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EXPRESSION OF FATTY ACID-BINDING PROTEINS IN THE DEVELOPING MOUSE MAMMARY GLAND

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Proteins of 14,000 daltons (SLP-14) were isolated and partially characterized from mouse mammary glands of different developmental stages. The purified proteins were partially sequenced at the amino acid level. The SLP-14 belong to the family of fatty acid-binding (FABP) proteins. The major SLP-14 expressed was a reflection of the differentiation stage of the mammary gland. Within the regions sequenced for each protein, virgin mouse mammary gland (primarily adipocytes) expressed a protein 100% homologous to adipocyte lipid binding protein whereas pregnant and lactating mouse mammary glands expressed a protein highly homologous to mouse heart fatty acid binding protein. In the lactating gland, the isolated protein was 97% identical to the heart FABP over a 98 amino acid stretch. We could not detect the 14Kd protein identified as mammary gland growth inhibitor (MDGI) which is also a member of the FABP family and highly homologous to heart FABP. A rabbit antiserum to the rat mammary SLP-14 recognized the SLP-14 proteins in mouse and rat mammary gland, skeletal muscle and heart, whereas it failed to recognize the SLP-14 in liver, intestine and other organs. These data indicate that the SLP-14 detected in rodent mammary gland is of the fatty acid-binding protein family. On the basis of amino acid sequence, the major form in the differentiated mouse mammary gland is apparently FABP and not MDGI. MDGI may be a protein in low abundance and/or localized to a specific group of mammary epithelial cells.

Recently, we isolated and partially characterized several liver proteins (SLP) on the basis of their ability to retain selenium through electrophoresis (1). One protein, SLP-14, appears to be fatty acid-binding protein (FABP) (2). FABP belongs to a family of proteins which include growth regulatory proteins in bovine mammary gland (mammary-derived growth inhibitor, MDGI) (3) and in fibroblasts (fibroblast growth regulator, FGR) (4). Additionally, Sorof and co-workers (5, 6) have shown that liver FABP concentration is increased in mitotic hepatocytes and in preneoplastic liver nodules. Thus, it appears that these proteins, termed FABP, may have several functions besides binding fatty acids and metabolites of arachidonic acid. Interestingly, the liver SLP-14 does not contain an in-frame TGA codon, which is specific for selenocysteine,

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the selenoamino acid in the selenoproteins, glutathione peroxidase (GSH-Px) and type 1 iodothyronine deiodinase (7).

The antibodies to liver SLP-14 (FABP) are tissue-specific and do not recognize a mammary gland SLP-14 (8). Since prior experiments had demonstrated that mammary gland contains a 14Kd protein which labels with ^{75}Se, we undertook the isolation and partial characterization of this protein. The rodent mammary gland theoretically could contain two other closely related proteins, MDGI and adipocyte lipid binding protein (ALBP), thus we determined the amino acid sequence of proteinase-generated polypeptides from the proteins isolated from virgin, pregnant and lactating mammary glands. The virgin mammary gland is comprised predominately of adipocytes in contrast to the midpregnant and lactating gland where the predominant cellular components are the functional epithelial cells.

MATERIALS AND METHODS

Introduction: In these experiments, ^{75}Se cpm was used as a marker to localize the fraction which contained the 14,000 dalton proteins. Although it appears that selenium labels proteins of this molecular weight which are FABP's (2), only a small percentage (<.01%) of the purified 14Kd protein retains the selenium moiety. The peptide sequencing was performed on 2 completely separate isolations of each of the 14Kd proteins from virgin and lactating mammary glands.

Protein isolation: Five each of virgin, pregnant and lactating BALB/c mice were injected i.p. with 50 μCi of ^{75}Se as Na_2SeO_3 (sp. act. 500 mCi/mg Se) twice at a two day interval. After 40 hrs of the second injection, the animals were anesthetized with ether, exsanguinated by cardiac puncture and processed as described in (1,2). A 30% homogenate of pooled mammary glands was prepared in 20 mM Tris acetate buffer, pH 7.4, centrifuged at 10,000g and then at 100,000g. Cytosol proteins were fractioned sequentially on Sephadex G-150, DEAE-Sephadex A50 and oleate affinity columns as described in (2). The major peaks containing ^{75}Se activity were pooled and analyzed by 1D PAGE (2).

Protein analysis: Discontinuous SDS-PAGE was performed as described by Laemmli (9) with minor modifications (1,2). Fifteen percent separating gels were allowed to polymerize for 16 h. The protein concentration in the samples was determined by the method of Bradford (10). The samples after DEAE and oleic acid gel fractionation were each further analyzed by 2-dimensional electrophoresis using the procedure of O'Farrell et al. (11) with slight modifications as described in (2).

To prepare samples for amino acid analysis, material isolated from SDS-PAGE gels was digested separately in situ using sequence grade endoproteinase Glu-c (EC 3.4.21.19) and endoproteinase Lys-c (Boehringer Mannheim) essentially as described by Cleveland et al. (12) with few modifications (2). Peptides were blotted onto a polyvinylidine difluoride membrane (Millipore), which was then stained with Coomassie Blue essentially as described by Matsudaria (12). Electroblotting was carried out in a Hoefer transfer electrophoresis unit at 1.5 A for 45 min (Hoefer Scientific Instruments). After transfer, the blots were washed with deionized water, stained with 0.1% Coomassie Blue and then destained. After washing with deionized water, the blots were air dried, and the peptide bands (8 to 12Kd) were cut out and sequenced using an automated pulsed liquid phase sequenator (Applied Biosystems model 477A protein sequenator with an in-line 120-A PTH analyzer) (2). The sequence data were checked for homology to known proteins (GenBank database).

Isolation of SLP-14 from pregnant rat mammary gland, antibody production and characterization: In order to assure that we would obtain sufficient purified protein to immunize rabbits, we

isolated the 14Kd proteins from rat mammary gland. Since the homology between rat and mouse FABP's was greater than 90%, we reasoned that antibodies to rat mammary gland 14Kd protein would easily recognize the homologous mouse protein. Four 10-days pregnant Sprague-Dawley rats were injected with a single injection of 100 μci of ^{75}Se as Na_2SeO_3 (Sp. Act. 100 mCi/mg Se). Purification procedure was the same as described above. Primary immunization of two New Zealand rabbits was initiated with total of 100 μg/rabbit of purified rat mammary gland SLP-14, suspended in Complete Freund's Adjuvant, injected intradermally with 20 μg of antigen per site. Three booster immunizations followed at monthly intervals with 30 μg each of purified protein suspended in Incomplete Freund's Adjuvant. The animals were bled 10 days after each booster injection. The specificities of the antisera were determined by Western immunoblot analysis as described in (2).

RESULTS

Isolation and biochemical characterization of mammary gland SLP-14: The separation of the 14Kd proteins in mammary gland cytosols by chromatography and electrophoresis was followed by ^{75}Se labeling. The separation of lactating mouse mammary gland cytosol on Sephadex G-150 column chromatography is illustrated in Figure 1. The chromatography patterns of the three tissues were virtually identical. Only peak III was further analyzed. Peak II had been shown previously to contain GSH-Px. Peak III contained 15% in VMG, 10% in LMG and 17% in PMG of the ^{75}Se cpm loaded onto the column. The separation of peak III proteins (fractions 55-74) on DEAE-Sephadex is illustrated in Figure 2. Two major ^{75}Se peaks were detected. The leading peak contained 60% in VMG, 61% in LMG and 54% in PMG of the resolved ^{75}Se and, on 1D-

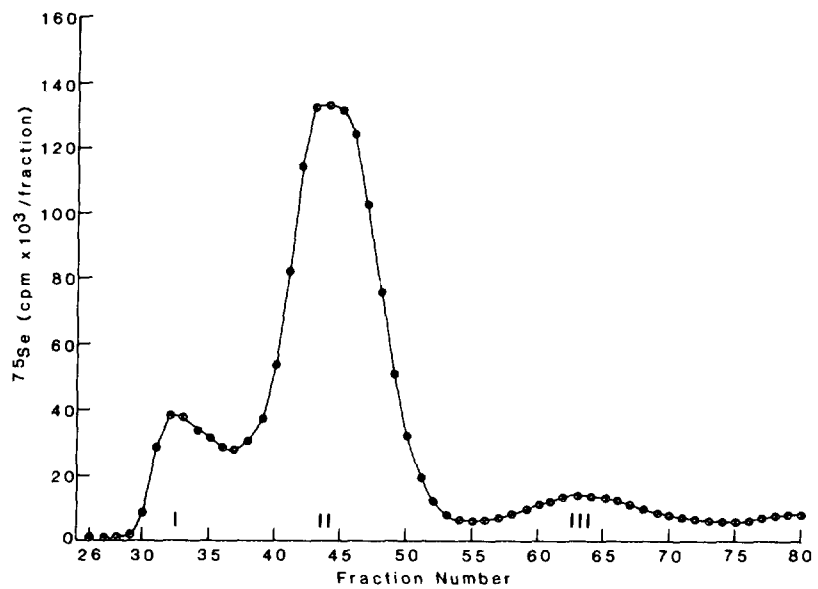

Figure 1. The separation of selenium labeled proteins from lactating mouse mammary gland cytosol by Sephadex G-150 column chromatography. Peak III contained 10% of the ^{75}Se cpm loaded onto the column and was subjected to further fractionation.

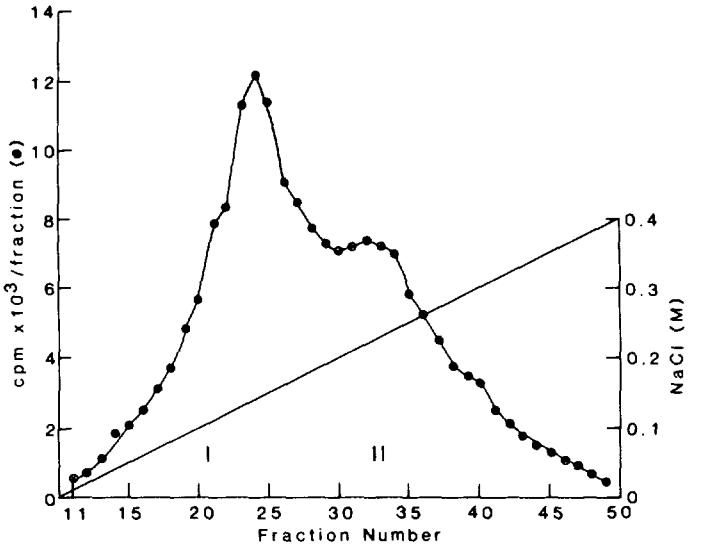

Figure 2. DEAE-Sephadex fractionation of peak III from the Sephadex G-150 column shown in Figure 1. Peaks I and II from this column were analyzed by 1D-PAGE.

PAGE, contained the 14Kd protein band. The second peak of ^{75}Se on 1D-PAGE contained the 26Kd protein band, the same position that the GSH-Px monomer consistently runs on our 1D-PAGE.

The first peak from the DEAE-Sephadex column was run on an oleate affinity column and the bound proteins at 14Kd eluted by 50% alcohol and subsequently by acid alcohol. As in our previous experiments on mouse liver cytosol, the ^{75}Se colocalized with the 14Kd band protein and not with the other protein bands. 1D-SDS-PAGE analysis of the eluted proteins showed intense protein bands at 14Kd (Figure 3). The protein bands in lanes C and F just below the 14Kd band are often present on 1D-PAGE gels of DEAE and oleic acid fractionations. We did not focus on this band for these experiments because previous experiments demonstrated this band to be a truncated (or degraded) version of the 14Kd band (2). The fractions after DEAE chromatography or the oleic acid elution were further analyzed on 2-D PAGE (Figure 4). The patterns of the 14Kd proteins were very similar for the DEAE and the oleic acid column fractions. Figure 4 illustrates the 2D PAGE patterns of the 14Kd band isolated from DEAE fractions. The 14Kd fractions after DEAE and oleic acid always showed three spots at 4.3, 5.2 and 5.6 PIs for the LMG and PMG, whereas the VMG showed a predominant spot at 4.3 pI and a minor spot at 5.2 pI. The spot at 4.3 pI ran at a slightly higher molecular weight (14.4-14.7Kd) in all preparations..

The 14Kd protein bands eluted from the oleic acid columns with 50% alcohol were digested in situ with endoproteinases Glu-c and Lys-c, as described in Material and Methods. The amino acid sequences of the major polypeptide bands from each protein were analyzed on an automated pulsed liquid phase sequenator and the sequences data checked for homology to known proteins on Gen Bank database. The amino acid sequences are illustrated in Table 1.

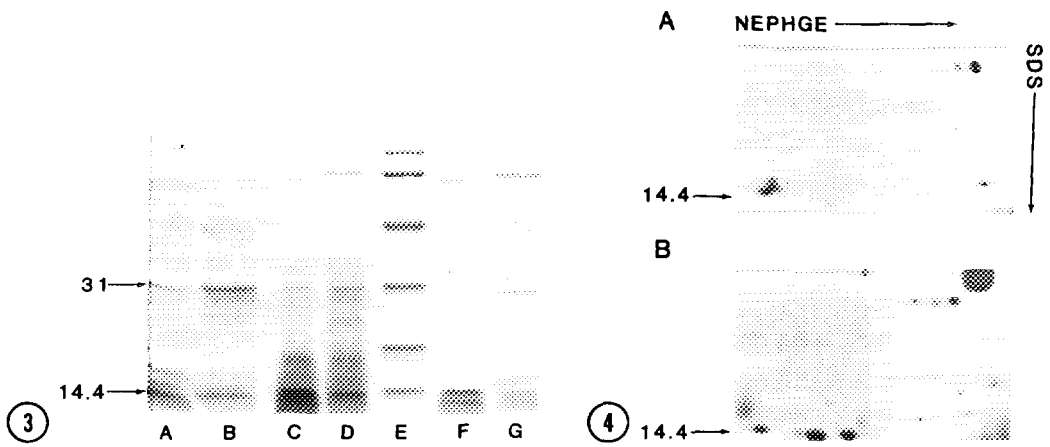

Figure 3. 1D-PAGE separation of mammary gland preparations stained with Coomassie Blue. Lane A represents 25 µg protein eluted with 50% alcohol from an oleic acid-affinity column after application of peak I sample of VMG DEAE-Sephadex fractionation. Lane B represents material eluted with acid alcohol. Lanes C and D represent protein from LMG eluted from an oleic acid-affinity column with 50% alcohol and acid-alcohol, respectively. Lane E represents molecular weight markers. Lanes F and G represent protein from PMG eluted from an oleic acid affinity column with 50% alcohol and acid-alcohol, respectively.

Figure 4. 2D-PAGE separation of peak I of DEAE-Sephadex fractionation of mammary gland preparations. Panel 4A represents VMG and shows a predominant spot at a pI of 4.3 and a minor spot at a pI of 5.2. Panel 4B represents LMG and shows prominent spots with pI's of 5.2 and 5.6 as well as 4.3.

Four sequences are shown: mouse heart fatty acid binding protein (H-FABP), mouse lactating mammary gland 14K protein (LMG-14), mouse adipocyte lipid-binding protein (ALBP) and mouse virgin mammary gland 14K protein (VMG-14). The H-FABP sequence shown represent amino acids 23-120 inclusive which comprise 74% of the total sequence. In the 3 other sequences, all (ALBP, LMG-14) or part (VMG-14) of the amino acid sequences are shown. Only the divergent amino acids are shown for these 3 sequences. The identical sequences are shown as dots. Amino acids not sequenced are shown as blank spaces. The mouse LMG-14 protein

Table I. Amino Acid Sequences of Mouse Fatty Acid Binding Proteins

	23 60 90 120
H-FABP (ref 19)	SLGVGFATRQVGSMTKPTTIIEKNGDTITIKTQSTFKNTEINFQLGIEFDEVTADDRKVKSLVTLDGGKLIHVQKWDGQETTLTRELVDGKLILTLT
LMG-14	.....A.....N.....R.....(95/98,97%)
ALBP (ref. 17)	EV.....K.AG.A..NM..SV...LV..RSE.....S.K..V....I.....II.....A.VQ.....KS..IK.KRDGD..VVECV(60/98,61%)
VMG-14	.....K.AG A..NM..SV...LV. .....S.K..V....I.....II...

showed 97% (95/98) amino acid homology to mouse H-FABP over this region. The divergent amino acids (A in place of G at position 34, and N in place of D at position 100) appeared in both of the lactating mammary gland preparations. The amino acid arginine at position 110 was found in one preparation and was not reached in any peptide band in the second preparation. Over this same region, another 14Kd protein termed mammary-derived growth inhibitor (MDGI-14) contains an additional 5 amino acids different from LMG-14. One peptide band from the pregnant mammary gland yielded a sequence. This sequence represented amino acids 74-93 and was identical to LMG-14 and H-FABP. Over this 20 amino acid region, MDGI contains 3 divergent amino acids (numbers 80, 85, 90). The mouse VMG-14 was 100% homologous to mALBP over 2 stretches of 57 amino acids between positions 25-90. The mouse ALBP exhibits only 61% homology to H-FABP over the same span of 98 amino acids. The results suggest that the major 14Kd protein in lactating and pregnant mammary gland is homologous to heart fatty acid binding protein. We could not find evidence of an MDGI protein. In contrast to the pregnant and lactating mammary glands, the virgin mammary gland where 90% of the cells are adipose or fibroblasts, rather than parenchymal epithelial, the major 14Kd-related protein was ALBP. Because we did not encounter heterogeneity in the amino acid sequence analysis of the LMG or PMG proteins, we assume that the proteins at 5.2 and 5.6 pIs represent charge heterogeneity and are the same protein.

Immunological Characterization of Polyclonal Antibody to rPMG-SLP14: Fractions of pregnant rat mammary gland containing the 14Kd protein were run on an oleate affinity column and the bound proteins at 14Kd eluted by acid alcohol. 1D-PAGE analysis of the eluted proteins showed intense protein bands at 14Kd. This protein band was electroeluted and used as an immunogen in rats. This protein was also digested by proteases and yielded overlapping sequences which were 100% identical to the rat H-FABP between amino acids 63-94. The rat sequence EISFQLGVEFDEVTADDRKVKSVVTLDGGKLV exhibits 4 different amino acids from the mouse sequence over this region.

Figure 5 illustrates the Western immunoblot data of the reactivity to various tissues of antibody generated against rPMG-SLP14. Initial experiments indicated the antibody recognized purified SLP-14 from mouse and rat mammary gland but not from liver. The antibody was active

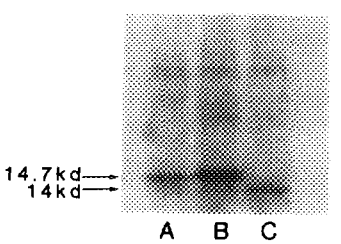

Figure 5. Immunoblot of mouse tissues using rabbit antiserum raised against electroeluted rat mammary gland SLP-14. Antiserum was used at 1:3000 dilution. Lanes A, B and C represent skeletal muscle, heart and pregnant mammary gland, respectively. Two protein bands of approximately 14,700 and 14,000 daltons were recognized by the antiserum.

at a titer of 1:3000. The data illustrated in Figure 5 indicated that the antiserum (1:3000) recognized 2 proteins (14,000 and 14,700) in mouse heart, skeletal muscle and female pregnant mammary gland. The higher molecular weight form was prominent in heart and skeletal muscle; the lower molecular weight form was prominent in female mammary gland. Proteins were present also in the male mammary fat pad where only rudimentary mammary epithelium is present (14,000, 14,700) and in ovary (14,000) whereas the 14Kd proteins in liver, small intestine, large intestine, kidney, uterus, testes, spleen and brain were not recognized by the immunoreactive antiserum (data not shown).

DISCUSSION

The proteins of 14,000 daltons in the mouse and rat mammary gland which were recognized by selenium-labeling belong to the family of fatty acid binding proteins. The mouse LMG-14Kd protein exhibited 97% amino acid homology to mouse heart FABP over a region of 98 amino acids. The mouse and rat PMG-14Kd proteins exhibited 100% homologies to mouse and rat heart FABP, respectively, over a region of 20-32 amino acids, respectively. The mouse VMG-14Kd protein was 100% homologous to mouse ALBP over a 57 amino acid span. These results clearly indicate that the mammary gland 14Kd proteins are members of the FABP family. In this respect, the data are consistent with our previous results that the selenium-labeled 14Kd protein in liver is FABP (2). Also consistent with the known properties of this family of proteins is the ability to bind fatty acids and the immunological specificity of the proteins. Thus, the polyclonal antibodies raised against rat mammary gland 14Kd protein recognized 14Kd proteins in muscle, mammary gland and mammary adipose tissues but failed to recognize the 14Kd proteins in liver and intestine. This result is consistent with the immunological results using liver (8) and cardiac (4) FABP's.

One of the questions at the onset of these experiments was the identity of the 14Kd protein. Other investigators have demonstrated that a 14Kd protein isolated from the bovine mammary gland belonged to the family of FABP's and in addition, was a potent inhibitor of human mammary epithelial cell growth (3,15). This protein increased in concentration in the fully differentiated bovine lactating mammary gland. This protein, termed MDGI, is also expressed in the differentiated mouse mammary gland, as determined by Northern blot analysis using a full length cDNA to MDGI isolated from a cDNA library of pregnant mouse mammary gland (14). Antibodies to the bovine protein, MDGI, recognized rat heart FABP, mouse adipocyte lipid binding protein (ALBP) and a growth inhibitory protein from fibroblasts (3). A second protein, ALBP, has been purified from 3T3-L1 cells (16). Although the presence of ALBP has not, to our knowledge, been demonstrated in mouse mammary adipose tissue, it appeared likely that this protein would also be present in cell homogenates prepared from virgin mammary gland. The mouse MDGI protein and mouse ALBP exhibit 93% and 61% amino acid homology to mouse heart FABP (Table 1), (16). The MDGI from pregnant mouse mammary gland has growth inhibitory activity (17). It was of interest then that we were able to demonstrate ALBP only in virgin mouse mammary glands. In pregnant and lactating gland which contains predominately epithelial cells, a protein with great identity to heart FABP was readily

identifiable. MDGI, which has been detected in mouse pregnant and lactating gland by RNA analysis, was not isolated in our preparations. Since MDGI and the mammary gland 14Kd proteins are highly homologous (only 5 amino acids are different), this result is puzzling, at first glance. The 2 proteins should run at similar molecular weights unless one is preferentially modified by phosphorylation or glycosylation. In our preparations from either the DEAE-Sephadex or oleic acid columns analyzed on 2D-PAGE gels, there are 2 spots of 14,000 daltons with slightly different pI's and one spot at 14,400-14,700 daltons. It is conceivable the 2 spots at 14,000 daltons represent charge-modified proteins which were not differentially identified in amino acid sequence analysis.

Another intriguing result from the Western immunoblot analysis was the recognition of 2 proteins at 14,000 and 14,700 daltons. It was evident that the 14,700 protein was prominent in heart and skeletal muscle and adipose tissue whereas it was a minor constituent in female pregnant mammary gland and ovary. The results of Hresko, et al. (18) suggested that a single tyrosine is phosphorylated on ALBP isolated from 3T3-L1 adipocytes. The status of phosphorylation on FABP's isolated from mouse heart and mammary gland is not known. It is possible that the higher molecular weight proteins recognized by the antiserum reported herein are secondarily modified FABP's. Future experiments should clarify this issue.

In summary, the results demonstrated that the expression of mammary gland FABP's is developmentally regulated and is dependent upon the particular cell type dominant in each particular stage of the gland. In mouse virgin mammary gland where adipose tissue is predominant, the isolated FABP was ALBP, whereas in mouse pregnant and lactating mammary gland where epithelium from secretory alveoli are predominant, the isolated 14Kd protein was a very close homologue of cardiac FABP. It is possible that an MDGI is expressed as a minor protein in the pregnant gland of the mouse since this cDNA was isolated in mouse pregnant gland by Grosse and coworkers (14,17). The antibodies against the rat mammary gland 14Kd protein detected the mouse mammary gland protein at high titer (1:3000) and should prove useful in examining cell expression of the protein during mammary gland development and tumorigenesis.

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