Regulation of protein synthesis in the mammary gland

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This thesis is dedicated to my parents

Mandi Hayashi and Ivaci Hayashi

For your unconditional love

"It is the simple things in life that are the most extraordinary, only wise men are able to understand them."

Paulo Coelho

ABSTRACT

This thesis examines the signaling pathways involved in the regulation of milk protein synthesis in the lactating mammary gland and their control. The protein synthetic machinery can be regulated during the transcription, translation and degradation stages of mRNA processing. Translation control in eukaryotes involves changes in the activity or other functional properties of the translation factors. These include proteins involved in initiation, peptide-chain elongation and termination of mRNA processing. Changes in the nutritional, physiological and hormonal status of the body are sensed by receptors that signal to a central protein, known as mammalian target of rapamycin (mTOR). The mTOR signaling pathway then activates or inhibits the activity of translation factors and kinases involved in the initiation and elongation stage of translation.

A major objective of this thesis was to elucidate which genes and pathways are involved in the regulation of milk protein synthesis in the mammary gland and the mechanism(s) that regulate their action. The results presented here show that changes in milk protein production occurring during lactation in response to external stimuli are potentially regulated at the level of translation or subsequent processing rather than by transcriptional regulation (mRNA abundance).

The results also show that in response to growth hormone (GH) treatment, which increased the yield of milk protein, the phosphorylation status of the ribosomal protein S6 (S6) is increased as well as the protein abundance of eukaryotic elongation factor 2 (eEF2) and eukaryotic initiation factor 4E (eIF4E). These results suggest an important

relationship between milk protein yield and changes in the initiation and elongation stages of translation.

Another major finding was the elucidation that mTOR is involved in the signaling pathways activated by GH and that this effect involves signaling through the PI-3 kinase pathway. In these experiments, increased protein synthesis was potentially achieved with the use of GH. Thus, this study suggests the mTOR signaling pathway is a key mediator of the GH effects in protein synthesis stimulation.

Finally, the requirement for a functional mTOR signaling (TOS) motif in the eukaryotic initiation factor 4E binding protein (4E-BP1) was identified. This finding could help the identification of other proteins that may be controlled by mTOR and consequently are regulators of mRNA translation.

In summary, this thesis unveils key signaling pathways involved in the regulation of milk protein synthesis and provides further insight into the control of the mTOR signaling pathway. These findings open new frontiers for the manipulation of milk composition.

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ABBREVIATIONS

4E-BP1	Eukaryotic initiation factor 4E-binding protein 1
ATP	Adenosine triphosphate
BES	N, N-bis [2-Hydroxyethyl]-2 aminoethanesulfonic acid
BSA	Bovine serum albumin
cDNA	Complementary DNA
Ct	Cycles to threshold
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetraacetic acid
eEF2	Eukaryotic elongation factor 2
eEF2K	Eukaryotic elongation factor 2 kinase
eIF	Eukaryotic initiation factor
eIF4E	Eukaryotic initiation factor 4E
eRF	Eukaryotic release factor
ERK	Extracellular signal-regulated protein kinase
EST	Expressed sequence tag
FCS	Foetal calf serum
FDR	False discovery rate
GDP	Guanosine diphosphate
GH	Growth hormone
GHR	GH receptor
GO	Gene ontology
GTP	Guanosine triphosphate
IGF	Insulin-like growth factor
IGFBP	Insulin-like growth factor binding protein
IPA	Ingenuity pathway analysis
IPTG	Isopropyl-b-thiogalactopyranoside

IRES	Internal ribosomal entry segments
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
m^7GTP	7-methyl-GTP
MAPK	MAP kinase
mRNA	Messenger RNA
mTOR	Mammalian target of rapamycin
NADPH	Nicotamide adonine dinuclootide phosphate
NCBI	National Center for Biotechnology Information
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI 3-kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidynositol 4,5-biphosphate
PKB	Protein kinase B
PTEN	Phosphatase and tensin homologue deleted on chromosome 10
PVDF	Polyvinylidene difluoride
qRT-PCR	Quantitative real time PCR
REST	Relative expression software tool
RNA	Ribonucleic acid
S6	Ribosomal protein S6
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
STAT	Signal transduction and transcriptional activation
Rheb	Ras homolog enriched in brain
dsRNA	Double-stranded RNA
RNAi	RNA interference
$P90^{RSK}$	P90 ribosomal S6 kinase
RNAi	RNA interference
siRNA	Small interfering RNAs
EGF	Epidermal growth factor
CoREs	Composite response elements
C/EBP	CAAT\enhancer binding protein
NF-1	Nuclear factor 1
YY-1	Yin Yang-1

GSK3	Glycogen synthase kinase 3
S6K	Ribosomal S6 kinase
S6	Ribosomal protein S6
P70S6K	P70S6 kinase
TCA	Trichloroacetic acid
GPAM	Mitochondrial glycerol-3-phosphate acyltransferase
MGEA5	Meningioma-expressed antigen 5
KRT15	K15 intermediate filament type I keratin
PKC	Protein kinase C
Raptor	Regulatory associated protein of mTOR
TOS	TOR signaling motif
HPLC	High pressure liquid chromatography
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
Mac-T	Mammary alveolar cells with large-T antigen
H4IIE	Rat hepatoma cell line
HEK293	Human embryonic kidney cell line
TPA	Phorbol-12-myristate-13-acetate
t-RNA	Transfer RNA
t-RNA$_i$	Initiator tRNA
TSC	Tuberous sclerosis complex
UTR	Untranslated region

GENERAL INTRODUCTION

Background

The New Zealand dairy industry is the country's biggest agricultural industry, accounting for 23% of New Zealand's total export earnings (www.investnewzealand.govt.nz/common/files/Dairy_Feb06.pdf). The application of new biotechnologies in the dairy industry is essential for sustaining the competitiveness and profitability of New Zealand in the global market. A number of studies have successfully demonstrated the physiological changes that occur in response to treatments that perturb the milk synthetic machinery. However, the elucidation of the molecular mechanisms, specifically those of the signaling pathways and genes, involved in the regulation of protein synthesis in the mammary gland are still being explored.

Protein synthesis is mainly regulated through the phosphorylation or binding ability of the initiation and elongation components of the mRNA translational machinery. Although there is ample evidence that certain exogenous stimuli change milk protein synthesis in lactating cows and it is clear that mRNA translation is important for the regulation of protein synthesis, there are no studies demonstrating that these stimuli affect the translation initiation and elongation factors and hence protein synthesis in the mammary gland.

During protein synthesis a tightly regulated step occurs during the initiation and elongation of the mRNA translation which involves the mammalian target of rapamycin (mTOR) signaling pathway. The start of initiation is controlled by the formation of the eukaryotic initiation factor (eIF) 4F complex, which requires the eIF4E protein. The

biological activation of the eIF4E protein is regulated by a family of translation repressor proteins, the eIF4E binding proteins (4E-BPs) proteins. mTOR regulates the phosphorylation of 4E-BP1, which occurs in multiple sites. However, the residues involved in the interaction between mTOR and 4E-BP1 and the nature of the interaction are still unclear.

Objectives

The main objectives of this thesis were to identify the pathways and genes involved in the regulation of milk protein synthesis and to understand their roles in the mammary gland during lactation in response to exogenous stimuli like growth hormone and atropine. A second objective was to study the regulation and interaction of 4E-BP1 and mTOR, the main identified regulators of protein synthesis in other tissues. A better understanding of the 4E-BP1-mTOR interaction will help to identify new downstream targets for the mTOR signaling cascade and consequently new proteins involved in the protein synthesis machinery.

It is anticipated that the knowledge gained from this research will open up possibilities to develop new technologies to manipulate milk composition and yield with potential economic benefits to the dairy industry.