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BARD

FINAL REPORT

PROJECT NO. IS-3105-99

**Translational Mechanisms that Govern Milk
Protein Levels and Composition**

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Project Title: Translational Mechanisms That Govern Milk Protein Levels And Composition

Investigators

Institutions

Principal Investigator (PI):

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Collaborating Investigators:

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Mammary gland, protein synthesis, translation control, polyadenylation, amino acids

Abbreviations commonly used in the report, in alphabetical order: AA, amino acid; BLG β -lactoglobulin; eIF eukaryotic initiation factor; Leu Leucine PA polyadenylation; PHAS-I phosphorylated heat and acid-stable substrate of insulin, also known as 4E-BP1; S6K1 p70 S6 kinase; UTR untranslated region;

Budget: IS: \$ 150,000

US: \$ 150,000

Total: \$ 350,000

Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1		1	2
Submitted, in review, in preparation	1			1
Invited review papers				
Book chapters				
Books				
Master theses			1	1
Ph.D. theses				
Abstracts				
Not refereed (proceedings, reports, etc.)	1			

Postdoctoral Training: List the names and social security/identity numbers of all postdocs who received more than 50% of their funding by the grant.

Kyoung Moo Choi . Social security Number: 435-99-6514

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	1	1		2
Longer Visits (Sabbaticals)				

Description of Cooperation:

During the first year of our cooperation, the US team acquired the techniques, protocols and reagents necessary to grow and differentiate mammary cells from the Israeli team. The US team used this system to discover that mRNA specific mRNA cytoplasmic polyadenylation is the mechanism responsible for the synergism between insulin and prolactin in inducing milk protein synthesis. During the second year, the Israeli team learned from the US team the method of polysome preparation and Northern RNA. Both teams exchanged information and advices regularly during the research period.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted				
Issued (allowed)				
Licensed				

ABSTRACT

Original objectives: The long term objective of the project is to achieve higher content of protein in the milk of ruminants by modulating the translational machinery in the mammary gland. The first specific aim of the BARD proposal was to characterize responsiveness of various experimental systems to combination of lactogenic hormones and amino acids with particular emphasis on discrimination between the control of total protein synthesis and milk protein synthesis. Based on the results, we planned to proceed by characterizing the stage of protein synthesis in which the stimulation by lactogenic hormones and amino acid occur and finally we proposed to identify which components of the translation machinery are modified.

Background to the topic: Milk protein is the most valuable component in milk, both for direct human consumption and for manufacturing cheese and other protein-based products. Attempts to augment protein content by the traditional methods of genetic selection and improved nutritional regimes have failed. The proposal was based on recent results suggesting that the limiting factor for augmenting protein synthesis in the bovine mammary gland is the efficiency of converting amino acids to milk proteins.

Major conclusions, solutions, achievements: **Insulin and prolactin synergistically stimulate β -casein mRNA translation by cytoplasmatic polyadenylation.** The interaction between insulin and prolactin was demonstrated two decades ago as crucial for milk-protein synthesis, but the molecular mechanisms involved were not elucidated. We found in differentiated CID 9 mouse mammary epithelial cells line that insulin and prolactin synergistically increases the rate of milk protein mRNA translation. We focused on β -casein, the major milk protein, and found that the increase in β -casein mRNA translation was reflected in a shift to larger polysomes, indicating an effect on translational initiation. Inhibitors of the PI3K, mTOR, and MAPK pathways blocked insulin-stimulated total protein and β -casein synthesis but not the synergistic stimulation. Conversely, cordycepin, a polyadenylation inhibitor, abolished synergistic stimulation of protein synthesis without affecting insulin-stimulated translation. The poly(A) tract of β -casein mRNA progressively increased over 30 min of treatment with insulin plus prolactin. The 3'-untranslated region of β -casein mRNA was found to contain a cytoplasmic polyadenylation element (CPE), and in reporter constructs, this was sufficient for the translational enhancement and mRNA-specific polyadenylation. Furthermore, insulin and prolactin stimulated phosphorylation of cytoplasmic polyadenylation element binding protein (CPEB) but did not increase cytoplasmic poly(A) polymerase activity. This study represents a clear yet unexpected solution for the enigma concerning the synergistic stimulation of milk-protein synthesis by insulin and prolactin. It also documents the first instance of hormone regulated cytoplasmatic polyadenylation in a somatic cell type.

Amino acid regulation of protein synthesis may account for the higher level of milk protein in rodents compared to ruminants. The role of amino acids as signaling molecules for regulation of protein synthesis has been fully recognized only over the past few years. We hypothesized that amino acid regulation may account, at least in part, for the 3-fold higher level of milk protein content in mice compared to ruminants. To provide a faithful cell culture model for the bovine mammary gland that reproduces its function in vitro, we established a bovine cell line (L-1), derived from mammary gland, which is capable of differentiation on Matrigel and milk protein synthesis in response to lactogenic hormones stimulation. We used L-1 cells to show that bovine cells are more sensitive than their murine counterparts (CID 9 cells) to amino acids manipulation, especially leucine (Leu). Furthermore, translational mechanisms (e.g., phosphorylation of PHAS-I) may account for at least some of the difference. To our knowledge, this represents first attempt to examine post translation regulation of milk protein synthesis by amino acids.

Implications: Boosting specific milk protein production may be achieved by manipulating the kinase responsible for phosphorylating CPEB and mediating the polyadenylation process. Identification and manipulation the intracellular sensing system for amino acids (possibly mTOR) may improve amino acid utilization for milk proteins.

ACHIEVEMENTS

Significance of main scientific achievements or innovations.

Milk is often referred as the “nearly perfect food” and protein is the most valuable gross component in milk. This point is graphically underscored by the fact that dairies include protein in the calculation of payment to their producers. The value of milk protein, both nutritionally and economically continue to increase worldwide and cheese production in the US during 2003 exceeded one billion pounds. It has also been suggested that improving the efficiency with which amino acids are converted into milk will result in reduced amount of nitrogen-containing by-products entering the environment. Thus, increasing the content of milk protein from dairy cows is the interest of producers, processors, consumers and environmentalists alike.

Attempts to increase milk protein levels in dairy ruminants have taken three routes: Genetic selection of the herd, improved nutrition, and genetic manipulation of the bovine mammary gland via transgenesis. Progress in genetic selection has been relatively slow. The milk content of the Israeli herd increased from 2.97% to only 3.11% from 1993 to 2002. Since 2002 the trend has even reversed; milk protein content has actually decreased. Nor have altered nutritional regimens resulted in improved protein content. A recent attempt to increased casein concentration in bovine milk by genetic manipulation indicated the feasibility of the transgenic approach in bovine but also suggested quantitative limitations, since transgenic mice with the same genes express the respective protein at much higher levels.

Our proposal was based on the hypothesis that the mammary gland does not function at maximal capacity and that the amounts and activities of components of the protein synthesis machinery are limiting for the rate of milk protein synthesis. We proposed that identifying these limiting factors may lead to a breakthrough in increasing milk protein content.

Insulin and prolactin synergistically stimulate casein mRNA translation by cytoplasmatic polyadenylation. The interaction between these two lactogenic hormones was demonstrated more than two decades ago as crucial for milk protein synthesis, but the molecular mechanisms involved were not elucidated. In a model of differentiated mammary cell line CID-9 we demonstrated that indeed translation regulation is involved in mediating the synergistic effect of insulin and prolactin. The finding that selective polyadenylation of β -casein mRNA is responsible for the lactogenic hormone synergism and account for a 83-fold increase in β -casein synthesis was unique, totally unexpected, and of high importance. It documents the first instance of hormone-regulated cytoplasmatic polyadenylation in somatic cell type. Knowledge of the two

signaling pathway and their point of convergence may suggest strategies for further boosting specific milk protein production.

Bovine mammary epithelial cells are more sensitive to amino acid manipulation compared to their murine counterparts. While anabolic properties of amino acids have long been appreciated, it has only recently become apparent that amino acids act as signaling molecules to regulate the protein synthesis apparatus. Branched chain amino acids, particularly Leu, can reproduce the stimulatory effect of amino acids on protein synthesis by regulating translation initiation. Rodent milk contains a three-fold higher level of milk protein than bovine milk. Our data suggest that translation mechanisms may account for at least some of the difference. To compare post-transcription regulation in murine and bovine cells we established a unique bovine cell-line (L-1) which is capable of milk protein synthesis and responsive to lactogenic hormones. By comparing milk-protein synthesis and regulation in the L-1 bovine cells to the murine CID-9 cells we made the first attempt, to our knowledge, to examine post transcription regulation of milk protein synthesis by amino acids.

Three important observations were made in this study:

- (i) The synthesis of the milk protein BLG is more sensitive to amino acid deprivation than total cellular [protein synthesis.
- (ii) It is possible to induce specific translation of milk protein by Leu in a rapamycin- and Ly294002-dependent manner.
- (iii) The synthesis of the milk protein BLG is more sensitive to amino acid manipulation, particularly that of Leu, in bovine compared to mouse mammary gland, and this is correlated with the phosphorylation status of PHAS-I, a regulatory protein involved in translation initiation.

These findings make the translational machinery, and especially the intracellular sensor of amino acid levels (probably the mTOR complex, upstream of PHAS-I), a unique and feasible target for manipulation in order to increase bovine milk protein level.

DETAILS OF COOPERATION: whether and how project objectives were promoted as a result of cooperation

The proposal was promoted by collaborative efforts of the U.S. and Israeli teams which contributed different expertise to the project. The U.S. laboratory has long studied the mechanism of translation in eukaryotes. It published one of the first articles identifying the initiation factor eIF4E and was the first to clone the cDNA for eIF4E and eIF4G. It discovered that eIF4E was a phosphoprotein and determined the site of modification. It determined the

domain structure of eIF4G and proposed a model for the interaction of mRNA-binding initiation factors. Finally, it established pathways for the regulation of protein synthesis by insulin.

The Israeli team has a long record of accomplishments in the area of protein expression in the mammary gland. It was the first to show hormonal regulation of translation initiation in the mammary gland.

The collaboration was initiated by a visit of the Israeli investigator to the U.S. lab and a critical examination by the U.S. co-PI of data obtained in transgenic mice and in vitro which suggested translation regulation of milk protein syntheses by lactogenic hormones.

BARD funding allowed initiation of the project and a visit of the U.S. co-PI to the Israeli lab. The U.S. team acquired the techniques necessary to cultivate mammary cells in culture from the Israeli team and identified mRNA polyadenylation to be the mechanism responsible for the synergism between insulin and prolactin in inducing milk protein synthesis. The Israeli team chose to focus on amino acid regulation of milk protein synthesis. During the second year of the project the Israeli PI visited the U.S. lab, learned the method of real-time PCR on polysome fractions, and discussed the results from both teams. The coming report will be the first attempt, to our knowledge to examine post-transcription regulation of milk protein by amino acids.

LIST OF PUBLICATIONS

German, T., and Barash I. (2002) Characterization of an epithelial cell line from bovine mammary gland. *In Vitro Cellular and Developmental Biology* **38**:282-292

Choi, K.M., Barash, I., and Rhoads, R.E. (2004) Insulin and prolactin synergistically stimulate β -Casein mRNA translation in mouse mammary epithelial cells by cytoplasmic polyadenylation. *Molecular Endocrinology*. **18**: 1670-1686.