ELSEVIER

In vitro phosphorylated bovine milk fat globule membrane proteins

V.L. Spitsberg and R.C. Gorewit

Department of Animal Science, Cornell University, Ithaca, NY USA

Incubation of the milk fat globule membrane (MFGM) with [γ-^{32}P]ATP, in the presence of protein phosphatase inhibitors and $MgCl_2$ and/or $MnCl_2$, led to protein phosphorylation within this membrane. Analysis of the ^{32}P-labeled proteins of bovine MFGM, by SDS/PAGE and autoradiography, showed at least 15 radioactive bands with relative molecular masses in the range of 10 to 200 kDa. Bovine MFGM had four bands (66–67, 51, 33, and 24 kDa) labeled more intensively than others. Calcium could not substitute for Mg^{2+} and Mn^{2+} in the phosphorylation reaction. Genistein (100 μmol/L) reduced protein phosphorylation by 50 to 70%, whereas daidzein and heparin did not. The 66–67 and 15 kDa in vitro *phosphorylated bovine MFGM proteins were butyrophilin and fatty-acid-binding protein (FABP), respectively. Phosphoamino acid analysis of ^{32}P-labeled 66, 51, and 15 kDa polypeptide acid hydrolyzates revealed radiolabeled phosphotyrosine, -threonine, and -serine in the 66 kDa protein, radiolabeled phosphotyrosine and -threonine in the 51 kDa protein, and radiolabeled phosphotyrosine in the 15 kDa protein. FABP appeared to be in a complex with its putative kinase. The analysis of* in vitro *phosphorylated human MFGM revealed 13–15 radioactive protein bands in the range of 20 to 200 kDa. The 65 and 50 kDa radiolabeled bands were prominent in all five individual samples of human MFGM analyzed. FAK, insulin β-subunit receptor, c-src^{60} and MAPK, were detected in the bovine and human MFGM using Western immunoblotting. MAPKAPK was also found in the bovine MFGM.* (J. Nutr. Biochem. 8: 181–189, 1997) © *Elsevier Science Inc. 1997*

Keywords: butyrophilin; fatty-acid-binding protein; milk fat globule membrane; MFGM; protein kinases; protein phosphorylation; genistein

Introduction

A remarkable feature of milk is the presence of lipid droplets coated with proteo-lipid material, the latter designated as milk fat globule membrane (MFGM).[1] MFGM is involved in the packaging and secretion of milk lipids,[1] and, as a natural emulsifier and stabilizer of lipid droplets, has an important nutritional role.[2] MFGM can be isolated, and is composed of four layers: the thin coating membrane derived from intracellular lipovesicles; the protein coat; the bilayered membrane primarily derived from the apical plasma membrane of mammary secretory epithelial cells and partially from the secretory vesicle membrane; and the glycocalyx.[1,3] Electron microscopy of MFGM has revealed that the majority of it appears as membranous sheets with associated coat material. However, some MFGM is in the form of vesicles with little or no coat material.[4] It is very likely that milk protein synthesis is simultaneously accomplished with intensive synthesis of MFGM components. This is necessary to replenish their loss by extrusion from mammary gland secretory cells during milk secretion. In this sense, milk is a unique depot of biological membranes synthesized inside mammary secretory epithelial cells.

MFGM proteins have received much attention in recent years and many of them have been well characterized. Moreover, several genes of these proteins have been cloned.[5–10] Some MFGM proteins are of special interest because of their involvement in critical cell processes. One of the major components of MFGM is the 66–67 kDa glycoprotein butyrophilin (Bph). Despite extensive biochemical studies of Bph,[8] its physiological role is unknown. Because Bph is specifically synthesized in the mammary gland, it may serve as a marker for mammary development and differentiation.[11] Other MFGM proteins, such as the recently described low molecular mass GTP-binding proteins, may play specific roles in directing lipid droplets to the apical membrane of epithelial cells, bypassing the Golgi

Address reprint requests to V.L. Spitsberg at 439 Morrison Hall, Department of Animal Science, Ithaca, NY 14853, USA.
Received October 2, 1996; accepted December 19, 1996.

© Elsevier Science Inc. 1997
655 Avenue of the Americas, New York, NY 10010

0955-2863/97/$17.00
PII S0955-2863(97)00001-6

apparatus-*trans*-Golgi route.[12] Another major component of MFGM is guinea pig glycoprotein GP55 or component 15/16 found in bovine milk. This protein has an epidermal growth factor-like domain, and is thought to be involved in mammary physiology and neonatal development.[13] Other proteins of MFGM, such as glycoprotein CD36,[6] fatty-acid-binding protein (FABP),[10,14] originally named "mammary-derived growth inhibitor" (MDGI),[10] and insulin receptor,[15] are involved in cellular processes, including growth, differentiation, and metabolic regulation.

Although the biochemical study of bovine MFGM spans over 20 years, an analysis of protein phosphorylation and protein kinases within these membranes has not been carried out. In the work of Bingham and Farrell,[16] preparations of MFGM proteins were used as substrates for Ca^{2+}/Mg^{2+}-dependent Golgi-apparatus associated kinase. These authors found that MFGM proteins were weakly labeled with [γ-^{32}P] ATP by the enzyme. Analysis of the phosphorylated proteins was not attempted. We report for the first time that MFGM isolated from bovine and human milk contains intrinsic protein phosphorylation activity.

Methods and materials

FAK mAb, panERK mAb, polyclonal anti-insulin receptor (β-subunit), caveolin mAb, and anti-alfa-PKC antibodies were obtained from Transduction Laboratories (Lexington, KY USA). The β-actin mAb (clone AC-74) was from Sigma Chemical Co. (St. Louis, MO USA); the v-src^{60} mAb was from Oncogene Science, Inc. (Uniondale, NY USA); the human thrombospondin mAb (clone 1, A4.1) was from Gibco BRL (Gaithersburg, MD USA). The CD36 polyclonal Ab was donated by Dr. Dale Greenwalt (American Red Cross, Rockville, MD USA). Polyclonal anti-MAPKAPK antibody was a gift from Drs. Rapp and Lerman (NCI, Frederick, MD USA). Rabbit polyclonal antibody to bovine mammary gland FABP was prepared in our laboratory.[14] Polyclonal antipeptide antibodies, specific for Bph, were kindly provided by Dr. I.H. Mather. These antibodies were separately generated to peptides encompassing residues 178 to 198 and residues 506 to 526 of bovine Bph.[9] The PVDF membrane was obtained from Bio-Rad Laboratories (Hercules, CA USA). Sodium heparin was from LypoMed, Inc. (Rosemount, Inc., IL USA). Alfa-, beta-caseins and goat antirabbit IgG-HRPO were from Sigma. All reagents for immunoblotting and SDS/polyacrylamide gel electrophoresis (SDS/PAGE) were from Bio-Rad Laboratories. Phosphatase inhibitors, genistein, EDTA, sodium deoxycholate, and *Staphylococcus aureus* cells were purchased from Sigma. Daidzein and Triton X-100 were obtained from Calbiochem (San Diego, CA USA). All other chemicals were of analytical grade from standard supply houses.

Preparation of the MFGM

Milk fat globule membranes were prepared from cream of fresh bovine or human milk.[14] Lactating cows and five human female volunteers were healthy and mastitis-free. Cream was obtained by centrifugation of milk at 3,000 g for 20 min. It was then suspended in two volumes of 50 mmol/L Tris-HCl, pH 7.5, containing 0.15 mmol/L NaCl (TBS), and homogenized in a Waring blender for 45 sec. The homogenate was centrifuged at 100,000 g for 90 min. The MFGM pellet was washed once by resuspending it in an initial volume of TBS, followed by centrifugation at 100,000 g for 90 min. No somatic cells were found in the MFGM, as determined by light microscopy.

Protein phosphorylation of the MFGM

Protein phosphorylation of the MFGM was conducted under conditions commonly used for detection of protein kinases,[17,18] with some modification. The phosphorylation was done as follows: 40 to 50 μg of MFGM in 50 μL of TBS was added to 550 μL of 50 mmol/L Tris/HCl buffer, pH 7.5, containing 1 mmol/L EDTA and 10 μmol/L of each phosphatase inhibitor (vanadate, β-glycerophosphate, phosphotyrosine, phosphoserine, phosphothreonine, *p*-nitrophenylphosphate), 10 μmol/L Na-salt of ATP, 2 μCi of [γ-^{32}P]ATP (3000–6000 Ci/mmole; Amersham), 5 mmol/L $MgCl_2$ and/or 3 mmol/L $MnCl_2$. The MFGM was incubated for 30 min at room temperature. After incubation, the mixture was immediately ultracentrifuged for 30 min at 100,000 g. The pellet was then analyzed by (12%) SDS-PAGE,[14] followed by autoradiography.

In the studies of kinetics and in the analysis of the activity of detergent-solubilized fractions, the kinase reaction was stopped by adding an equal volume of cold 10% TCA. After 10 to 15 min, precipitated proteins were collected by centrifugation at 10,000 g for 10 min or filtered through glass microfiber filters (type GF/C, Whatman). Filters were washed with cold 5% TCA, 70% ethanol, dried, and then counted in a liquid scintillation analyzer. Pellets from TCA precipitates were also solubilized in 50 to 70 μL of SDS-sample buffer, neutralized by adding 2 to 4 μL of 0.5 mol/L NaOH, and analyzed by (12%) SDS/PAGE.[14] After SDS/PAGE, gels were fixed in 40% methanol-10% acetic acid for at least 4 hr, dried, and autoradiographed with X-OMAT film (Kodak) for 2 to 4 days at −70°C.

In experiments dealing with the identification of phosphorylated proteins and phosphoaminoacid analysis of ^{32}P-labeled 15 kDa protein, labeling of MFGM was carried out using 10 μCi of [γ-^{32}P]ATP with no addition of cold ATP.

Immunoprecipitation

[^{32}P]-phosphorylated MFGM (~500 μg protein) was suspended in 500 μL of lysis buffer comprising 50 mmol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, 1% (v/v) Triton X-100, 50 mmol/L dithiothreitol (DTT), and 2 mmol/L phenylmethylsulphonyl fluoride (PMSF), on ice. After mixing for 10 to 15 min, samples were centrifuged at 100,000 g for 30 min, and the solubilized proteins were dialyzed for 4 hr against TBS, containing 0.5% (v/v) Triton X-100, to remove the reducing agent. Dialyzed protein solutions were then clarified by centrifugation at 100,000 g for 30 min. Alternatively, [^{32}P]-labeled MFGM proteins were solubilized with the radioimmunoprecipitation assay (RIPA) buffer comprising 10 mmol/L Tris-HCl (pH 7.2), 158 mmol/L NaCl, 1% (v/v) Triton X-100, 1% (w/v) sodium deoxycholate, 0.1% (w/v) SDS, and 1 mmol/L PMSF. Solubilized proteins were recovered by centrifugation at 100,000 g for 30 min and used for immunoprecipitation directly. One hundred microliters of a 1:1 mixture of affinity-purified rabbit antipeptide antibodies (see Methods and materials) were added to 500 μL (200 μg protein) of detergent-solubilized extracts (~0.050 μg antibody/μg solubilized protein) and incubated for 2 hr at 4°C. A 20 μL suspension of 10% (v/v) *S. aureus* cells was then added to the mixture. It was incubated for an additional hour while being stirred. The *S. aureus* cells were then collected by centrifugation and washed four times with RIPA buffer and finally once with distilled water. The adsorbed proteins were solubilized in 50 μL of the SDS-PAGE sample buffer,[13] containing 3% (w/v) SDS and 20 mmol/L β-mercaptoethanol at 100°C for 3 min and analyzed by (12%) SDS/PAGE.[14]

Western immunoblotting

Electrotransfer of proteins from the SDS/PAGE gels, to PVDF membrane, was performed as described by Towbin et al.[19] Immunodetection of proteins (50 to 70 μg of MFGM/lane) was performed as described by Spitsberg et al.,[14] and in accordance with recommendations provided by the antibody supplier.

Treatment of bovine MFGM by detergents

The MFGM (5 to 10 mg of protein) was homogenized in 1 to 2 mL of: (1) 50 mmol/L Tris-HCl buffer (pH 7.5), containing 0.15 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L PMSF, 1% Triton X-100 and 50 mmol/L DTT, or (2) RIPA-buffer (1% Triton X-100, 1% deoxycholate, 0.1% SDS, 158 mmol/L NaCl, 10 mmol/L Tris-HCl, pH 7.2, 5 mmol/L EDTA, 1 mmol/L PMSF). After mixing for 5 to 10 min, the homogenate was ultracentrifuged at 100,000 g for 60 min at 4 to 5°C. The 100,000 g pellets and supernatants were analyzed for protein autophosphorylation activity as described, using 50 μg of protein per assay.

Protein sequence analysis of the 66 kDa protein

Non-labeled and labelled samples of bovine MFGM were separated by SDS/PAGE.[14] Fifty micrograms of protein/lane was used for SDS/PAGE. Proteins were electrophoretically transferred to PVDF membrane.[19] The membranes were stained with 0.1% Ponceau S in 1% acetic acid and washed in deionized water. The stained band corresponding to the radiolabeled 66 kDa zone (determined by autoradiography) was excised for amino acid sequence analysis. A partial N-terminal sequence was obtained using an Applied Biosystems protein sequencer 470 A, equipped with an online PTH sequencing program 03RPTH. Sequence analysis was performed at the Cornell Biotechnology Analytical/ Synthesis Facility, Ithaca, NY.

Analytical procedures

The protein content of the samples (MFGM or its fractions) was measured after their solubilization with 0.5 mol/L NaOH according to the procedure of Bradford.[20] Phosphoamino acids in acid hydrolyzates of proteins, immobilized on a PVDF membrane,[21] were analyzed by thin-layer chromatography (TLC), as described by Munoz and Marshall.[22] Briefly: [^{32}P]-labeled bovine MFGM was separated by (12% gel) SDS/PAGE.[14] Proteins were transferred to the PVDF membrane,[19] which was then exposed to x-ray film for 1 to 2 days. Bands corresponding to Bph and the 51 and 15 kDa proteins were cut out and hydrolyzed with 5.7 mol/L HCl for 3 hr at 110°C. The hydrolysate was dried, and the residue dissolved in NH_4OH/ethanol (1:2) and separated by ascending thin-layer chromatography on a Whatman silica gel sheet (AL Sil G) for Bph and 51 kDa protein and on an Eastman silica gel sheet (type R 301R) for 15 kDa protein in the same solvent. Phosphotyrosine, phosphothreonine, and phosphoserine were also applied as standards. Standards were detected with ninhydrin solution and the labeled amino acids by autoradiography.

Results

Protein phosphorylation of the MFGM: General procedures and conditions

Optimal conditions for MFGM protein phosphorylation were based on preliminary experiments. The maximum incorporation of radioactive phosphate (^{32}P) into TCA-precipitated MFGM proteins was achieved on incubation of 15 to 20 μg of MFGM with [γ-^{32}P]ATP (2 μCi) and Mg^{2+} (5 mmol/L) for 20 to 30 min at 20°C. This maximum incorporation constituted about 200 pmol/min/mg of protein. We also analyzed the ^{32}P-labeled bovine MFGM proteins by SDS/PAGE and autoradiography after incubation of 50 μg of MFGM with [γ-^{32}P]ATP and Mg^{2+} for 1, 5, and 30 min. This experiment clearly showed that labeling of individual MFGM proteins was time-dependent. A 30-min incubation period was sufficient to observe the complete ^{32}P-labeled protein pattern of MFGM, using SDS/ PAGE, followed by autoradiography.

Effect of protein phosphatase inhibitors

Incubation of MFGM with [γ-^{32}P]ATP, in the presence of protein phosphatase inhibitors (vanadate, beta-glycerophosphate, phosphotyrosine, phosphoserine, phosphothreonine, *p*-nitrophenyl phosphate) and 5 mmol/L $MgCl_2$ and/or 3 mmol/L $MnCl_2$, led to phosphorylation of several MFGM proteins (*Figure 1*). Inclusion of protein phosphatase inhibitors into kinase incubation media was necessary for maximum ^{32}P-labeling of MFGM proteins (*Figure 1B*). The main 15 radioactive protein bands, in the bovine MFGM, ranged from 10 to 200 kDa (200, 150, 130, 100, 75, 66, 51, 45, 39, 35, 32, 24, 21, 15, and 13) (*Figure 1A, lane 1*). Bands corresponding to 66, 51, 32 to 35, and 24 kDa proteins were predominantly labeled in the bovine MFGM. The SDS/PAGE-pattern of radiolabeled bovine MFGM proteins was highly reproducible with preparations obtained from individual cows of the same breed. This indicated that the observed radiolabeling pattern reflected the specific protein composition of the MFGM. The 10 to 15 in vitro ^{32}P-labeled protein bands, ranged from 20 to 200 kDa, were found in individual human MFGM samples (*Figure 1C*). The two bands, 65 kDa and 50 kDa, were prominent in all five individual samples of human MFGM analyzed.

Role of divalent metals

The MFGM protein phosphorylation was dependent on divalent metals. Practically no protein phosphorylation was observed when Mg^{2+} and Mn^{2+} were excluded from the incubation media (*Figure 1A, lane 4* versus *lane 1*). This indicated that phosphorylation activity within the MFGM was not related to the phosphorylation system described by West and Clegg for rat mammary gland Golgi vesicles.[23] Calcium (5 mmol/L) could not substitute for Mg^{2+} and Mn^{2+} in the phosphorylation reaction within the MFGM (*Figure 1A, lane 2*); thus, indicating that the Ca^{2+}/Mg^{2+}-dependent casein kinase, normally associated with the Golgi apparatus of the lactating mammary gland cell,[16] is not present in the MFGM and is not involved in protein phosphorylation.

Protein phosphorylation in the MFGM is not a result of loosely bound protein kinases

The MFGM, treated with RIPA-buffer containing 1% deoxycholate and 0.1% SDS or with 1% Triton X-100 − 50 mmol/L DTT solution, continued to be labeled with [γ-^{32}P]ATP to the extent comparable to non-treated MFGM (*Figure 6, lanes 2* and *3* versus *Figure 1A, lane 1*). Radiosignals of the 32 to 35 kDa protein bands [caseins,[24]

Figure 2A] in RIPA-treated MFGM (*Figure 6, lane 2*) were significantly diminished relative to those from non-treated MFGM, as a result of the partial removal of caseins by detergents.

Effects of protein kinase inhibitors

Genistein is a specific inhibitor of protein tyrosine kinase (PTK).[26] Genistein (100 μmol/L) caused a 50 to 70% inhibition of protein phosphorylation in the bovine MFGM (*Figure 1A, lane 3*). The inhibitor had a similar effect on phosphorylation of proteins in the human MFGM. We also found that genistein strongly inhibited protein phosphorylation of the bovine MFGM proteins solubilized with detergents or 100 mmol/L DTT, at pH 8, in 50 mmol/L imidazole buffer. We suggest that PTK and presumably other types of protein kinases, which require genistein-sensitive phosphorylation of tyrosine residues for their activity, play key roles in protein phosphorylation within the MFGM. There is an indication that genistein can affect not only PTKs, but other protein kinases as well.[27]

Daidzein is an inactive analog of genistein, but an inhibitor of protein (casein) kinase CKII activity.[28] Protein phosphorylation in both human and bovine MFGM was unaffected by treatment with 100 μmol/L daidzein. Additional evidence that CKII is not present in the MFGM was obtained in experiments using heparin as an inhibitor of CKII. Heparin strongly inhibits this kinase at a concentration of 1.4 nmol/L.[25] We did not observe any change of the MFGM protein phosphorylation pattern (including 32 to 35 kDa proteins, i.e., caseins) in the presence of up to 1 μmol/L heparin.

Identification of the phosphorylated MFGM proteins and radiolabeled phosphoamino acids

Our work represents the first attempt to precisely identify proteins of the MFGM that undergo in vitro phosphorylation after incubation with ATP. Comparison of the in vitro phosphorylated protein pattern of bovine MFGM (*Figure 1*) with the Coomassie blue staining pattern of proteins of the same MFGM (*Figure 2A*) showed that the ^{32}P-labeled 66 kDa protein corresponded to one of the major glycoproteins of MFGM known as Bph.[8] Western immunoblotting analysis, in conjunction with autoradiography (*Figure 3A, B*), supported our suggestion that the labeled 66 kDa protein of the bovine MFGM was in fact Bph. A similar result was obtained with a human MFGM sample.

Microsequencing amino acid analysis (up to 20 cycles) of the bovine 66 kDa protein, corresponding to the ^{32}P-labeled 66 kDa band, provided unequivocal evidence that the ^{32}P-labeled 66 kDa protein was Bph. Finally, a radiolabeled 66 kDa protein was detected by specific immunoprecipitation of supernatant samples, obtained from the ^{32}P-labeled bovine MFGM (*Figure 4A, B, lane 1*).

The in vitro ^{32}P-labeled 15 kDa protein of the bovine MFGM was identified as FABP. *Figure 3* demonstrates that the 15 kDa radioactive protein band, in *panel D*, exactly corresponds to the 15 kDa protein (*panel C*) that positively reacted with anti-FABP IgG (see also *Figure 2*). Amino acid

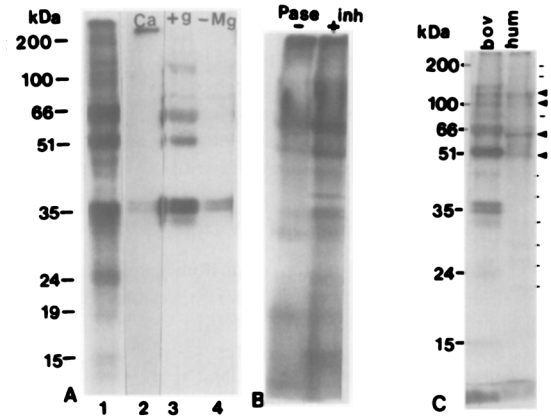

Figure 1 In vitro phosphorylation of MFGM proteins. Bovine and human MFGM (40 μg in each case) were incubated with protein kinase media and [γ-^{32}P]ATP, and processed for 12% SDS/PAGE and autoradiography. *A*, *Lane 1*, bovine MFGM with 3 mmol/L Mn^{2+} and 5 mmol/L Mg^{2+}; *Lane 2*, Bovine MFGM with 5 mmol/L Ca^{2+}, no Mg^{2+} or Mn^{2+}, EDTA was omitted from kinase media; *Lane 3*, Bovine MFGM with the addition of 100 μmol/L genistein (+g); Lane 4, MFGM with no Mg^{2+} and Mn^{2+}. *B*, Bovine MFGM with (+) and without (−) phosphatase inhibitors. *C*, Bovine MFGM versus human MFGM; major and minor bands of human MFGM are marked by arrows and lines. The MWr's, in kDa, of major radiolabeled protein bands are indicated to the left of the figures. Protein standards were prestained SDS/PAGE standards (Bio-Rad Lab.).

sequencing analysis of the 15 kDa protein was not performed, because this analysis was performed previously.[40]

Phosphoamino acid analysis of two of the most prominent radiolabeled polypeptides (66 kDa or Bph and 51 kDa) confirmed that the observed radiolabeling of MFGM proteins was a result of protein phosphorylation (kinase) activity within the MFGM (*Figure 5*). (^{32}P)-tyrosine, -threonine and -serine were detected in the 66 kDa protein or Bph. (^{32}P)-tyrosine and -threonine were detected in the

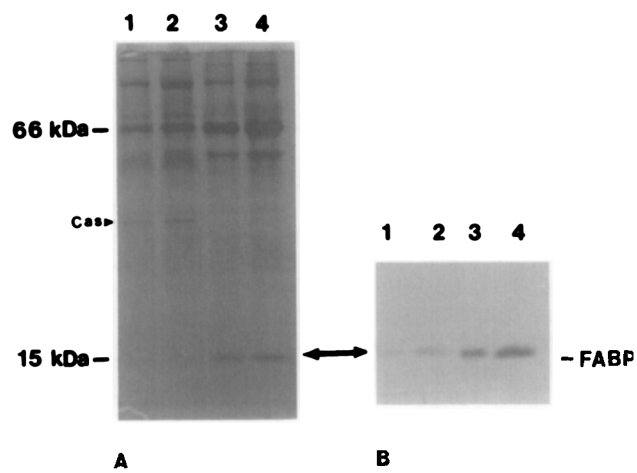

Figure 2 SDS/PAGE (12%) and Western immunoblotting with anti-FABP of the detergent-solubilized and non-solubilized proteins of bovine MFGM. *A*, The gel stained with Coomassie blue R-250. *B*, The Western immunoblot. "Cas" denotes the electrophoretic position of casein. *Lanes 1* and *2*, 100,000 g-supernatant obtained after treatment of bovine MFGM with 1% Triton X-100 + 10 mmol/L β-mercaptoethanol. *Lanes 3* and *4*, 100,000 g pellets of non-soluble MFGM proteins. In *lanes 1* and *3*, 10 μg of protein/lane was applied. In *lanes 2* and 4, 20 μg of protein/lane was applied.

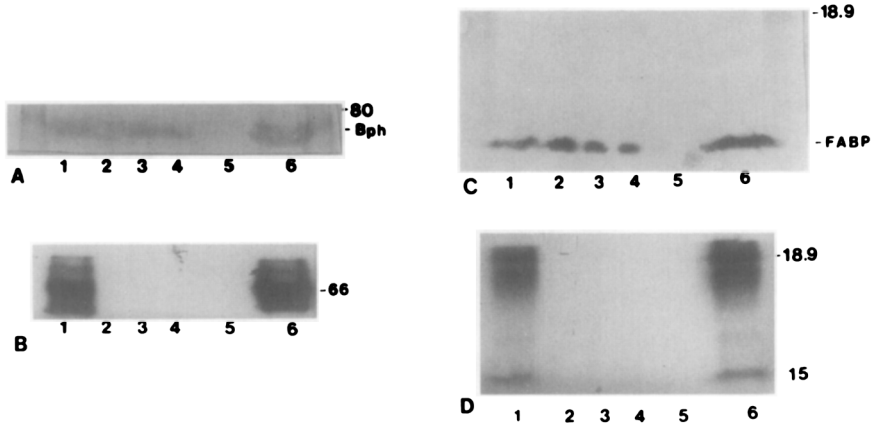

Figure 3 Identification of 66–67 kDa and 15 kDa proteins of bovine MFGM as Bph and FABP, respectively by Western immunoblotting and autoradiography. *A*, Detection of Bph by Western immunoblotting with rabbit polyclonal anti-C-terminal peptide of Bph. *B*, Autoradiograph of the A-blot. *C*, Detection of FABP by Western immunoblotting with rabbit polyclonal antibody to bovine FABP. *D*, Autoradiograph of the C-blot. *Lane 1*, 40 μg of ^{32}P-MFGM; *lane 2*, 20 μg of non-labeled MFGM; *lane 3*, 10 μg of non-labeled MFGM; *lane 4*, 5 μg of non-labelled MFGM; *lane 5*, none; *lane 6*, 60 μg of ^{32}P-MFGM. The MFGM labeling was done with 10 μCi of [γ-^{32}P]ATP, 5 mmol/L Mg^{2+}, and 3 mmol/L Mn^{2+}/50 μg of protein. Goat anti-rabbit IgG-HRPO served as secondary antibody. The MWr's were obtained from a calibration curve made with the prestained protein broad range standards (Bio-Rad Lab).

51 kDa protein. A similar analysis was performed for the 15 kDa protein or FABP. Labeling of MFGM in this case, however, was carried out with a higher concentration of radioactive ATP (10 μCi/50 μg of protein). This analysis revealed a radioactive phosphotyrosine spot in its acid hydrolyzate (*Figure 5, panel* "15 kDa").

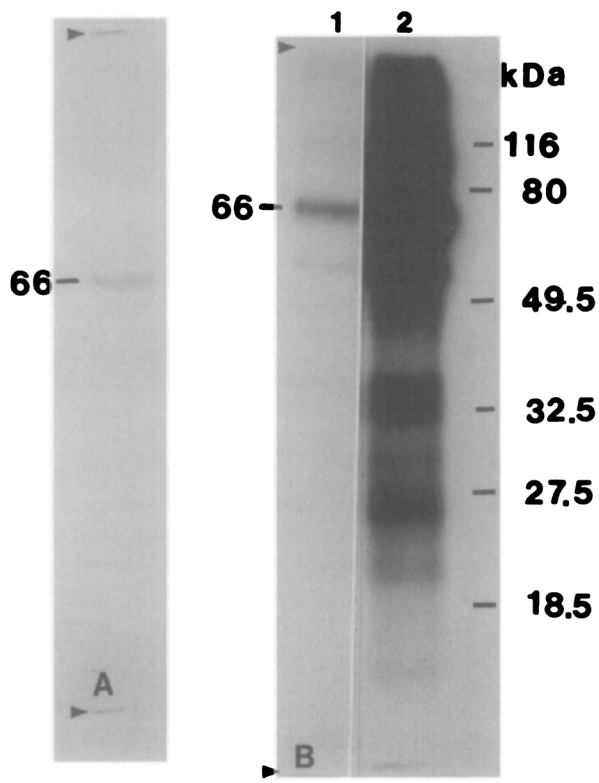

Figure 4 Autoradiograms of SDS/PAGE immunoprecipitate of [^{32}P]-labeled Bph. *A*, *Lane 1*, Immunoprecipitate from RIPA-buffer extract of [^{32}P]-labeled MFGM was analyzed in 10% gel. *B*, *Lane 1*, Immunoprecipitate from 1% Triton X-100 + 50 mmol/L DTT-extract of [^{32}P]-labeled MFGM was analyzed in 12% gel; *Lane 2*, [^{32}P]-labeled MFGM in 12% gel. The MWr's of low range prestained SDS/PAGE standards (Bio-Rad Lab) are shown to the right of the figure. The autoradiogram (*A*) was obtained after 2 days. Autoradiogram (*B*) was obtained after 5 days. *Arrows* indicate upper and lower edges of the separating gel.

Phospholabeled 15 kDa protein as a protein/kinase complex

An attempt to detect the labeled 15 kDa protein (FABP) using 2D gel electrophoresis of MFGM, labeled as indicated in Methods and materials, i.e., with 2 μCi [γ-^{32}P]ATP/assay, was not successful. However, Bph species were clearly seen (Mather and Spitsberg, unpublished observation). The absence of the radiosignal in the 15 kDa zone could be explained by insufficient labeling of this protein and/or a limited solubilization of radiolabeled membrane proteins in the solubilizing solution, i.e., in 8 M urea-containing the solution used for isoelectrofocusing. Therefore, we examined the solubilization characteristics of the MFGM proteins with an emphasis on those labeled in kinase media. Treatment of ^{32}P-labeled bovine MFGM with 1% Triton X-100 + 50 mmol/L DTT or 10 mmol/L β-mercaptoethanol or with RIPA-buffer led only to partial solubilization of radiolabeled MFGM proteins. Despite the solubilization of ~50% of the total proteins, radiolabeled 15 kDa protein (or FABP) was still detected in the residual MFGM along with other proteins (*Figure 6, lane 4*). As well, the 15 kDa protein was clearly phosphorylated in both detergent-treated samples of MFGM (*Figure 6, lanes 1–3*). These findings indicated that the detergents did not completely remove the 15 kDa protein from MFGM and did not alter the relationship between this protein and its putative kinase. It appeared that other MFGM proteins besides 15 kDa protein (or FABP) also form tight association (complexes) with their kinases and are not well solubilized by detergents.

Milk fat globule membranes and cellular signal transduction pathway components

The commercial availability of a large number of highly affinity-purified antibodies allowed us to partially identify some of the MFGM protein kinases using Western immunoblotting. This analysis demonstrated that MFGM may contain a number of membrane proteins related to signal transduction pathways, i.e., FAK, c-src[60], MAPK, β-insulin receptor subunit, β-actin, caveolin, thrombospondin, and MAPKAPK (*Figure 7* and *Table 1*). The specificity of

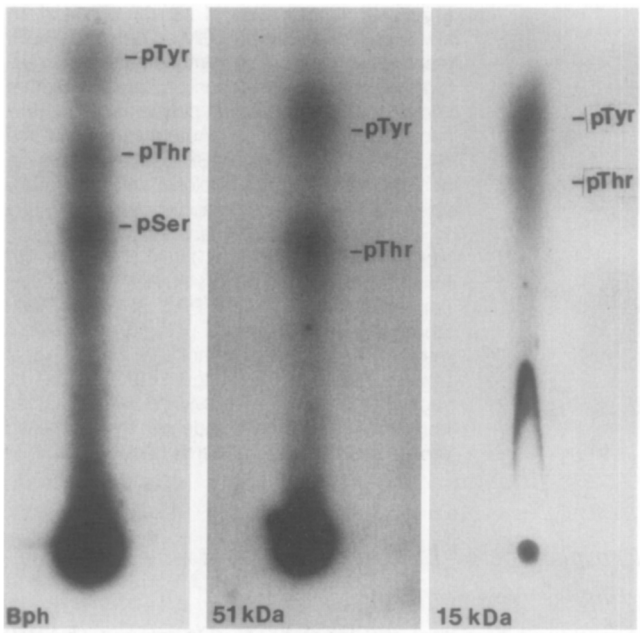

Figure 5 Identification of [^{32}P]-labeled amino acids of Bph, 51 and 15 kDa proteins by TLC. Analysis of Bph and the 51 and 15 kDa proteins was performed in three independent TLC experiments. pTyr-phosphotyrosine, pThr-phosphothreonine, pSer-phosphoserine.

Western immunoblotting was confirmed by the use of non-immune IgG fractions in control experiments. The appearance of protein kinases on immunoblots as thin bands indicated that these kinases can be considered as minor components of the MFGM proteins. However, they may be partially responsible for the protein phosphorylation pattern of the MFGM.

Discussion

In this work we demonstrated for the first time that the MFGM from both bovine and human possesses intrinsic protein phosphorylation activity, i.e., incubation of MFGM with ATP and Mg^{+2} or Mn^{+2} leads to phosphorylation of a number of proteins of this membrane. Our goal was to determine whether the observed phosphorylation activity of MFGM is a result of the presence in MFGM of protein kinases as its integral proteins, or this activity is a result of the presence in the prepared MFGM of kinases as contaminating cellular proteins.

Because of complexity in the formation of the MFGM,[1] the proteins of the isolated MFGM are not solely the proteins specific to apical plasmalemma of mammary gland secretory epithelial cells, but also the proteins derived from the secretory vesicles, as well as the cytoplasmic proteins occasionally entrained into secreted fat globules. In this work we showed that the observed in vitro phosphorylation of the proteins within MFGM can not be explained by the presence of loosely bound "contaminating cytoplasmic kinases." Namely, the detergent-treated ("washed") MFGM had a protein phosphorylation pattern quite similar to that of the untreated MFGM. Moreover, the potentially contaminating kinases, such as protein kinase C[38,39] and protein (casein) kinase II,[25,28] in the MFGM were not detected in

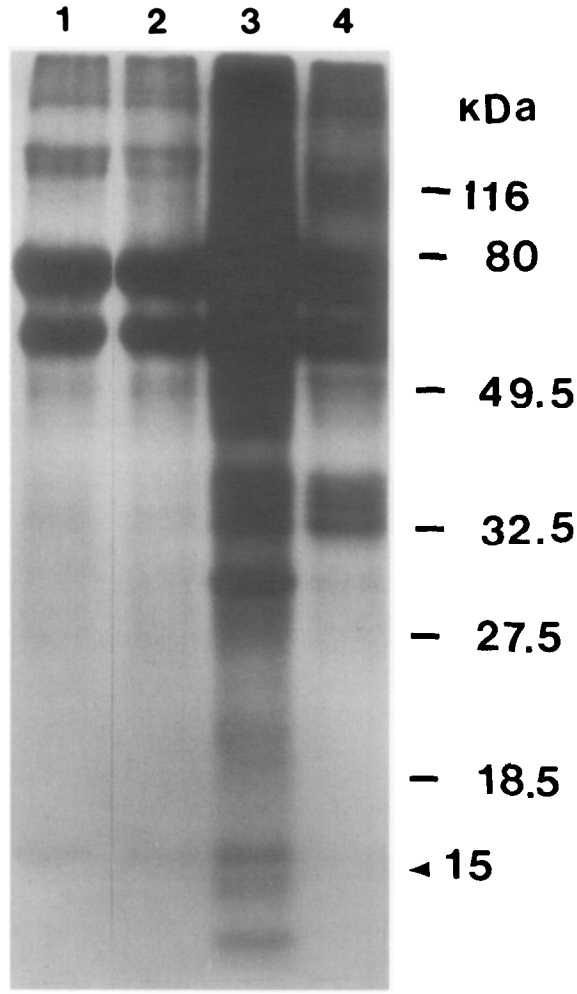

Figure 6 Protein phosphorylation pattern of detergent treated bovine MFGM. *Lane 1*, MFGM (50 μg) incubated in RIPA-buffer for 15 min with subsequent labeling with [γ-^{32}P]ATP; *lane 2*, in vitro ^{32}P-labeled 100,000 g-pellet (50 μg), obtained from MFGM treated with RIPA-buffer; *lane 3*, in vitro ^{32}P-labeled 100,000 g-pellet obtained from treated with 1% Triton X-100 + 50 mmol/L DTT MFGM; *lane 4*, 100,000 g-pellet obtained from ^{32}P-labeled treated with RIPA-buffer MFGM.

our preparation of MFGM. In vitro protein phosphorylation of MFGM cannot be also explained by the presence in this preparation of the specific protein kinases characteristic to mammary gland Golgi-apparatus.[16,23] We showed that the protein phosphorylation of the MFGM was not supported by Ca^{+2} and did not occur unless Mg^{+2} or Mn^{+2} were added. These findings clearly demonstrated that the Ca^{+2}/Mg^{+2}-dependent kinase of mammary gland Golgi-apparatus,[16] or protein phosphorylation system of mammary gland Golgi vesicles, described by West and Clegg,[23] were not present in the preparations of MFGM used in our study. According to West and Clegg[23] protein phosphorylation in mammary gland Golgi vesicles proceeds without addition of bivalent cations (Ca^{2+} or Mg^{2+}), because an endogenous pool of these cations, present in these vesicles at very low concentrations, was quite sufficient to support protein phosphorylation. West and Clegg[23] also showed that the addition of 100 μmol/L Ca^{2+} or Mg^{2+}, to the kinase media caused a noticeable inhibition of autophosphorylation in Golgi vesi-

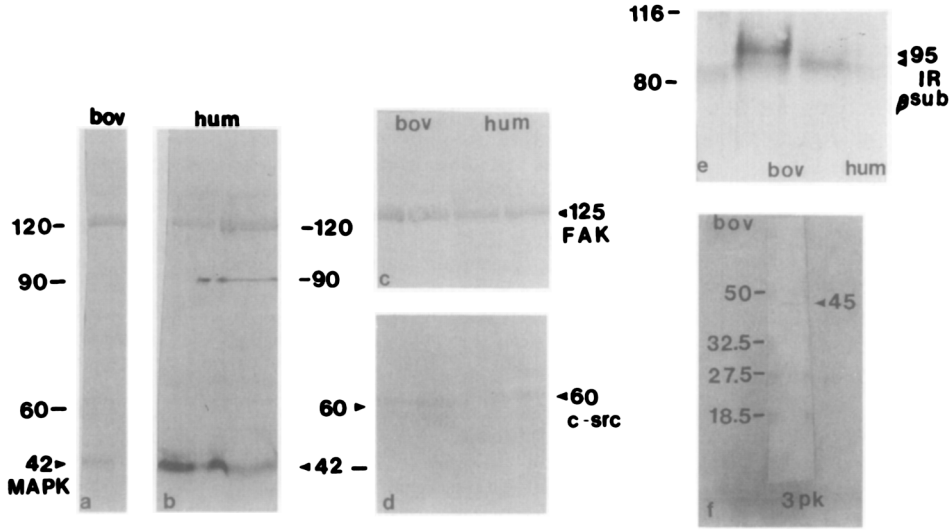

Figure 7 Signal transduction proteins of bovine and human MFGM detected by Western immunoblotting. The antibodies used were against: MAPK in (*a*) and (*b*), FAK in (*c*), c-src[60] in (*d*), insulin β-subunit receptor in (e); "3-pk" or MAPKAPK in (*f*). Approximately 100 μg of protein/lane was applied.

cles. Our work, therefore, shows that the in vitro phosphorylation of MFGM proteins is more likely attributable to protein kinases different from the aforesaid kinases and representing integral membrane proteins.

Two in vitro phosphorylated proteins were identified in the MFGM preparations. These were FABP (15 kDa) and Bph (66–67 kDa). The fact that the 15 kDa bovine protein FABP, also known as MDGI,[10] was phosphorylated after incubation of MFGM with ATP and Mg^{2+}/Mn^{2+} indicated that this protein is associated with its kinase in the MFGM. It is highly probable that a similar association of FABP takes place with its kinase in mammary gland cells. This would explain the detection of the phosphorylated form of FABP in primary epithelial cells obtained from the bovine mammary gland.[41] It is likely that the phosphorylating FABP kinase is the β-subunit of the insulin receptor. The presence of the β-subunit of the insulin receptor has been shown by Western immunoblotting (*Table 1*); insulin binding sites have been previously detected in bovine MFGM.[15] There is also evidence that FABP [or 422(aP2)] is phosphorylated in 3T3-L1 adipocytes by the insulin receptor and that this phosphorylation takes place at tyrosine-19.[42] We clearly demonstrated the phosphorylation of a tyrosine residue of MFGM-derived FABP. However, the exact phosphorylation site of bovine MFGM-derived FABP still has to be determined.

Table 1 Positively detected protein kinases and other membrane-associated proteins in bovine and human MFGM by Western immunoblotting

Protein	MW, kDa*	Bovine	Human
FAK[29]	125	+	+
Insulin receptor β-subunit[30]	95	+	+
c-src[60 31]	55	+	+
MAPKAP kinase[32]	45	+	nd
MAPK[33]	42	+	+
β-Actin[4,34]	56bov or 46hum	+	+
Thrombospondin[14,35]	120–130	+	+
CD36[6]	80	+	+
Caveolin[36,37]	27	+	nd
FABP[10,14]	15	+	+
PKC[38,39]	84	none	slightly positive

*The numbers indicate the MW of the proteins detected in this work by Western immunoblotting; nd = not determined.

If FABP phosphorylation can be interpreted as resulting from the association of FABP with kinase, the mechanism whereby Bph is phosphorylated is unclear. Bph was shown to be phosphorylated on serine, threonine, and tyrosine residues. This shows that such phosphorylation can take place as a result of one or more dual-specificity kinases tightly associated with Bph.[43] Whether Bph is phosphorylated by specific kinase(s) or Bph represents a new form of kinase is a question that needs to be answered if we are to properly understand the function of Bph in mammary gland secretory epithelial cells.

The strong inhibition (up to 50 to 70%) of ^{32}P-labeling of bovine MFGM proteins, by genistein, makes it apparent that the protein tyrosine kinase(s) plays a key role in the protein phosphorylation within MFGM. The residual protein phosphorylation that was resistant to genistein can be explained either by the inability of genistein to completely inhibit the activity of protein kinases,[26] or by the preexistence in MFGM of already activated forms of protein kinases.

The whole scenario of protein phosphorylation in the MFGM can be presented as a result of *cis-* and *trans-*activation of protein kinases in these membranes, with subsequent phosphorylation of their substrates. Detection of proteins related to protein tyrosine kinases, like FAK, the β-insulin receptor subunit, c-src[60], and the Ser/Thr kinases like MAPK and MAPKAPK, fits a general pattern of in vitro protein phosphorylation within the MFGM. However, one alternative explanation for the noticeable pleiotropic effect of genistein on MFGM protein phosphorylation is suggested by the presence of the putative genistein-sensitive protein kinase as the most predominant kinase, one presumably associated with many other MFGM proteins.

The findings presented here provide evidence that the MFGM can serve as a suitable source for biochemical studies on the relationships between certain kinases and

their substrates. The knowledge gained from such studies should lead to a better understanding of the role of kinases in mammary cell proliferation, differentiation, metabolism as well as in milk fat secretion. Along these lines, the role of protein kinases in lipid secretion by mammary epithelial cells was recently demonstrated.[44]

Acknowledgments

We thank Dr. Ian H. Mather for his help with the 2D electrophoresis experiment and providing antibutyrophilin antibody, Dr. H. Farrell for providing valuable information regarding caseins in the MFGM, and Dr. E. Matitashvili and Mr. S. Satti for their assistance with some of the experiments. This work was supported by grants from the USDA, New York Agricultural Experiment Station, and Dairy Management, Inc.

References

1 Kanno, C. (1990). Secretory membranes of the lactating mammary gland. *Protoplasma* **154,** 184–208

2 Kanno, C. (1991). Physicochemical properties of milk fat emulsions stabilized with bovine milk fat globule membrane. *J. Food Sci.* **56,** 1219–1223.

3 Bucheim, W., Weslch, U., and Patton, S. (1988). Electron microscopy and carbohydrate histochemistry of the human milk fat globule membrane. In: *Biology of Human Milk.* (L.A. Hanson, ed.) vol. 15, p. 27–44, Raven Press, New York, NY USA

4 Keenan, T.W., Frendenstein, C.R., and Franke, W.W. (1977). Membranes of mammary gland. XIII. A lipoprotein complex derived from bovine milk fat globule membrane with some preparative characteristics resembling those of actin. *Cytobiologie* **14,** 259–278

5 Stubbs, J.D., Lekutis, C., Singer, K.L., Bui, A., Yuzuki, D., Srinivasan, U., and Parry, G. (1990). cDNA cloning of a mouse mammary epithelial cell surface protein reveals the existence of epidermal growth factor-like domains linked to factor VIII-like sequence. *Proc. Natl. Acad. Sci. USA* **87,** 8417–8421

6 Greenwalt, D.E., Lipsky, R.H., Ockenhouse, C.F., Ikeda, H., Tandon, N.N., and Jamieson, C.A. (1992). Membrane glycoprotein CD36: A review of its role in adherence, signal transduction, and transfusion medicine. *Blood* **80,** 1105–1115

7 Wreschner, D.H., Hareuveni, M., Tsafoty, I., Smorodinsky, N., Horev, J., Zaretsky, J., Kotkes, P., Weiss, M., Latke, R., Dion, A., and Kyedar, J. (1990). Human epithelial tumor antigen cDNA sequences. Differential splicing may generate multiple protein forms. *Eur. J. Biochem.* **189,** 463–473

8 Mather, I.H. and Jack, L.J.W. (1993). A review of the molecular and cellular biology of butyrophilin, the major protein of bovine milk fat globule membrane. *J. Dairy Sci.* **76,** 3832–3850

9 Jack, L.J.W. and Mather, I.H. (1990). Cloning and analysis of cDNA encoding bovine butyrophilin, an apical glycoprotein expressed in mammary tissue and secreted in association with the milk-fat-globule membrane during lactation. *J. Biol. Chem.* **265,** 14481–14486

10 Grosse, R. and Langen, P. (1990). Mammary-derived growth inhibitor. In *Handbook of Experimental Pharmacology* 95/II. (Sporn, M. and Roberts, A., eds.) p. 249–265, Springer Verlag, Heidelberg

11 Mather, I.H. (1987). Proteins of the milk-fat-globule membrane as markers of mammary epithelial cell and apical plasma membrane. In *The Mammary Gland: Development, Regulation and Function* (Neville, M.C. and Daniel, C.W., ed.) p. 217–267, Plenum Publishing Corp., New York, NY USA

12 Ghosal, D., Ankrapp, D., and Keenan, Th.W. (1993). Low molecular mass GTP-binding proteins are secreted from mammary epithelial cells in association with lipid globules. *Biochim. Biophys. Acta* **1168,** 299–306

13 Mather, I.H., Banghart, L.R., and Lane, W.S. (1993). The major fat-globule membrane proteins, bovine components 15/16 and guinea-pig, are homologous to MFG-E8, a murine glycoprotein containing epidermal growth factor-like and factor V/VIII-like sequences. *Biochem. Mol. Biol. Intern.* **29,** 545–554

14 Spitsberg, V.L., Matitashvili, E., and Gorewit, R.C. (1995). Association and coexpression of fatty-acid-binding protein and glycoprotein CD36 in the bovine mammary gland. *Eur. J. Biochem.* **230,** 872–878

15 Smith, D.H., Roehrig, K.L., Palmquist, D.L., and Schanbacher, F.L. (1987). Effect of dietary fat and lactation statue on insulin binding to bovine milk fat globule membranes. *J. Dairy Sci.* **70,** 1537–1562

16 Bingham, E.W. and Farrell, H.M. (1974). Casein kinase from the Golgi apparatus of lactating mammary gland. *J. Biol. Chem.* **249,** 3647–3651

17 Huang, M.M., Bolen, J.B., Bernwell, J.W., Shattil, S.J., and Brugge, J.S. (1991). Membrane glycoprotein IV (CD36) is physically associated with the Fyn, Lyn, and Yes protein-tyrosine kinases in human platelets. *Proc. Natl. Acad. Sci. USA* **88,** 7844–7848

18 Moodie, S., Willumsen, B.M., Weber, M.J., and Wolfman, A. (1993). Complexes of Ras.GTP with Raf-1 and mitogen-activated protein kinase. *Science* **260,** 1658–1661

19 Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 5350–5354

20 Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72,** 248–254

21 Kamps, M.P. and Sefton, B.M. (1989). Acid and base hydrolysis of phosphoproteins bound to immobilon facilitates analysis of phosphoamino acids in gel-fractionated proteins. *Anal. Biochem.* **176,** 22–27

22 Munoz, G. and Marshall, S.H. (1990). An alternative method for a fast separation of phosphotyrosine. *Anal. Biochem.* **177,** 233–237

23 West, D.W. and Clegg, R.A. (1982). Transient formation of a phosphoprotein during autophosphorylation of rat mammary gland Golgi vesicles. *Biophys. Acta* **690,** 290–295

24 Basch, J.J., Greenberg, R., and Farrell, H.M., Jr. (1985). Identification of the milk fat globule membrane proteins. II. Isolation of major proteins from electrophoretic gels and comparison of their amino acid compositions. *Biochim. Biophys. Acta* **830,** 127–135

25 Hathaway, G. and Truagh, J.A. (1982). Caseins kinases-multipotential protein kinases. *Curr. Top. Cell. Regul.* **21,** 101–127

26 Akiyama, T., Ishida, J., Nakagawa, S., and Fukami, Y. (1987). Genistein, a specific inhibitor of tyrosine-specific kinases. *J. Biol. Chem.* **262,** 5592–5595

27 Constantinou, A. and Huberman, E. (1995). Genistein as an inducer of tumor cell differentiation: Possible mechanisms of action. *Proc. Soc. Exp. Biol. Med.* **208,** 109–115

28 Higashi, K. and Ogawara, H. (1994). Diadzein inhibits insulin- or insulin-like growth factor-1-mediated signaling in cell cycle progression of Swiss 3T3 cells. *Biochim. Biophys. Acta* **1221,** 29–35

29 Shaller, M.D., Borgman, C.A., Cobb, B.S., Vines, R.R., Reynolds, A.B., and Parsons, J.T. (1992). ppFAK125, a structurally distinctive protein-tyrosine kinase associated with focal adhesions. *Proc. Natl. Acad. Sci. USA* **89,** 5192–5196

30 Kahn, C.R., White, M.F., Shoelson, S.E., and Kahn, S.A. (1993). The insulin receptor and its substrate. Molecular determinants of early events in insulin action. In: *Recent Progress in Hormone Research.* **48,** 291–339

31 Bolen, T.B., Rowly, R.B., Spana, C., and Tsyganov, A. (1992). The src family of tyrosine kinases in hemopoietic signal transduction. *FASEB J.* **6,** 3403–3409

32 Sithanandam, G., Latif, F., Duh, F.M., and Allikmets, R. (1996). 3pk, A new map kinase activated protein kinase, located in the small cell lung cancer tumor suppressor gene region. *Mol. Cell. Biol.* **16,** 868–876

33 Davis, R.J. (1993). The mitogen-activated protein kinase signal transduction pathways. *J. Biol. Chem.* **268,** 14535–14556

34 Janmey, P.A. and Chaponnier, C. (1995). Medical aspects of the actin cytoskeleton. *Curr. Opion. Cell Biol.* **7,** 111–117

35 Dawes, J., Clezardin, P., and Pratt, D.A. (1987). Thrombospondin in milk, other breast secretion, and breast tissue. *Semin. Thromb. Hemostas.* **143,** 378–384

36 Glenny, J.R. (1992). The sequence of human caveolin reveals

identity with VIP21, a component of transport vesicles. *FEBS Lett.* **314,** 45–48

37 Lisanti, M.P., Sargiacomo, M., and Scherer, P.E. (1995). Caveolin, a principal component of caveolae membranes. Transduction Laboratories Insights **1,** 1–2

38 Kolch, W., Heldecker, G., Kochs, G., Hummel, R., Vahidl, H., Mischak, H., Finkenzeller, G., Marme, D., and Rapp, U.R. (1993). Protein kinase C activates RAF-1 by direct phosphorylation. *Nature* **364,** 249–252

39 Coussens, L., Parker, P.T., Rhee, L., Yang-Feng, T.L., Chen, E., Waterfield, M.D., Francke, U., and Ulrich, A. (1986). Multiple, distinct forms of bovine and human protein kinases C suggest diversity in cellular signalling. Pathways. *Science* **233,** 859–866

40 Brandt, R., Peppezle, M., Otto, A., Kraft, R., Boehmer, F.O., and Grosse, R. (1988). A 13-kilodalton protein purified from milk fat globule membrane is closely related to a mammary-derived growth inhibitor. *Biochemistry* **27,** 1420–1425

41 Spitsberg, V.L., Matitashvili, E., and Gorewit, R.C. (1994). Bovine mammary cells contain a low molecular weight phosphorylated protein that is immunoreactive to MDGI/FABP. *J. Anim. Sci.* **72** (**Suppl. 1**), 75

42 Hresko, R.C., Hoffman, R.D., Flores-Riveros, J.R., and Lane, M.D. (1990). Insulin receptor tyrosine kinase-catalyzed phosphorylation of 422-AP2 protein substrate activation by long-chain fatty acid. *J. Biol. Chem.* **265,** 21075–21085

43 Spitsberg, V.L. and Gorewit, R.C. (1996). Is the MFGM associated protein butyrophilin (Bph) a kinase? The FASEB J. **10,** A684, 3946.

44 Rohlfs, E.M., Louie, D.S., and Zeisel, S.H. (1993). Lipid synthesis and secretion by primary cultures of rat mammary epithelial cells. *J. Cell. Physiol.* **157,** 469–480