Increased milk protein synthesis in response to exogenous growth hormone is associated with changes in mechanistic (mammalian) target of rapamycin (mTOR)C1-dependent and independent cell signaling

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ABSTRACT

The objective of this study was to determine if increased milk protein synthesis observed in lactating dairy cows treated with growth hormone (GH) was associated with mechanistic (or mammalian) target of rapamycin complex 1 (mTORC1) regulation of downstream factors controlling nucleocytoplasmic export and translation of mRNA. To address this objective, biochemical indices of mammary growth and secretory activity and the abundance and phosphorylation status of mTORC1 pathway factors were measured in mammary tissues harvested from nonpregnant lactating dairy cows 6 d after treatment with a slow-release formulation of GH or saline ($n = 4/\text{group}$). Treatment with GH increased mammary parenchymal weight and total protein content and tended to increase ribosome number and cell size, whereas protein synthetic efficiency, capacity, and cell number were unchanged. Cellular abundance of the mTORC1 components mTOR and (phosphorylated) mTOR^{Ser2448} increased, as did complex eukaryotic initiation factor 4E:eukaryotic initiation factor 4E binding protein 1 (eIF4E:4EBP1), whereas no change was observed for mTORC1-downstream targets 4EBP1, 4EBP1^{Ser65}, p70/p85^{S6K} and p70^{S6K}/p85^{S6K} Thre389/p85^{S6K} Thre412. Changes in activation were not observed for any of the targets measured. These results indicate that GH treatment influences signaling to mTORC1 but not downstream targets involved in the nucleocytoplasmic export and translation of mRNA. Increased eIF4E:4EBP1 complex formation indicates involvement of the mitogen-activated protein kinase (MAPK) pathway. Abundance of MAPK pathway components eIF4E, eIF4E^{Ser209}, eIF4E:eIF4G complex, MAP kinase-interacting serine/threonine-protein kinase 1 (MKNK1), MKNK1^{Thr197/202}, and ribosomal protein S6 kinase, 90kDa, polypeptide 1 (RPS6KA1) increased significantly in response to GH, whereas relative activation of the proteins was unchanged. Expres-

sion of IGFBP3 and IGFBP5 increased, that of IGF1R decreased, and that of IGF1 remained unchanged in response to GH. PatSearch analysis of the milk caseins α_{S1} -casein, α_{S2} -casein, and β -casein, MAPK signaling target RPS6KA1, and proliferation gene IGFBP3 mRNA indicated that all contained putative eIF4E-sensitivity elements. In response to GH, these genes were all upregulated, suggesting that increased abundance of eIF4E and eIF4E^{Ser209} plays a role in mediating their nucleocytoplasmic export. We propose that, in response to GH, the IGF1-IGF1R-MAPK signaling cascade regulates eIF4E-mediated nucleocytoplasmic export and translation of mRNA, whereas mTOR controls cell renewal, cell turnover, and rRNA transcription through an alternative signaling cascade.

Key words: lactation, growth hormone, mechanistic (or mammalian) target of rapamycin (mTOR)

INTRODUCTION

Cellular regulation of protein synthesis is a tightly coordinated system that utilizes common factors located in the cytoplasm and nucleus (Culjkovic et al., 2008). Cytoplasmic factors are widely recognized as regulators of mRNA translation, whereas their nuclear counterparts control the export of specific mRNAs linked to cell survival, proliferation, and growth (Culjkovic et al., 2005). In the ruminant mammary gland, coordination of these factors relies upon a complex endocrine, mitogen, and nutritional signaling cascade that has yet to be fully elucidated (Briskin and O'Malley, 2010). Identifying and understanding the molecular mechanisms that underpin this coordination may provide fundamental knowledge that leads to the development of novel technologies to increase dairy cow lactation performance. A well-established treatment model used to study increased milk protein synthesis by the ruminant mammary gland is growth hormone (GH). The galactopoietic effect of GH has been well investigated; however, cell signaling mechanisms that mediate its action on the mammary gland are not fully understood (McDowell et al., 1987; Capuco et al., 2001).

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Studies with in vitro models suggest that GH may signal directly to mammary epithelial cells via the growth hormone receptor, through pathways linked to protein synthesis and cellular proliferation (Johnson et al., 2010). Mammary explant studies show that GH alone increases cell survival by suppressing expression of the pro-apoptosis gene insulin-like growth factor-binding protein (**IGFBP**) 5 (Sakamoto et al., 2007). Evidence from in vivo studies postulates that GH can also act indirectly on the mammary gland via stimulation of liver IGF1 secretion or by increasing blood flow and nutrient availability to the gland (Mephram et al., 1984; Davis et al., 1988; Capuco et al., 2001). These physiological stimuli increase epithelial cell proliferation or secretory activity in the lactating mammary gland (Capuco et al., 2001), indicating the involvement of signaling pathways regulating cell turnover and renewal, RNA transcription, and protein synthesis in mediating the effects of GH on milk production. We have previously reported that treatment with GH increases plasma IGF1 concentrations, increases ribosomal protein S6 (**RPS6**) abundance and phosphorylation, and increases eukaryotic initiation factor 4E (**eIF4E**) abundance in the mammary gland of lactating dairy cows (Hayashi et al., 2009). Furthermore, Burgos and Cant (2010), using the bovine mammary epithelial cell line MAC-T, showed that IGF1 increases formation of the eIF4E:eukaryotic initiation factor 4G (**eIF4G**) complex and p70^{S6K} phosphorylation, and decreases formation of the eIF4E:eukaryotic initiation factor 4E binding protein 1 (**4EBP1**) complex. Interestingly, p70^{S6K} phosphorylation and eIF4E:4EBP1 complex formation can be influenced by the mechanistic (or mammalian) target of rapamycin (**mTOR**) pathway (Gingras et al., 2001; Ruvinsky and Meyuhas, 2006).

The mTOR protein is an evolutionarily conserved serine/threonine kinase that exists in 2 known complexes, mTORC1 and mTORC2. The mTORC1 complex responds to 4 major signals: nutrients, growth factors, energy, and stress to control protein synthesis, cell size, cellular proliferation, and gene expression, whereas mTORC2 responds to nutrients and growth factors to regulate cytoskeleton formation (Jacinto et al., 2004). Activation of mTORC1 signaling elevates protein synthesis by 2 distinct mechanisms that are cytoplasmic and nuclear in origin. Cytoplasmic mTORC1 phosphorylation of 4EBP1^{Ser65} uncouples it from eIF4E, allowing the formation of the 5'-m7G cap-dependent eIF4E:eIF4G protein synthesis initiation complex (Gingras et al., 2001). Once protein synthesis has begun, mTORC1 regulates the speed of translation by phosphorylating p70^{S6K}, which in turn phosphorylates its downstream target RPS6 (Schmelzle and Hall, 2000). Nuclear signaling by mTORC1 involves the phosphory-

lation of MAF1 to increase rRNA transcription and p85^{S6K} to increase mRNA transport (Kantidakis et al., 2010).

The aim of this study was to determine if the increased milk protein synthesis observed in dairy cows treated with GH is associated with changes in mTORC1 signaling and downstream factors regulating nucleocytoplasmic export and translation of mRNA.

MATERIALS AND METHODS

Animals and Treatments

All procedures involving animals were carried out in compliance with the guidelines of the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand), in accordance with the 1999 Animal Welfare Act of New Zealand. The trial design and sample collection methodology have been described in detail elsewhere (Hayashi et al., 2009). Briefly, 8 non-pregnant, lactating second-parity Jersey cows (178–200 d postpartum) were housed in separate indoor stalls and fed a diet formulated to exceed requirements for ME, protein, and essential amino acids. Cows were fed and milked twice daily. A single subcutaneous injection of either a slow-release formulation of commercially available GH (500 mg; Lactatropin, Elanco Animal Health, Bryanston, South Africa) or saline ($n = 4/\text{group}$) was administered. Six days after injection, cows were milked and then euthanized, mammary glands were trimmed (skin, lymph nodes, fat, and teats removed), and parenchymal tissue (alveolar tissue free of large ducts and blood vessels) was collected and snap frozen in liquid nitrogen (-80°C) within 5 min for subsequent analyses.

Biochemical Indices

Mammary tissue from each cow was homogenized in TRIzol reagent (Invitrogen, Auckland, New Zealand), and total RNA, DNA, and protein were extracted according to the manufacturer's protocol. Nucleic acids were quantified using a NanoDrop spectrophotometer (ND-1000; Nanodrop Technologies, Wilmington, DE) and protein was quantified using the Bradford assay (Bradford, 1976). Then, DNA, RNA, and protein were used to determine biochemical indices of cell size (protein:DNA ratio), protein synthetic efficiency (protein:RNA ratio), protein synthetic capacity (RNA:DNA ratio), DNA, RNA, protein concentration, and total parenchymal DNA, RNA, and protein contents. Differences between treatment groups were determined using the T-TEST procedure (SAS Inst. Inc., Cary, NC). Differences between means were considered significant at $P \leq 0.05$.

Quantitative Real-Time PCR

RNA Extraction and cDNA Synthesis. Total RNA extracted for biochemical indices was purified by use of RNeasy minikits, with on-column DNase I treatment (Qiagen, San Diego, CA) to remove any residual DNA contamination. Purified RNA was quantified using a NanoDrop spectrophotometer (ND-1000; Nanodrop Technologies), and RNA quality assessed by running 1 µg on a 1% nondenaturing agarose gel, stained with SYBR Safe (Invitrogen). Total RNA (500 ng) was reverse transcribed to make cDNA using the SuperScript VILO cDNA Synthesis Kit and the manufacturer's modified protocol of 120 min at 42°C (Invitrogen).

Primer Design. Primers were designed against mRNA sequences from both *Bos taurus* and *Ovis aries* using publicly available data at National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and the Commonwealth Scientific and Industrial Research Organisation (CSIRO; www.livestockgenomics.csiro.au/sheep/), using the Roche Universal ProbeLibrary assay design center (www.roche-applied-science.com/sis/rtpcr). Primers for IGF1 had been published previously (Pfaffl et al., 2002a). Primers were made by Integrated DNA Technologies (Antwerp, Belgium) and purified using desalting to remove short truncated products and small organic contaminants. Primer sequences are presented in Table 1.

Quantitative PCR Assay. Quantitative real-time PCR (qPCR) was performed using the SensiFAST SYBR No-ROX Kit (Bioline, London, UK) in a Rotor-Gene 6000 (Qiagen), with the template and all reagents used at half of the manufacturer's recommended volume. The PCR efficiency and quantification cycle values were obtained for each sample using LinRegPCR (Ramakers et al., 2003). Two reference genes, *MAPK1* and *SHC1*, were identified using NormFinder (<http://www.mdl.dk/publicationsnormfinder.htm>; Andersen et al., 2004). Reactions were performed in triplicate with all data entered into REST 2009 (<http://www.gene-quantification.de/rest-2009.html>; Pfaffl et al., 2002b) and the fold change in expression ratios between the means of 2 treatment groups determined. All qPCR data were reported according to published guidelines (Bustin et al., 2009).

Protein Precipitation and Immunoblotting

Protein Extraction. Tissue was homogenized in cold protein extraction buffer (20 mM Tris-HCl, pH 7.4, 1 mM EDTA, pH 8, 1 mM ethylene glycol tetraacetic acid (EGTA), pH 8, 1% Triton X-100, 0.5 mM sodium vanadate, 1 mM benzamidine hydrochloride,

Table 1. Name, symbol, gi number (<http://www.ncbi.nlm.nih.gov/Sitemap/sequenceIDs.html>), primer sequence, and amplicon size of genes analyzed by quantitative PCR

Gene name	Symbol	gi number	Hybridization	Primer (5' to 3')	Amplicon size (bp)
Reference gene					
Mitogen-activated protein kinase 1	<i>MAPK1</i>	31343545	Forward	TGCGAGGAAGACCTGAATTG	165
			Reverse	TCCTCTTGTGAGGGTTGAACG	
SHC – both variants (Src homology 2 domain containing transforming protein 1	<i>SHC1</i>	255759964	Forward	CAGTCCATCTCGTTTGCATC	260
Target gene					
Insulin-like growth factor 1	<i>IGF1</i>	255759963	Reverse	GGCTCTTCTCCTCCTCATC	
Insulin-like growth factor 1 receptor	<i>IGF1R</i>	148747337	Forward	TGCGATCTCTTCTATCTGGCCCTGT	240
			Reverse	GCAGTACATCTCCAGCCTCTCAGA	
Insulin-like growth factor binding protein 3	<i>IGFBP3</i>	347446670	Forward	CTGTATGCCTCTGTGAACCC	197
			Reverse	TCGTTACCCGCTTAAATGGC	
Insulin-like growth factor binding protein 5	<i>IGFBP5</i>	27807006	Forward	AGGAATGGCAGTGAGTCG	172
			Reverse	CTCGTAGTCAACCTTGTAGCG	
Ribosomal protein S6 kinase, 90 kDa, polypeptide 1	<i>RPS6K1</i>	157427781	Forward	GACCCAGTCCAAGTTGCTG	200
			Reverse	TCTGTAGAACCCTTTGGCGG	
18S Ribosomal RNA	<i>18S</i>	139948214	Forward	CTTCTACTTTTGACACTGAGTTCACG	108
			Reverse	TGGCCACGAACTGAAGC	
		307691242	Forward	AAACGGCTACCACATCCAAAG	250
			Reverse	CGCTCCCAAGATCCAACTAC	

1 mM dithiothreitol, 0.1 mM phenylmethanesulfonyl fluoride, 1 µg/mL pepstatin, 1 µg/mL antipain, 1 µg/mL leupeptin), centrifuged at $4,820 \times g$ for 10 min at 4°C. The supernatant was collected and protein concentration determined using the Bradford assay (Bradford, 1976).

Protein Precipitation. For analysis of eIF4E complex, protein supernatants were treated with 7-methyl guanosine-5'-triphosphate (GTP) sepharose beads following the manufacturer's protocol (GE Healthcare, Auckland, New Zealand). Immunoprecipitation of total mTOR was performed with the primary antibody (Cat. No. 2672, Cell Signaling Technologies, Boston, MA) using the protein G-Dynabead magnetic separation kit and DynaMag-2 magnetic separator according to the manufacturer's protocol (Invitrogen).

SDS-PAGE and Western Blotting. All protein samples were separated via reducing SDS-PAGE. Protein targets mTOR, mTOR^{Ser2448}, eIF4G, p70^{S6K}/p85^{S6K}, and p70^{S6K} Thre389/p85^{S6K} Thre412 (Cat. Nos. 2672, 2971, 2469, 9202, 9205, respectively; Cell Signaling Technologies) were separated on 3 to 8% Tris-acetate gels (Invitrogen), whereas eIF4E, eIF4E^{Ser209}, 4EBP1, 4EBP1^{Ser65}, RPS6KA1, MKNK1, and MKNK1^{Thr197/202} (Cat. Nos. 9742, 9741, 9452, 9451, 9333, 2195, 2111, respectively; Cell Signaling Technologies) were separated on a 4 to 12% Bis-Tris gradient gel (Invitrogen), according to manufacturer's instructions. Proteins were then transferred using an iBlot Gel Transfer Device to a polyvinylidene difluoride membrane (Invitrogen). The membrane was then incubated with primary antibodies, followed by a horseradish peroxidase-conjugated secondary antibody. Proteins were then visualized using SuperSignal West Pico enhanced chemiluminescence reagents (BioRad, Auckland, New Zealand) and Kodak Biomax XAR film (Rochester, NY). At least 3 repeat films were scanned using an HP Photosmart B110 (Hewlett Packard, Auckland, New Zealand) set at 300 dpi, and the signal intensity of individual bands was determined by densitometric measurement using ImageJ software (National Institutes of Health, Bethesda, MD). Within each film, individual band intensities were calculated per unit of DNA to express relative abundance per cell. The calculated values were then expressed as a ratio of the total signal from all bands on the film to reduce band intensity variation between repeat films. To determine activation, the intensities of phosphorylated signals were normalized to total protein intensities. Differences between treatment groups were analyzed using the T-TEST procedure (SAS Inst. Inc.). Differences between means were considered significant at $P \leq 0.05$.

Analysis of mRNA 3' Untranslated Region for Putative eIF4E Sensitivity Element

The publicly available Ensembl (www.ensembl.org) bovine database was used to obtain the 3' untranslated region (UTR) sequences of α_{S1} -casein (gi: 31341348; <http://www.ncbi.nlm.nih.gov/Sitemap/sequenceIDs.html>), α_{S2} -casein (gi: 31341749), β -casein (gi: 31341343), κ -casein (gi: 27881411), α -lactalbumin (gi: 31342165), β -lactoglobulin (gi: 81294269), IGFBP3 (gi: 27807006), IGFBP5 (gi: 157427781), RPS6KA1 (gi: 139948214), and G1/S-specific cyclin-D1 (gi: 114053226). The PatSearch algorithm (<http://itbtools.ba.itb.cnr.it/pat-search>) was used to identify putative eIF4E-sensitivity elements (4E-SE) corresponding to the conserved sequence element UX2UX2A within the 3' UTR.

RESULTS

Effect of GH Treatment on Mammary Gland Weight and Biochemical Measures

Total parenchymal RNA (33%, $P = 0.10$) and protein (34%, $P = 0.06$) tended to be increased, whereas DNA concentration tended to decrease (20%, $P = 0.09$) in GH-treated compared with control cows (Table 2). Cell size tended to be increased by 30% ($P = 0.14$) in GH-treated cows compared with control cows, whereas total parenchymal DNA content (cell number) did not change with treatment. Mammary parenchymal weight was increased such that GH-treated cows had a 33% ($P = 0.03$) heavier mammary parenchyma compared with control cows. These results indicate greater total ribosome number (protein synthetic capacity), which supports increased mammary parenchymal protein content leading to increased cell size, mammary parenchymal weight, and decreased DNA concentration.

Effect of GH Treatment on the mTORC1 Signaling Pathway

The mTOR pathway has been identified in mammalian cells to regulate ribosomal RNA transcription (Iadevaia et al., 2012) and mRNA nucleocytoplasmic export and translation (Ma et al., 2008). Treatment with GH increased the abundance of total and mTOR^{Ser2448} (Figure 1) and the abundance of complexed 4EBP1:eIF4E compared with control cows (Figure 2). No change in the abundance of total 4EBP1, 4EBP1^{Ser65}, total p70/p85^{S6K}, and p70^{S6K} Thre389/p85^{S6K} Thre412 (Figure 1) was observed between treatment groups. No change in relative activation was observed for any of the mTOR pathway proteins measured between treatment groups.

Table 2. Mammary gland weight, total parenchymal content, and parenchymal ratio in cows treated with growth hormone (GH) or untreated (control)¹

Measurement	GH (n = 4)	Control (n = 4)	SEM	P-value
Parenchymal weight, g	9,336	7,000	472	0.03
Total parenchymal content, mg				
DNA	17.8	16.5	1.9	0.68
RNA	35.3	26.6	2.8	0.10
Protein	806	601	52	0.06
Parenchymal concentration, mg/g				
DNA	1.9	2.4	0.1	0.09
RNA	3.8	3.9	0.4	0.91
Protein	86.8	85.6	6.8	0.91
Parenchymal ratio				
RNA:DNA	2.13	1.63	0.29	0.32
Protein:DNA	47.1	36.1	4.0	0.14
Protein:RNA	24.0	22.8	2.5	0.76

¹Each control and GH value (excluding mammary gland weight) represents the average of 3 independent extractions and their pooled SEM; $P \leq 0.05$ = significant; $P \leq 0.15$ = trend.

Effect of GH Treatment on MAPK Signaling Pathway

The MAPK pathways regulate proliferation and differentiation, in part by controlling the protein translation machinery (Ueda et al., 2004). They are activated by mitogenic stimuli such as growth factors and cytokines, including IGF1. The abundance of MAPK pathway proteins total eIF4E, eIF4E^{Ser209}, total MKNK1, and MKNK1^{Thr197/202}, and total RPS6KA1 (Figure 3) and the formation of the eIF4E:eIF4G complex (Figure 2) were increased in GH-treated compared with control

cows. No change in relative activation was observed for any of the MAPK pathway proteins measured between treatment groups.

Effect of GH Treatment on Mammary Gland Gene Expression

The mRNA abundance of *IGFBP3*, *IGFBP5*, *18S rRNA*, and *RPS6KA1* were increased by 1.4-, 1.6-, 1.8-, and 1.3-fold, respectively, in mammary tissue

Figure 1. Growth hormone (GH) treatment increased the abundance of total and Ser2448 phosphorylated mechanistic target of rapamycin (mTOR) compared with controls. No treatment effect was observed for 4EBP1, 4EBP1^{Ser65}, p70S6K, p70^{S6K}Thre389, p85S6K, or p85^{S6K}Thre412. The figure shows values for each animal measured (•, n = 4/treatment), the mean of those values (bars) ± standard errors (lines); * $P \leq 0.05$, † $P \leq 0.15$.

Figure 2. Growth hormone (GH) treatment increased the association of total 4EBP1 and eIF4G with total eIF4E. The figure shows values for each animal measured (•, $n = 4/\text{treatment}$), the mean of those values (bars) $\pm$ standard errors (lines); $*P \leq 0.05$.

of GH animals compared with controls. The mRNA abundance of *IGF1R* decreased by 1.3-fold and that of *IGF1* remained unchanged in the mammary tissue of cows treated with GH compared with controls (Figure 4).

Effect of GH Treatment on eIF4E-Mediated Nucleocytoplasmic Export of mRNA

The growth-promoting IGFBP3 and RPS6KA1 and the milk proteins α_{S1} -casein, α_{S2} -casein, and β -casein

Figure 3. Growth hormone (GH) treatment increased the abundance of MAPK signaling proteins eukaryotic initiation factor 4E (eIF4E), eIF4E^{Ser209}, MKNK1, MKNK1^{Thre197/202} and RPS6KA1. The figure shows values for each animal measured (•, $n = 4/\text{treatment}$), the mean of those values (bars) $\pm$ standard errors (lines); $*P \leq 0.05$.

Figure 4. Growth hormone (GH) treatment increased the expression of ribosomal RNA gene *18S* and the IGF binding protein (*IGFBP*)*3* and *IGFBP**5* and decreased expression of the IGF1 receptor (*IGF1R*). The mitogen-activated protein kinase (MAPK) signaling gene ribosomal protein S6 kinase, 90 kDa, polypeptide 1 (*RPS6KA1*) was increased, whereas *MAPK* and Src homology 2 domain containing 1 (*SHC1*) had the most stable expression of all the genes analyzed and were used as reference genes. The figure shows the fold change in gene expression in GH animals compared with control animals ($n = 4/\text{treatment}$); $**P \leq 0.01$.

were shown to contain putative 4E-SE, linking their increased expression in mammary glands of GH cows to the increased abundance of eIF4E and eIF4E^{Ser209} (Table 3). Pro-apoptosis marker IGFBP5 and milk protein κ -casein and α -lactalbumin mRNA were elevated in mammary glands of GH compared with control cows, but contained no 4E-SE, indicating that their export is driven by an eIF4E-independent mechanism.

DISCUSSION

This study provides new knowledge of potential molecular mechanisms regulating milk protein synthesis in the mammary gland of lactating dairy cows treated with GH. We postulate that in response to GH, the IGF1-IGF1R-MAPK signaling cascade regulates eIF4E-mediated nucleocytoplasmic export and translation of mRNA, whereas an as yet unidentified signaling cascade mediated by mTOR controls cell renewal, cell turnover, and rRNA transcription.

GH Treatment Increases Mammary Gland Protein Synthetic Capacity

In mammalian cells, the most abundant RNA species is rRNA, accounting for >80% of the total cellular

RNA content (Iadevaia et al., 2012). Three subspecies of RNA exist: 5S, 18S, and 28S, which are used by cells to manufacture ribosomes, the factories used to translate mRNA into protein. In this study, we demonstrated that GH treatment potentially increased the protein synthetic capacity of the lactating mammary gland by increasing the abundance of *18S rRNA* mRNA, total parenchymal RNA, and by association total parenchymal rRNA. We observed no effect on parenchymal cell number, consistent with previous cow and goat lactation studies, which showed that GH does not exert its effect on the mammary gland by increasing secretory cell number, but rather by increasing cellular hypertrophy (Capuco et al., 2001; Boutinaud et al., 2003).

Elevated Total and Activated mTOR Abundance Is Associated with Increased Mammary Gland Protein Synthetic Capacity

The observed increase in total parenchymal RNA was associated with an increase in total parenchymal protein, indicating that increased protein synthesis occurs in response to GH treatment. The regulation of protein synthesis is a highly coordinated and intricately linked 3-step process involving the expression and nucleocytoplasmic export of specific mRNA and their

Table 3. Increased expression of growth-promoting insulin-like growth factor binding protein (IGFBP) 3, ribosomal protein S6 kinase, 90 kDa, polypeptide 1 (RPS6KA1), and milk proteins α _{SI}-casein, α _{S2}-casein, and β -casein mRNA was associated with presence of putative eukaryotic initiation factor 4E (eIF4E)-sensitivity element (4E-SE)

Target RNA (gi number)	Function/growth-promoting property	4E-SE Sequence ¹
IGFBP3 (27807006)	Cellular growth control and proliferation	ACT TA ATGT GGAGCTCAAATA T GCCT TATT TTGCAC AAAA GAC TGC
IGFBP5 (157427781)	Apoptosis	None identified
RPS6KA1 (139948214)	Cellular growth control and proliferation	ACT GT ACAA ACTCTTTATCTA T TTTT AAGG ACAAA ATGG CA TCA
α _{SI} -Casein (31341348)	Micelle formation in milk	ACU CC ACAG UUAUGG U CUUU GAUG GUUCUG AAAA U UCC
α _{S2} -Casein (1341749)	Micelle formation in milk	ACU U AGCU GAUGAUUA U UUAU AAUC CUUCCCUAC UCUU UC UGA
β -Casein (31341343)	Micelle formation in milk	ACU A AGAU AAUUUU U AAU GAGU AUACAUGGAA CAAA AAA UGA
κ -Casein (27881411)	Prevents precipitation of other caseins in milk	None identified
α -Lactalbumin (31342165)	Lactose production	None identified

¹The pro-apoptosis marker IGFBP5 and milk proteins κ -casein and α -LA mRNA were elevated but contained no 4E-SE

translation into protein by ribosomes. Ribosomal RNA transcription (Iadevaia et al., 2012) and mRNA nucleocytoplasmic export and translation (Ma et al., 2008) in mammalian cells are regulated by mTOR signaling. In this study, the abundance of total and mTOR^{Ser2448} was increased in response to GH compared with controls, indicating that the mTOR pathway plays a potential role in mediating the effect of GH in the lactating mammary gland.

The nucleocytoplasmic export of specific mRNA and their translation into protein is regulated by the mTOR pathway and mediated by several downstream factors common to each process: 4EBP1, p70^{S6K} and p85^{S6K} (Ma et al., 2008; Rong et al., 2008). The 4EBP1 protein has an estimated nuclear to cytoplasmic ratio of 30:70, where its principal role in both sub-cellular compartments is to bind eIF4E (Rong et al., 2008). Phosphorylation of 4EBP1 by mTOR at Ser65 releases eIF4E. In the nucleus, this leads to downregulation of eIF4E-mediated mRNA export, whereas in the cytoplasm, it leads to increased initiation of protein synthesis. In response to GH treatment, 4EBP1:eIF4E complex formation was increased, but no change in the abundance of total and activated 4EBP1 was observed. This result agrees with the findings of Toerien and Cant (2007), who reported increased 4EBP1:eIF4E complex in lactating dairy cows compared with nonlactating cows and concluded that this was due to the glands of lactating cows not operating at maximum capacity. Based on observations from the present study, it is more likely that in response to increasing levels of protein synthesis, secretory cells sequester more eIF4E into the nucleus via interaction with 4EBP1 to support elevated nucleocytoplasmic export of specific mRNA.

Regulation of protein synthesis by mTOR signaling also occurs via Thre^{389/412} phosphorylation of p70/p85^{S6K}, enhancing the transcription of specific mRNA, and phosphorylation of RPS6 at Ser235/236 (Wickenden and Watson, 2010). We showed previously that GH treatment increases phosphorylation of RPS6^{Ser235/236} (Hayashi et al., 2009), a well-established change associated with increased cell size (Ruvinsky et al., 2005). In this study, cell size was increased; however, the abundance of total and p70/p85^{S6K} Thre^{389/412} was unaffected. These results indicate that the regulation of RPS6^{Ser235/236} phosphorylation, and potentially cell size, occurred via an mTOR-independent mechanism. The phosphorylation of RPS6^{Ser235/236} can also be regulated by RPS6KA1, a downstream target of the MAPK signaling cascade (Roux et al., 2007). We observed that the abundance of RPS6KA1 increased in response to GH treatment, suggesting that MAPK signaling also controls RPS6^{Ser235/236} phosphorylation.

The relative abundance of phosphorylated mTOR^{Ser2448}, 4EBP1^{Ser65}, or p70/p85^{S6K Thre389/412} to total abundance (activation) did not change in GH compared with control cows, in contrast to other ruminant lactation studies in which elevated protein synthesis was associated with increased activation of signaling components upstream and downstream of the mTOR pathway (Toerien and Cant, 2007; Hayashi et al., 2009; Burgos et al., 2010). We postulate, based on the results from this study, that in response to GH treatment, the protein synthetic capacity of the mammary gland is increased via mTOR regulation of factors that control rRNA transcription, consistent with the model of chronic increased protein synthesis proposed by Wang and Proud (2006).

Changes in MAPK Signaling Are Associated with Increased Nucleocytoplasmic mRNA Export and Translation

Located at the 5'-end of an mRNA molecule is the m7G cap, a structure required for the mRNA's cytoplasmic translation into protein (Culjkovic et al., 2006). In the nucleus, a subset of mRNA molecules use the 5'-m7G cap and an extra stem-loop pair in the 3' UTR known as the 4E-SE to mediate their export to the cytoplasm (Culjkovic et al., 2006). Each process is mediated by the protein eIF4E, which is bound to the 5'-m7G cap. Cytoplasmic eIF4E initiates mRNA translation by binding eIF4G and recruiting the multiprotein eIF4F complex to the 5'-m7G cap (Avdulov et al., 2004). We observed that formation of the eIF4E:eIF4G complex was increased in response to GH, suggesting that the observed increase in total parenchymal protein could be a function of elevated mRNA translation. Nuclear eIF4E binds both the 5'-m7G cap and 4E-SE to mediate the export specific mRNA molecules involved in cellular growth and proliferation (Culjkovic et al., 2005). Results from this study using a PatSearch analysis of genes known to be upregulated in response to GH showed growth-promoting IGFBP3 and RPS6KA1 and the milk proteins α_{S1} -casein, α_{S2} -casein, and β -casein mRNA, which contained putative 4E-SE. Therefore, nuclear eIF4E may play a role in mediating the export of mRNA molecules that encode proteins used for cell survival and milk protein synthesis in response to GH. This is the first study to show that milk protein mRNA molecules contain 4E-SE and that their presence is associated with increased expression in the lactating mammary gland of the GH-supplemented cow.

The abundance of total and eIF4E^{Ser209} and the eIF4E:eIF4G complex increased in response to GH. These results suggest that rather than utilizing the mTOR signaling pathway to phosphorylate 4EBP1^{Ser65}

and relieve the repression of protein synthesis, secretory cells in the mammary gland increase eIF4E production. This method of elevating protein synthesis has been well characterized in mammalian cells undergoing oncogenic transformation (De Benedetti and Graff, 2004). Evidence also shows that mammalian cells may increase eIF4E production in response to increased expression of mRNA containing 4E-SE to mediate their export from the nucleus (Culjkovic et al., 2005). The role of eIF4E^{Ser209} is still under investigation, but its elevation in response to GH fits with the 2 mechanisms proposed in the literature. First, Zuberek et al. (2003), using intein-mediated protein ligation, generated pure eIF4E selectively phosphorylated at Ser 209. Results from that study showed that eIF4E^{Ser209} has less affinity for 5'-m7G. Those authors proposed that the reduced interaction allowed eIF4E to use a "skipping mechanism." In this model, eIF4E recruits an eIF4F complex to the 5'-m7G. Once translation has begun, eIF4E can dissociate from the complex and skip to the 5'-m7G of another mRNA molecule, recruiting another eIF4F complex, leading to increased protein translation initiation. Second, and in agreement, Topisirovic et al. (2004) reported that nuclear eIF4E^{Ser209} has less affinity for 5'-m7G, and proposed that this feature aids in eIF4E-mediated transport of mRNA from the nucleus of NIH3T3 cells. Interestingly, eIF4E:eIF4G complex formation and elevation of eIF4E^{Ser209} phosphorylation are controlled by MKNK1, a kinase in the MAPK pathway (Ueda et al., 2004). We observed that the abundance of total and MKNK1^{Thre197/202} was increased in response to GH, providing further support to our hypothesis that elevated eIF4E^{Ser209} and eIF4E:eIF4G formation occur via MAPK signaling in response to GH treatment.

Changes in IGF1-IGF1R Signaling Potentially Regulate the MAPK and mTORC1 Pathways

Previously, researchers reported that circulating levels of IGF1 increase in response to GH treatment (Prosser et al., 1989; Hayashi et al., 2009), which suggests that GH may exert its influence on the mammary gland by utilizing the IGF1-IGFR signaling cascade. The IGF1R auto-phosphorylates and activates downstream signaling pathways in response to IGF1 binding (Fleming et al., 2005). The 2 classical signaling pathways activated by IGF1 are the phosphatidylinositol-3 kinase (PI3K) and MAPK pathways. The IGF1-IGFR-MAPK signaling cascade regulates nucleocytoplasmic export and translation initiation of mRNA, whereas the IGF1-IGFR-PI3K-mTOR signaling cascade regulates IGFBP3 and IGFBP5 expression (Fleming et al., 2005). Increased IGFBP3 expression aids cell survival and proliferation, whereas increased IGFBP5 has been

Figure 5. Proposed model of the results presented in this study. Solid arrows represent data with direct links to results generated in this study, whereas dashed arrows represent data with indirect links supported by the literature. mTORC1 = mechanistic (mammalian) target of rapamycin complex 1; RPS6KA1 = ribosomal protein S6 kinase, 90 kDa, polypeptide 1; IGFBP3 and IGFBP5 = IGF binding protein 3 and 5; SHC1 = Src homology 2 domain containing 1; MAPK = mitogen-activated protein kinase; MKNK1 = MAP kinase-interacting serine/threonine-protein kinase 1; eIF4E = eukaryotic initiation factor 4E; eIF4G = eukaryotic initiation factor 4G; 4EBP1 = eukaryotic initiation factor 4E binding protein 1; P = phosphorylated form. Color version available in the online PDF.

linked to apoptosis and remodeling of the mammary gland (Flint et al., 2000). In vitro studies with the bovine mammary epithelial cell line MAC-T show that IGF1 stimulates mTOR pathway activation and protein synthesis (Burgos and Cant, 2010). We observed that expression of both IGFBP3 and IGFBP5 were increased in the mammary gland of GH-treated cows, suggesting that mTOR signaling may play a role in maintaining cell number in the mammary gland through increased cell turnover and renewal (Capuco et al., 2001). This agrees with our biochemical indices results, which showed that cell number was unaffected by GH treatment. Gene expression analysis also showed that IG1R mRNA was decreased, whereas autocrine expression of IGF1 was unchanged. This may indicate that IGF1 signaling did not occur through an autocrine mechanism but rather

through a para- or endocrine mechanism. Expression of IGF1R decreased when the levels of IGF1 increased, an action that stimulated apoptosis and was associated with increased IGFBP3 expression (Chi et al., 2000). It is important to note that previous reports state that activation of the IGF1-IGFR-MAPK signaling cascade reduces the expression of IGFBP3 and IGFBP5 (Fleming et al., 2007). This reduction could indicate a potential uncoupling of mTORC1 and MAPK signaling via regulation from another pathway and warrants further investigation.

The findings from this study indicate that increased milk protein production in response to GH is potentially mediated by mTORC1 and MAPK signaling, stimulated by IGF1-IGF1R activation (Figure 5). Increased abundance of total and mTOR^{Ser2448} is associ-

ated with elevated total parenchymal RNA, an indicator of protein synthetic capacity. Increased abundance of total and mTOR^{Ser2448} is also linked to the maintenance of cell number (indicated by total parenchymal DNA content), potentially operating via regulation of increased expression of IGFBP3 and IGFBP5, which are measures of cell renewal and turnover. Increased abundance of total and phosphorylated components of MAPK signaling pathway are associated with elevated eIF4E-mediated nucleocytoplasmic mRNA export and the initiation of mRNA translation in the cytoplasm. Decreased expression of IGF1R indicates increased levels of circulating IGF1 are directly signaling to the lactating gland through the MAPK and mTORC1 pathways. Although the MAPK pathway principally responds to mitogens and hormones, mTORC1 has a far wider range of signaling inputs, including energy, hypoxia, and amino acids (Wang and Proud, 2006). Further research will be required to determine the potential role that IGF1-IGF1R activation plays in regulating mTORC1 and MAPK signaling and to identify if other signaling pathways stimulate mTORC1 and MAPK.

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