of IGFBP-6 has not been extensively explored in the mammary gland, however, an *in vivo* study using the *N*-nitrosomethylurea-induced rat mammary tumor model demonstrated an increase in IGFBP-6 mRNA in the stromal compartment of the gland during tumor regression (Manni *et al.* 1994). These data, along with several reports demonstrating an association with IGFBP-6 in quiescent or non-proliferating cells, indicate a possible role as an autocrine/paracrine inhibitor of cell proliferation (Bach 1999). Further studies will explore the role of IGFBP-6 in regulating the IGF axis in primary mammary cells.

In the present study, IGF-I stimulated both an increase in IGFBP-4 mRNA levels, as well as proteolysis of IGFBP-4 protein through the protease, PAPP-A. IGF-dependent proteolysis of IGFBP-4 by PAPP-A has been demonstrated in several bovine tissues and species; however, it has not been previously reported in the mammary gland (Lawrence *et al.* 1999, Mazerbourg *et al.* 2001). PAPP-A has also been reported to proteolyse IGFBP-2 and -5 (Byun *et al.* 2001, Monget *et al.* 2003), although the present data suggest that PAPP-A proteolysis is specific to IGFBP-4 in the mammary gland. *In situ* hybridization analysis in the murine mammary gland has found IGFBP-4 to be specifically localized to stromal cells surrounding the epithelial structures (Allar & Wood 2004). Given the distinct location of IGFBP-4 expression, it has been proposed that IGFBP-4 is necessary to maintain a boundary between stromal and epithelial IGF action (Zhou *et al.* 2003, Allar & Wood 2004). The present study supports this hypothesis.

Among the IGFBPs, IGFBP-4 is unique in that in every *in vitro* study thus far, encompassing multiple cell types, IGFBP-4 inhibited IGF-stimulated proliferation (Zhou *et al.* 2003). *In vivo* studies have also demonstrated that IGFBP-4 is a functional antagonist of IGF action. For example, local administration of IGFBP-4 inhibited IGF-stimulated increases in bone formation in mice (Miyakoshi *et al.* 1999). Additionally, transgenic mice overexpressing IGF-I in smooth muscle cells exhibited smooth muscle hypertrophy (Wang *et al.* 1997), while smooth muscle hypoplasia was observed in transgenic mice overexpressing a protease-resistant IGFBP-4 mutant in smooth muscle cells (Wang *et al.* 1998, Zhang *et al.* 2002).

In contrast to these inhibitory actions of IGFBP-4, several studies have suggested that IGFBP-4 enhances the bioavailability of IGF *in vivo*. The majority of IGF-I in circulation is bound in a 150 kDa complex with IGFBP-3 and the acid-labile subunit. This complex is unable to cross vascular/endothelial barriers; however, the IGFBP/IGF 50 kDa binary complex has been shown to freely cross vascular endothelium and deliver IGF to cells (Adams *et al.* 1995, Rajaram *et al.* 1997). Miyakoshi *et al.* (2001) reported that in mice, systemic administration of native IGFBP-4, but not a protease-resistant IGFBP-4, significantly increased free IGF serum levels and subsequently bone formation. They therefore concluded that IGFBP-4 increased IGF bioavailability to cells by decreasing the amount of circulation-restricted IGF in the 150 kDa complex. Once out of circulation, an IGFBP-4 protease-dependent mechanism released IGF-I, allowing it to stimulate the cells (Miyakoshi *et al.* 2001). Additionally, preincubation of human skin fibroblasts with IGF-II was shown to enhance IGF-I-stimulated DNA synthesis. Analysis with protease-resistant IGFBP-4 demonstrated that the IGF-II-stimulated proteolysis of IGFBP-4 significantly contributed to the enhancement of IGF action (Conover *et al.* 1994, 1995b). Interestingly, in the present

study all the components of this regulatory mechanism are produced in the BMFs and were virtually undetectable in the BME cells. However, since proteolysed IGFBP-4 fragments retain some ability to bind IGF, further studies are needed to determine the role of IGFBP-4 proteolysis in mediating IGF action in the mammary gland.

In the present study, IGF-I and -II mRNA levels were readily detectable in BMFs, and undetectable in BME cells by RT-PCR. This supports the hypothesis that IGF is locally produced in the stroma of the mammary gland (Marshman & Streuli 2002). While we were unable to detect IGF-I or -II in BME cells, previous studies using qRT-PCR analysis detected both IGF-I and -II mRNA in the murine MEC line, HC11 (Boutinaud *et al.* 2004). Furthermore, *in situ* analysis of murine mammary glands demonstrated that IGF-I and -II were differentially expressed throughout the various stages of mammary development (Allar & Wood 2004). Whether IGF-I and/or IGF-II is produced in bovine MECs remains controversial; however, our data support the hypothesis that in addition to systemic IGF, local production of IGF is also important to mammary gland function.

The results of our studies clearly indicate substantial differences between epithelial and fibroblastic production of IGFBPs under basal conditions and in response to IGF-I stimulation. This unique, reciprocal relationship of IGFBP regulation supports the hypothesis that IGFBPs perform distinct roles within the mammary gland in a cell-type-specific manner. These responses are probably involved in the coordinated growth of stroma and epithelial tissues.

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