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308 Lasani Town, Sargodha Road, Faisalabad - Pakistan
Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

Isolation, Purification and Characterization of Fatty-Acid-Binding Protein from Milk Fat Globule Membrane: Effect of Bovine Growth Hormone Treatment

Vitaly L. Spitsberg¹ and R. C. Gorewit²,

¹BioVita Technologies, Inc., Ithaca, NY, ²Lactation Physiology Laboratory, Cornell University, Ithaca, NY 14853

Abstract: Fatty-acid-binding protein (FABP) was purified from bovine milk fat globule membrane (MFGM) by ion-exchange chromatography on DEAE-Sepharose and by gel-filtration on Sephadex G-50. Purified FABP was similar to bovine mammary gland heart (H)-type FABP/ mammary derived growth inhibitor (MDGI). It inhibited growth of mammary epithelial cells at nanogram concentrations, had a relative molecular mass of 15 kDa, as determined by SDS-PAGE, had two isoforms with pI around 5.0 and cross-reacted with antibody to mammary gland H-FABP. The content of FABP in MFGM, obtained from growth hormone (GH)-treated cows, was not essentially different from that of MFGM obtained from untreated cows. However, the level of *in vitro* phosphorylated FABP of MFGM, obtained from GH-treated cows, was diminished in comparison to the sample of MFGM, obtained from untreated cows. The role of the insulin receptor in the phosphorylation of FABP in mammary gland secretory epithelial cells is suggested.

Key words: Milk fat, globule membrane, bovine growth, protein

Introduction

The role of fatty-acid-binding protein (FABP) in fatty acid transport and lipid metabolism is well known (Bass, 1988; Spener *et al.*, 1985). However, recently accumulated experimental findings have allowed us to suggest that FABP can also play a role in regulation of cell proliferation and differentiation, at least, in the mammary gland (Grosse and Langen, 1990; Bohmer *et al.*, 1984; Bohmer *et al.*, 1987; Yang *et al.*, 1994).

It has been established that bovine mammary gland contains a 14.5 kDa protein that inhibits the growth of bovine mammary gland cells and some breast cancer cell lines. This protein was purified and named mammary derived growth inhibitor (MDGI) (Bohmer *et al.*, 1985; Bohmer *et al.*, 1987; Grosse and Langen, 1990; Grosse *et al.*, 1991). Direct protein sequence analysis of MDGI revealed 95 % homology to bovine H-FABP (Bohmer *et al.*, 1987). However, the sequence of MDGI, deduced from the presumably cloned bovine MDGI cDNA, was identical to bovine H-FABP (Grosse *et al.*, 1991). Despite this last finding and the fact that recombinant H-FABP had a similar cell growth inhibiting effect as MDGI (Yang *et al.*, 1994), bovine mammary gland FABP, as isolated and purified according to the protocol of Bohmer *et al.* (1985) or to the improved procedure of Grosse *et al.* (1991), was still called MDGI, suggesting the existence of a specific message for MDGI.

Recent studies by Specht *et al.* (1996); Borchers *et al.* (1997) helped resolve the controversy of the primary structure of MDGI. These studies demonstrated that the bovine mammary gland contains at least two types of FABP, namely, H-FABP and adipocyte (A)-type FABP. Comparison of the sequences of H-FABP and A-FABP allowed researchers to conclude that the direct protein sequencing analysis of MDGI, reported previously, had led to the erroneous identification of amino acid residues in, at least, 7 positions. This was a result of the presence of contaminating amounts of the co-purified A-FABP in the analyzed MDGI preparation. However, this research group (Borchers *et al.*, 1997) demonstrated that H-FABP, but not A-FABP, was involved in growth inhibition and differentiation in the mammary cell, and was localized in secretory epithelial cells of mammary gland tissue. A-FABP was found only in myoepithelial cells of the mammary gland. Therefore, based on the study of Grosse's research group (Grosse *et al.*, 1991) and Specht *et al.* (1996) and Borchers *et al.* (1997), we have designated the bovine mammary gland 15 kDa FABP with cell growth inhibitory property as bovine mammary gland H-FABP/MDGI. FABPs related to bovine MDGI were also identified in mammary gland of humans (Shi *et al.*, 1998) and mice (Binas *et al.*, 1992; Bansal and Medina, 1993; Treuner *et al.*, 1994).

The inhibitory action of H-FABP, or MDGI, on cell proliferation can be mimicked by synthetic peptides related to the 11-amino acid C-terminus of this protein (Grosse *et al.*, 1991; Yang *et al.*, 1994). This C-terminus has structural homology to Type I repeat of thrombospondin (TSP) (Spitsberg *et al.*, 1995). It is thought that the Type I repeat domain of TSP is responsible for the physiological effects of TSP through its binding to surface glycoprotein CD36 (Greenwalt *et al.*, 1992). Glycoprotein CD36 is significantly expressed in epithelial cells of the lactating mammary gland (Greenwalt *et al.*, 1992). It is suggested that the cellular action of FABP/MDGI, as a differentiation factor, can be exerted through an apocrine loop mechanism (Brandt *et al.*, 1988), i.e. through the secretion by mammary gland cells of FABP/MDGI into extracellular spaces with subsequent binding of FABP/MDGI to CD36 (Spitsberg *et al.*, 1995). This would then lead to the triggering of specific signal transduction pathway(s) involved in cell differentiation. Recent studies also suggested that FABP/MDGI can function as a tumor suppressor gene product (Huynh *et al.*, 1996).

FABP, similar to mammary gland FABP/MDGI, has been found in bovine milk fat globule membrane (MFGM) (Brandt *et al.*, 1988). However, this membrane-associated FABP was not isolated from MFGM in its intact form, and, therefore, its physicochemical nature and biological activity has not been defined.

In this work we report the purification, characterization and biological activity of FABP from bovine MFGM. Amino acid analysis, SDS/PAGE, isoelectrofocusing, Western blotting, two-dimensional electrophoresis (2D-E) and inhibitory cell growth assay demonstrated the similarity of FABP from MFGM to H-FABP/MDGI of bovine mammary gland tissue. In this work, we also showed that treatment of cows with bovine growth hormone (GH) appears to influence the level of *in vitro* FABP phosphorylation within the MFGM.

Materials and Methods

Milk from non-hormone treated and bovine recombinant growth hormone (rGH, Posilac, Monsanto, St.Louis, MO, USA) treated cows was obtained from the Cornell University Dairy Teaching and Research farm. Milk from hormone-treated cows was collected from those cows which had received injections of rGH (500 mg of Somatitribove zinc) every two weeks over a five month period. MFGM was prepared according to Spitsberg *et al.* (1995). Rabbit polyclonal antibodies to bovine mammary gland-derived FABP/MDGI were obtained according to Spitsberg *et al.* (1995). DEAE-Sepharose CL-6B and Sephadex G-50 (fine) were from Sigma Chemical Co. (St.Louis, MO, USA). Precast polyacrylamide slabs for isoelectrofocusing (Ampholine PAGplate, pH 4.0 - 6.5; A = 5%,

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C=3%) were from Pharmacia (Uppsala, Sweden). Ultrafiltration membranes (pore size, 0.45 μm) were from Bio-Rad Laboratories (Hercules, CA, USA). Gamma- ^{32}P -ATP (3000-6000 Ci/mmol) was purchased from Amersham, Inc. All other reagents were high purity grade and were purchased from various vendors.

Isolation and purification of FABP from bovine MFGM: All purification procedures were carried out at 2-4 °C. MFGM was obtained from composite (bulk tank) milk according to the protocol of Spitsberg *et al.* (1995). The MFGM was suspended in 50 mM imidazole buffer (pH 8.0) at about 10 mg/ml. This suspension was ultrasonicated by four strikes (each of 15 sec duration) at maximum output. After that, β -mercaptoethanol was added to the suspension to a concentration of 15-20 mM. The suspension was mixed for 10-15 min and was subjected to centrifugation at 80,000 \times g for 1 h. The resulting supernatant was applied to a DEAE-Sepharose column (2 \times 15 cm) equilibrated with 50 mM imidazole buffer, pH 8.0 (IB). The FABP fraction was eluted from the column by IB, containing 50 mM NaCl. The FABP fraction was concentrated by ultrafiltration with membrane UM1 (molecular weight cut off < 3 kDa) to about 5 ml, and this protein solution was applied to a Sephadex G-50 column (90 \times 2.5 cm; V_0 = 150 ml), equilibrated with 50 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl (TBS-buffer). The eluted protein fraction/peak with K_d = 1.6 (Fig. 1) was collected and concentrated by ultrafiltration, and stored at -70 °C. The isolation and purification of FABP from MFGM was repeated three times using the protocol described above. The average yield of FABP was approximately 400 μg of protein per isolation procedure.

Isolation and purification of bovine mammary gland FABP/MDGI: Bovine mammary gland FABP/MDGI was prepared by the protocol, designated as Method B, of Grosse *et al.* (1991). This method is more advanced than the method previously published by Bohmer *et al.* (1985). In Method B, the following improvements were made: the concentration of reducing agent, β -mercaptoethanol, in the buffer, used for gel-filtration and ion-exchange chromatography, was increased from 10^{-4} M to 10^{-3} M, at the same time 10% glycerol was added to this buffer. The volume of the DEAE-Sepharose column was increased from 2 ml to 26 ml. The buffer, containing increased amount of β -mercaptoethanol and glycerol, provides conditions sufficient to prevent unnecessary aggregation of proteins during purification. The increased amount of the ion-exchanger provides a better condition for separation of the different types of FABP present in mammary gland tissue.

Amino acid analysis: Amino acid analysis of isolated and purified FABP from MFGM and bovine mammary gland tissue was done by the standard protocol at the Biotechnology Facilities of Cornell University.

Isoelectrofocusing: Isoelectrofocusing was done according to the procedure recommended by the vendor (Pharmacia) with precast polyacrylamide slabs, containing ampholines to support the pH gradient from pH 4.0 to pH 6.5. Protein (2-3 μg) was applied to each lane. After isoelectrofocusing, the slabs were fixed using 40% methanol, containing 7% acetic acid, for 16 h. The slabs were then processed for silver staining according to the procedure of Gooderham (1984).

Cell growth inhibitory activity of FABP derived from MFGM: The determination of biological activity, i.e. inhibition of cell growth, of MFGM-derived FABP was done essentially as described by Spitsberg *et al.* (1995). MAC-T cells and primary mammary gland epithelial cells were used in these experiments. The experiments were repeated four times and the standard mean error (SME) was about +5%.

Protein phosphorylation: *In vitro* protein phosphorylation of MFGM proteins was done according to Spitsberg and Gorewit

(1997). Samples of MFGM were prepared from the milk of cows treated with recombinant bovine GH and from milk of non-treated cows (controls), as indicated by Spitsberg *et al.* (1995). The examining of *in vitro* phosphorylation of 15 kDa protein within bovine MFGM was done in three different series of the experiments. In the first series we analyzed five samples of MFGM obtained from GH-treated cows and five samples from non-hormone treated cows. In the second series, three samples from the GH-treated cows and one sample of MFGM from non-hormone treated cow were analyzed; and we, also, analyzed two samples of MFGM prepared from the bulk milk, obtained from one of the local dairy farms, which was using injections of recombinant GH (Posilac, Monsanto) for increased milk production.

Other procedures: SDS/PAGE and Western immunoblotting were performed as described by Spitsberg *et al.* (1995). Non-denatured (12%) polyacrylamide gel electrophoresis was done with the buffer system used for SDS/PAGE, with no addition of SDS to the buffer and gel.

Two-dimensional electrophoresis (2D-E) was done according to Anderson (1991). After 2D-E the gel slabs were subjected to Western immunoblotting (Spitsberg *et al.*, 1995).

Protein concentration was determined by Bradford's method (Bradford, 1976). Densitometric analysis of the autoradiograms was done with the Saggita Color LE scanner (Qtronix) and with the image analysis program, developed by Molecular Dynamics (Sunnyvale, CA, USA).

Results

Isolation, purification and physicochemical properties of FABP from MFGM: The protocol described above for the isolation and purification of FABP from MFGM allowed us to obtain 300-500 μg of highly pure FABP from MFGM, obtained from 3 l of milk. The high purity of FABP was confirmed by the resolution of a single band after SDS-PAGE electrophoresis and Western blotting (Fig. 2A and B). Though the purification procedure employed in this work was quite similar to that used previously to purify FABP/MDGI from mammary gland tissue (Grosse *et al.*, 1991), the ultrasonication and addition of β -mercaptoethanol (15-20 mM) to the extraction solution were necessary for the maximum solubilization of FABP from MFGM. Attempts to improve the yield of FABP from MFGM by inclusion of Triton X-100 into extraction buffer were not successful.

The SDS-PAGE analysis of purified FABP from MFGM showed the presence of only one protein with a relative molecular mass of 15 kDa, identical to that of mammary gland FABP /MDGI (Fig. 2A). Western immuno-blotting analysis of FABP showed that FABP from MFGM was immunologically similar to mammary gland FABP/MDGI (Fig. 2B). Like FABP/MDGI from mammary gland, the purified FABP from MFGM had two components, fast and slow, when it was analyzed by PAGE at non-denatured conditions (Fig. 2C). It is likely that the appearance of these two components was a result of the existence of two isoforms of FABP (Fig. 3). It is unlikely that the slow and fast components were a result of the nonspecific self-aggregation of FABP (Fournier and Rahim, 1983), since the aggregation would lead to the appearance of numerous bands rather than only two bands, detected with different preparations of MFGM FABP and mammary gland FABP/MDGI. One of the isoforms of FABP from MFGM had a pI = 5.20, and the other had a pI = 5.35. These isoforms, appeared as a result of the charge heterogeneity of the same protein, were similar to isoforms of FABP/MDGI from mammary gland (Fig. 3, lanes 2 and 3 versus lane 1). In an additional experiments done by 2D-E, in which 8 M urea was used in the first dimension (isoelectrofocusing), only two isoforms of the purified FABP from MFGM were detected at pH around 5.0. Similarly, only two isoforms at pH around 5.0 were found in purified mammary gland FABP, analyzed by 2D-E. Therefore, the 2D-E analysis of MFGM FABP and mammary gland FABP, purified by the protocol as it is

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Table 1: Inhibition (in %) of MAC-T and primary bovine mammary epithelial cells with various doses of MFGM-derived FABP/MDGI. Data are based on four experiments performed in triplicate. The mean standard error was ± 5.0 .

Concentration, ng/ml	MAC-T cells (%)	Primary epithelial cells (%)
0.1	28.0	30.0
1.0	57.0	60.0
10	71.0	71.0
100	87.0	90.0

indicated in the Methods, did not reveal any noticeable amount of 15 kDa band related to the A-FABP (Specht *et al.* 1996). The different numbers in pI for two isoforms of FABP, reported in this study, and for bovine mammary gland H-FABP, reported by Specht *et al.* (1996), can be explained by inaccuracies in determination of pH gradient in isoelectrofocusing experiments rather than by differences in the nature of the investigated FABP's. What is essential in both cases, is that the two isoforms of FABP in this study and Specht's study had quite similar differences in their pI's, namely, 0.15 units in our study, and 0.2 units in study of Specht *et al.* (1996), and these two isoforms were positioned at pH around 5.0. The possibility that MFGM FABP or FABP/MDGI, purified in our study, could represent bovine brain (B)- type of FABP, which has a high homology (90-95%) with bovine H-FABP (Schoentgen *et al.*, 1989), can be excluded, since the B-FABP would have a pI around 6.0. Because the 11-amino acid C-terminus of B-FABP is quite different from that of H-FABP, it is unlikely that B-FABP would have cell growth inhibitory properties. On similar grounds, keratinocyte/epidermal lipid-binding protein with 48% homology to H-FABP and bovine MDGI (Krieg *et al.*, 1993) can not represent the MFGM FABP studied in this work.

In our work it was also found that the amino acid composition of purified MFGM FABP was quite similar to that of purified FABP/MDGI and was not significantly differed from the amino acid composition derived from the published bovine mammary gland H-FABP amino acid sequence (Borchers *et al.*, 1997).

The analytical parameters of MFGM FABP and bovine mammary gland FABP/MDGI, purified by the Method B (Grosse *et al.*, 1991), provided evidence that both preparations of FABP analyzed in our work belong to the H-type of FABP, i.e. they are identical to H-FABP, described by Borchers *et al.* (1997).

Cell growth inhibitory activity of FABP derived from MFGM : The examination of growth inhibitory activity of FABP from MFGM showed that this protein had biological activity quite similar to FABP prepared from mammary gland (Grosse *et al.*, 1991; Spitsberg *et al.*, 1995). A activity was exerted in the concentration range 1-10 ng/ml (Table 1).

***In vitro* phosphorylated FABP :** The level of *in vitro* phosphorylation of FABP or 15 kDa-protein (Spitsberg and Gorewit, 1997) within MFGM obtained from cows, treated with recombinant bovine growth hormone, were examined in this work. Fig. 4, Panel B, represents the autoradiogram of the *in vitro* phosphorylation of MFGM proteins obtained from the raw milk of GH-treated cows and from untreated cows. The level of phosphorylation of FABP, i.e. the densitometric density (DD) of the 15 kDa bands of the autoradiogram (Fig.4, Panel B) is presented in Fig 5. The fig. 4 and 5 clearly demonstrated that the *in vitro* labeling of 15 kDa protein of all five samples of MFGM, obtained from the GH-treated cows, was less than in the samples of MFGM obtained from non-treated cows. The labeling of 15 kDa protein was especially reduced in the samples of MFGM analyzed in lanes 2 and 6 in Fig.4B. The phosphorylation of other major bands of MFGM, such as the 66 kDa and 51-52 kDa bands, was not markedly affected by GH treatment. We found that the ratio of the DD of the 66 kDa band to the DD of the 15 kDa band was practically constant when it was measured in five different

samples of MFGM obtained from non-hormone-treated cows. Therefore, this ratio can serve as an indicator of the status of the phosphorylation level of 15 kDa protein. The lesser phosphorylation of 15 kDa protein/ FABP in MFGM could be explained by diminished content of FABP in MFGM obtained from hormone-treated animals. However, the amount of 15 kDa protein/FABP within MFGM, obtained from control and GH-treated cows, was quite similar in both types of MFGM, as determined by SDS-PAGE analysis (Fig. 4A). The possible reduction of the *in vitro* phosphorylation of 15 kDa protein within of MFGM, obtained from the GH-treated cows, was confirmed in the additional experiments with three individual samples of MFGM from GH-treated cows and with two samples of MFGM, prepared from the bulk milk of one of the local dairy farms, using GH for the increased production of milk.

Discussion

Previously it has been shown that bovine MFGM contains FABP which is related to bovine FABP/MDGI (Brandt *et al.*, 1988). However, in this study MFGM FABP was isolated from SDS-polyacrylamide gels, i.e. in a denatured form. Therefore, FABP obtained in this way is not suitable for examining its biological activity. Moreover, the biochemical characteristics of MFGM FABP were unknown. We felt it important to determine if MFGM associated FABP is structurally and functionally similar to FABP/MDGI. MFGM, as it is known, consists mostly of apical plasma membrane elements of secretory epithelial cells of the lactating mammary gland (Kanno, 1990). The discovery of FABP/MDGI in this membrane may support the suggestion of the involvement of FABP/MDGI in cell growth and differentiation through an apocrine loop mechanism (Brandt *et al.*, 1988).

In our work, we presented evidence that FABP, associated with the bovine MFGM, is identical to the soluble, cytosolic form of H-FABP or MDGI of bovine mammary gland tissue (Grosse *et al.*, 1991). Firstly, we demonstrated that purified 15 kDa protein from bovine MFGM and FABP/MDGI from mammary gland tissue (Grosse *et al.* 1991; Spitsberg *et al.*, 1995) had similar biological activities. Both FABPs were able to suppress growth of mammary-derived cells at nanomolar concentrations. Secondly, the identity of the isolated 15 kDa protein from MFGM and FABP/MDGI was confirmed by analysis of the macromolecular species of these proteins by SDS-PAGE, non-denaturing PAGE, isoelectrofocusing, Western immunoblotting and 2D-E. Since, in bovine mammary gland, only two types of FABP, namely, H-FABP, localized in secretory epithelial cells, and A-FABP, localized only in myoepithelial cells, were clearly identified, we feel strongly that the isolated and purified 15 kDa protein from MFGM is FABP of heart-type, and can be referred to MFGM-associated H-FABP/MDGI.

The identification of H-FABP/MDGI associated with MFGM, representing the apical plasma membrane of secretory epithelial cells of mammary gland, opens a new avenue for studying the expression and biochemical modification of MFGM associated H-FABP/MDGI in the mammary gland under various physiological conditions.

We analyzed whether GH-treatment of cows can effect the content of H-FABP/MDGI within the MFGM and/or the level of *in vitro* phosphorylation of MFGM-associated H-FABP / MDGI (Spitsberg and Gorewit, 1997). This work showed that the level of phosphorylation of 15 kDa FABP, within the MFGM obtained from GH-treated animals was effected by GH-treatment. The level of phosphorylation was less than that for MFGM obtained from non-treated animals. The content of H-FABP/MDGI of MFGM was not effected by prolonged GH-treatment of cows, although the effect of GH-treatment on total expression of H-FABP/MDGI in mammary gland tissue can not be ruled out. Here, it is worthy to mention the recent *in vitro* finding of Huynh and Beamer (1998) that GH and insulin-like growth factor 1 (IGF-1) can suppress MDGI gene expression.

The observed reduced level of *in vitro* phosphorylation of FABP within the MFGM, obtained from GH-treated cows, can be

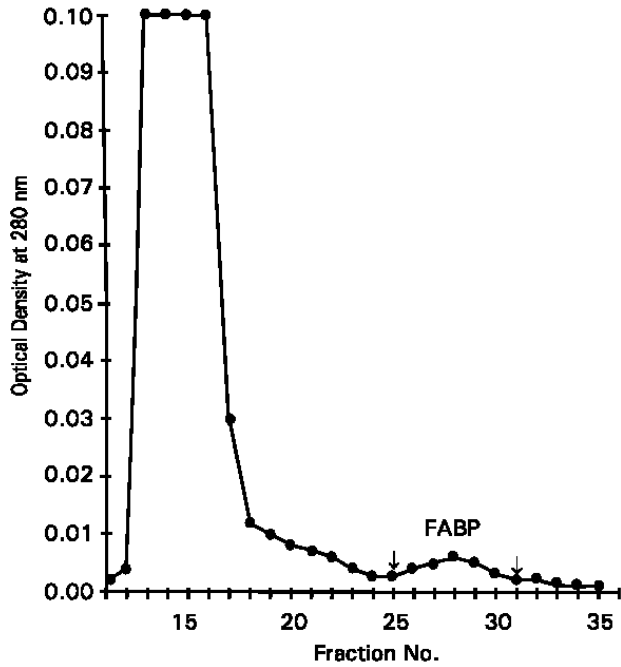


Fig. 1: Elution profile of the MFGM-FABP containing fraction, obtained from DEAE-Sepharose, on Sephadex G-50 (90x2.5 cm). Equilibration buffer: 50 mM Tris-HCl, pH 7.4, containing 140 mM NaCl. The protein peak with $V_e/V_0 = 1.5-1.6$ ($V_0 = 150$ ml; $V_e = 240$ ml) was collected and analyzed by SDS-PAGE, Western immunoblotting and cell growth inhibitory assay (see Methods). The first 10 fractions were collected at 14.5 ml/tube and subsequent fractions were collected at 5.3 ml/tube. The triangle pointers show the V_e (left) and V_0 (right).

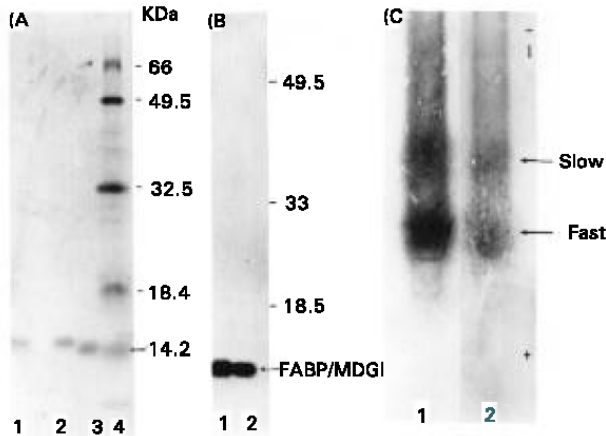


Fig. 2: Analysis of purified FABP from MFGM by PAGE and Western immunoblotting. Panel A: (12%) SDS-PAGE; lane 1- 100 ng of bovine mammary gland FABP; lane 2- 125 ng of bovine MFGM-FABP; lane 3- 100 ng β -lactalbumin (14.2 kDa); lane 4 - protein molecular weight standards. The gel was stained with silver (Gooderham, 1984). Panel B: Western immunoblotting analysis of purified bovine MFGM-FABP. The proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Lane 1- about 100 ng of bovine mammary gland FABP; lane 2- about 100 ng of MFGM-FABP. Primary antibody was anti-bovine mammary gland FABP (Spitsberg *et al.*, 1995). Secondary antibody was conjugated goat anti-rabbit IgG-HRPO. The blott was developed with 4-chloro-1-naphthol and H_2O_2 . Panel C: Western immunoblotting analysis after the separation of the proteins in nondenaturing (12%) PAGE. Lane 1: 100 ng of mammary gland FABP; 2- 100 ng of MFGM-FABP. Antibodies and detection were as it is described in panel B.

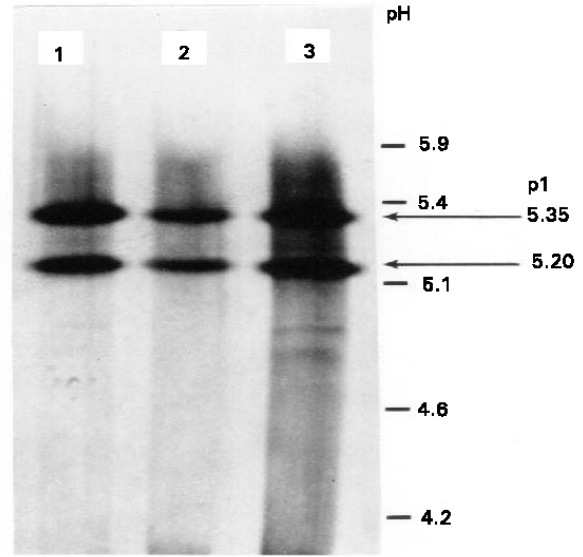


Fig. 3: Analysis of FABP by isoelectrofocusing. Lane 1: 3 μ g of bovine mammary gland FABP; lane 2- 1.8 μ g of MFGM-FABP; lane 3- 3.0 μ g of MFGM-FABP. The gel was stained by silver.

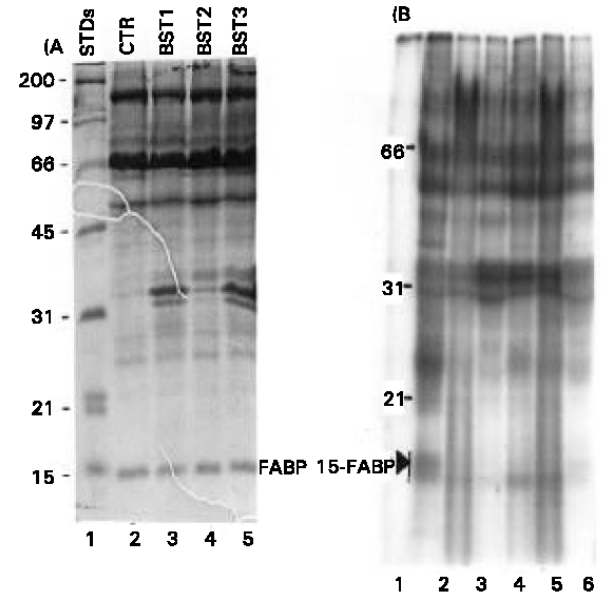


Fig. 4: Analysis of *in vitro* ^{32}P -labeled MFGM proteins. Panel A: (12%) SDS-PAGE of bovine MFGM, obtained from GH-treated and untreated cows. Lane 1: MW (kDa) standards; Lane 2: 50 μ g of MFGM obtained from milk of non-hormone-treated cow (CTR control); lanes 3-5: 50 μ g of MFGM obtained from three individual samples of milk of GH-treated cows (BST1, BST2, BST3). The gel was stained by Coomassie blue R-250. The 15 kDa protein, previously identified only as FABP (Brandt *et al.*, 1988; Spitsberg *et al.*, 1995; Spitsberg and Gorewit, 1997) is present in these samples of MFGM in equal amount. Panel B: Autoradiogram of (12%) SDS-PAGE of the *in vitro* ^{32}P -labeled MFGM proteins, obtained from GH-treated and non-hormone-treated cows. 50 μ g of proteins of MFGM were *in vitro* phosphorylated and subjected to SDS-PAGE (see Methods). Lane 1: MFGM from non-treated cow (control); lanes 2-6: individual MFGM from five GH-treated cows. The labelling pattern of other samples of MFGM from four untreated cows were similar to that shown in lane 1. Note that the *in vitro* labeling of 66 kDa protein (butyrophilin) (Spitsberg and Gorewit, 1997) was quite similar in all analyzed samples of MFGM.

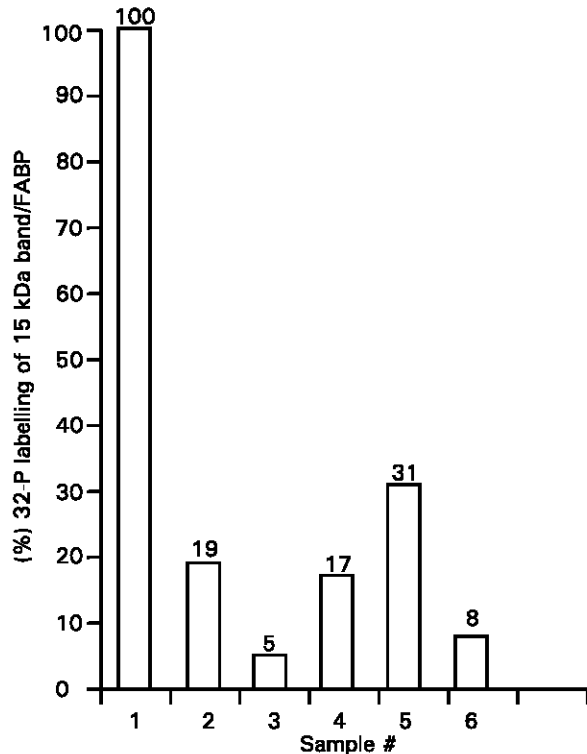


Fig. 5: Graph of the densitometric analysis of the autoradiogram in Fig. 4, Panel B. The graph demonstrates the decreased labeling of 15 kDa protein/FABP in five individual samples of MFGM obtained from GH-treated cows. The level of labelling of 15 kDa protein/FABP within control MFGM was taken as 100%.

interpreted as a result of diminished content or diminished activity of the phosphorylating FABP protein kinase in this membrane. One of the candidates for this kinase is the beta-subunit of insulin receptor since this polypeptide was detected in MFGM by Western immunoblotting (Spitsberg and Gorewit, 1997). In addition, the specific insulin-binding sites in bovine MFGM have been also reported (Smith *et al.*, 1987). In our previous work, we demonstrated that a tyrosine residue of the 15 kDa protein of bovine primary epithelial cells is phosphorylated and it reacted positively with FABP-antibody (Spitsberg *et al.*, 1994). There is evidence that FABP in mouse mammary gland epithelial cells is phosphorylated by the insulin receptor (Nielsen and Spener, 1993; Nielsen *et al.*, 1994). There are a few recent publications indicating the relationships between GH and insulin receptor (Balbis *et al.*, 1996, 1992; Leenanurksa and McDowell, 1988). Balbis *et al.* (1996, 1992) demonstrated that overexpression of bovine GH in transgenic mice is associated with down regulation of hepatic insulin receptor. In other work (Leenanurksa and McDowell, 1988), injection of recombinant bovine GH into aloxan-diabetic-insulin-stabilized ewes led to increased plasma glucose, which was decreased by a double-dosed insulin infusion.

If the phosphorylation of FABP takes place through the involvement of the beta-subunit of the insulin receptor, the association of FABP with membrane should occur on the cytoplasmic side of the plasma membrane and consequently on the cytoplasmic side of MFGM since MFGM represents the portion of plasma membrane encircling the secreted lipid droplets. The analysis of immunoprecipitates formed after the addition of anti-FABP or anti-CD36 to the solubilized proteins of MFGM showed that one of the candidates for the binding of FABP to the plasma membrane is the cytoplasmic domain of CD36 (Spitsberg *et al.*, 1995). Further study will be needed to show if the complex of FABP with CD36 is also associated with the beta-subunit of insulin

receptor.

The significance of the finding that the phosphorylation of FABP within the MFGM is under hormonal regulation may have a practical application. Our preliminary work (Spitsberg and Gorewit, unpublished data) indicates that there is a correlation between the level of phosphorylation of FABP within the MFGM and the level of milk production by individual animals. We suggest the degree of *in vitro* phosphorylation of FABP within the MFGM can serve as an indirect sign as to whether or not the animals were given biologically effective doses by GH. Therefore, the analysis of the *in vitro* phosphorylation of individual samples of MFGM could provide an understanding as to why some cows do not respond to the injection of GH with significantly elevated milk production.

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References

- Anderson, L., 1991. Two-dimensional electrophoresis: Operation of the Iso-Dalt System. Large Scale. Biology Press, Washington, DC.
- Bansal, M.P. and D. Medina, 1993. Expression of fatty acid-binding proteins in the developing mouse mammary gland. *Biochemical and Biophysical Research Communications*, 191: 61-69.
- Balbis, A., A. Bartke and D. Turyn, 1996. Overexpression of bovine growth hormone in transgenic mice is associated with changes in hepatic insulin receptors and in their kinase activity. *Life Science*, 59: 1363-1371.
- Balbis, A., J.M. Dellacha, A. Bartke and D. Turyn, 1992. Down regulation of masked and unmasked insulin receptors in the liver of transgenic mice expressing bovine growth hormone gene. *Life Science*, 51: 771-778.
- Bass, N.M., 1988. The cellular fatty acid binding proteins: aspects of structure, regulation and function. *International Review of Cytology*, 111: 143-185.
- Binas, B., E. Spitzer, B. Zschiesche, B. Erdmann, A. Kurtz, T. Muller, C. Niemen, W. Blenau and R. Grosse, 1992. Hormonal induction of functional differentiation and mammary-derived growth inhibitor expression in cultured mouse mammary gland explants. *In vitro Cellular and Developmental Biology*, 28A: 625-634.
- Bohmer, F.-D., W. Lehmann, H. Schmidt, P. Langen and R. Grosse, 1984. Purification of a growth inhibitor for Ehrlich ascites mammary carcinoma cells from bovine mammary gland. *Experimental and Cellular Research*, 150: 466-476.
- Bohmer, F.-D., W. Lehmann, F. Noll, R. Samtleben, P. Langen and R. Grosse, 1985. Specific neutralizing antiserum against a polypeptide growth inhibitor for mammary cells purified from bovine mammary gland. *Biochimica et Biophysica Acta*, 846: 145-154.
- Bohmer, F.-D., R. Kraft, A. Otto, C. Wernstedt, A. Kurtz, K. Muller, G. Rohde, G. Etzold, P. Langen, C.-H. Heldin and R. Grosse, 1987. Identification of a polypeptide growth inhibitor from bovine mammary gland. MDGI sequence homology to fatty acid and retinoid binding proteins. (1987) *J. Biol. Chem.*, 262: 15137-15143.
- Borchers, T., C. Hohoff, C. Buhlmann and F. Spener 1997. Heart-type fatty acid binding protein-involvement in growth inhibition and differentiation. *Prostaglandins, Leukotrienes and Essential Fatty Acids*, 57: 77-84.
- Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, 72: 248-254.
- Brandt, R., M. Papperle, A. Otto, R. Kraft, F.-D. Boehmer and R. Grosse 1988. A 13-kDa protein purified from milk fat globule membrane is closely related to a mammary-derived growth inhibitor. *Biochemistry*, 27: 1420-1425.
- Chen, N.Y., W. Y. Chen and J. J. Kopchick, 1997. Liver and kidney growth hormone (GH) receptors are differently in diabetic GH and GH antagonist transgenic mice. *Endocrinology*, 138: 1988-1994.

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- Fournier, N. and M. H. Rahim, 1983. Self-aggregation, a new property of cardiac fatty acid-binding protein. Predictable influence on energy production in the heart. *J. Biol. Chem.*, 258: 2929-2933.
- Gooderham, K., 1984. High-sensitivity silver staining of proteins following polyacrylamide gel electrophoresis. In: *Methods in Molecular Biology*. Vol. 1, Proteins. Edited by J.M. Walker ed., pp: 113-118. Humane Press, Clifton, NJ.
- Greenwalt, D. E., R.H. Lipsky, R.H. DeKenhause, H. Ikeda, N. N. Tandon and G.A. Jamieson, 1992. Membrane glycoprotein CD36 : A review its role in adherence, signal transduction, and transfusion medicine, *Blood*, 80: 1105-1115.
- Grosse, R., F.D. Boehmer, P. Langen, W. Lehmann, M. Mieth and G. Wallukat, 1991. Purification, biological assay and immunoassay of mammary-derived growth inhibitor. *Methods in Enzymology*, 198, 425-440.
- Grosse, R and P. Langen, 1990. Mammary derived growth inhibitor. In *Handbook of Experimental Pharmacology 95/II*. M. Sporn and A. Roberts, editors. Springer Verlag, Heidelberg. pp: 249-265.
- Huynh, H. , L. Alpert and M. Pollak, 1996. Silencing of the mammary-derived growth inhibitor (MDGI) gene in breast neoplasia is associated with epigenetic changes. *Cancer Research*, 56: 4865-4870.
- Huynh, H. and W. Beamer, 1998. Suppression of mammary-derived growth inhibitor gene expression by growth hormone and insulin-like growth factor 1. *International J. oncology*, 13: 577-582.
- Kanno, C., 1990. Secretory membranes of the lactating mammary gland. *Protoplasma*, 159: 184-208.
- Krieg, P., S. Feil, G. Furstenberger and G. T. Bowden, 1993. Tumor-specific overexpression of a novel keratinocyte lipid-binding protein. *J. Biol. Chem.*, 268: 17362-17369.
- Leenanuraksa, D. and G. H. McDowell, 1988. Control of glucose homeostasis in lactating ewes use of the alloxan-diabetic-insulin-stabilized ewe to study effects of insulin and growth hormone. *Australian J. Biol. Sci.*, 41: 421-434.
- Nielsen, S. U. and F. Spener, 1993. Fatty acid-binding protein from rat heart is phosphorylated on Tyr-19 in response to insulin stimulation. *J. Lipid Res.*, 34: 1355-1366.
- Nielsen, S. U., R. Rump, P. Hojrup, P. Roepstroff and F. Spener, 1994. Differentiation regulation and phosphorylation of the fatty acid-binding protein from rat mammary epithelial cells. *Biochimica et Biophysica Acta*, 1211: 169-197.
- Schoentgen, F., G. Pignete, L. M. Bonnanno and P. Jolles, 1989. Fatty-acid-binding protein from bovine brain. Amino acid sequence and some properties. *European. J. Biochem.* 185: 35-40.
- Shi, Y. E., J. Ni, G. Xiao, E. Liu, A. Fuchs, G. Yu, J. Su, J. M. Cosgrove, L. Xing, M. Zhang, J. Li, B.B. Aggarwal, A. Meager and R. Gentz, 1998. Antitumor activity of the novel human breast cancer growth inhibitor, mammary-derived inhibitor-related gene, MRG. *Cancer Research*, 57: 3084-3091.
- Smith, D.H., K. L. Roehring, D.L. Palmquist and F.L. Schanbacher, 1987. Effect of dietary fat and lactation status on insulin binding to bovine milk fat globule membranes. *J. Dairy Sci.*, 70: 1537-1562.
- Specht, B., N. Bartecko, C. Hohoff, H. Kuhl, R. Franke, T. Borchers and F. Spener, 1996. Mammary derived growth inhibitor is not a distinct protein but a mix of heart-type and adipocyte-type fatty acid-binding protein. *J. Bio. Chem.*, 271: 19943-19949.
- Spener, F., T. Borchers and M. Mukhejee, 1985. On the role of fatty acid-binding protein in fatty transport and metabolism. *FEBS Letters*, 244: 1-5.
- Spitsberg, V. L. and R. C. Gorewit, 1997. *In vitro* phosphorylated bovine milk fat globule membrane proteins. *J. Nutr. Biochem.*, 8: 181-189.
- Spitsberg, V. L. , E. Matitashvili and R. C. Gorewit, 1994. Bovine mammary cells contain a low molecular weight phosphorylated protein that is immunoreactive to MDGI/FABP. *J. Dairy Sci.*, 77 (Suppl.1), 75.
- Spitsberg, V. L., E. Matitashvili and R. C. Gorewit, 1995. Association and co-expression of fatty-acid-binding protein and glycoprotein CD36 in the bovine mammary gland. *European J. Biochem.*, 230: 872-878.
- Treuner, M., C.A. Kozak, D. Gallahan, R. Grosse and T. Muller, 1994. Cloning and characterization of the mouse gene encoding mammary-derived growth inhibitor heart-fatty acid-binding protein. *Gene (Amst.)* 147, 237-242, 1993
- Yang, Y., E. Spitzer, N. Kenny, W. Zschiescho, M. Li, A. Krominga, , T. Muller, F. Speher, A. Lezius, J. Veerkamp, G. Smith, D. Salomon and R. Grosse, 1994. Members of fatty acid binding protein family are differentiation factors for the mammary gland. *J. Cell Bio.*, 127: 1097-1109.
- Yang, C. W., G. E. Striker, W. Y. Chen, J. J. Kopchick and L. J. Striker, 1997. Differential expression of glomerular extracellular matrix and growth factor mRNA in rapid and slowly progressive glomerulosclerosis: Studies in mice transgenic for native or mutated growth hormone. *Laboratory Investigation*, 76: 467-476.

Abbreviations: MFGM - milk fat globule membrane; H-FABP - heart-type fatty acid binding protein; A-FABP - adipocyte-type fatty acid binding protein; MDGI - mammary derived growth inhibitor; GH - growth hormone; BST - bovine somatotropin, SDS - sodium dodecylsulfate; PAGE- polyacrylamide gel electrophoresis.