Mammary Derived Growth Inhibitor Is Not a Distinct Protein but a Mix of Heart-type and Adipocyte-type Fatty Acid-binding Protein*  

(Received for publication, July 10, 1995, and in revised form, April 29, 1996)

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The amino acid sequence of the mammary derived growth inhibitor (MDGI) from bovine mammary gland (Böhmer, F.-D., Kraft, R., Otto, A., Wernstedt, C., Hellman, U., Kurtz, A., Müller, T., Rohde, K., Etzold, G., Lehmann, W., Langen, P., Heldin, C.-H., and Grosse, R. (1987) J. Biol. Chem. 262, 15137-15143) revealed 95% identity to bovine heart fatty acid-binding protein (H-FABP), explaining the observed immunocross-reactivity. However, a cDNA encoding MDGI has not been found to date. Artificial MDGI cDNA was expressed in an in vitro transcription/translation assay. Analysis by isoelectric focusing of the immunoprecipitated in vitro translation products of lactating bovine mammary gland mRNA did not indicate a protein corresponding to the in vitro translation product of artificial MDGI mRNA. Moreover, two-dimensional electrophoresis of bovine mammary gland proteins confirmed the absence of a protein with the pl of the in vitro translated artificial MDGI mRNA in bovine mammary gland and instead revealed, apart from H-FABP, an unknown protein that was recognized by anti-H-FABP antibodies. From lactating bovine mammary gland the cDNA for adipocyte fatty acid-binding protein (A-FABP) was cloned. The in vitro translation of recombinant mRNA derived from this cDNA yielded a polypeptide that behaved like the unknown immunoreactive protein. Western blotting and immunofluorescence using monospecific antibodies demonstrated the coexistence of H-FABP and A-FABP in the lactating mammary gland. Taking into account that deviations of the MDGI sequence from the bovine H-FABP sequence correspond with A-FABP we attribute the structure originally reported as MDGI to a mix of these proteins.

A polypeptide designated as "mammary derived growth inhibitor" (MDGI)† had been purified from lactating bovine mammary gland using a proliferation assay with Ehrlich ascites carcinoma cells as target cells (1) and characterized by amino acid sequencing (2). The amino acid sequence of the 14.5-kDa MDGI revealed no homology to any of the hitherto known growth inhibitors (3); rather, MDGI belongs to a family of fatty acid-binding proteins (FABPs) capable of binding hydrophobic ligands with high affinity (4). The members of this family were named according to the tissue of their first isolation (i.e. heart, adipose, intestine, liver, myelin, epidermis, and brain). From numerous studies a role of FABP in intracellular transport and metabolism of fatty acids (5, 6) as well as in differentiation (7) and signal transduction was inferred (4).

In addition to growth inhibition of Ehrlich ascites carcinoma cells and of mammary epithelial cell lines by MDGI (8, 9), Yang et al. (10) recently reported that MDGI as well as H-FABP can act as growth inhibitors of primary mouse mammary epithelial cells. In mammary gland organ culture, growth inhibition was associated with functional differentiation in the presence of H-FABP or MDGI (10).

The literature abounds with partially conflicting data about presence and identity of FABP in the mammary gland. MDGI exhibits a striking 95% identity to bovine heart FABP (H-FABP) and on the basis of amino acid sequence differs from H-FABP in only seven positions (2, 11). Correspondingly, both proteins show immunocross-reactivity (12). Screening of a cDNA library derived from bovine lactating mammary gland revealed a cDNA sequence (13) that was identical to the H-FABP cDNA sequence from bovine heart (14). J. ones et al. (15) isolated two FABPs from a lactating rat mammary gland that differed in their isoelectric points (pl 4.8 and 4.9). The major form (pl 4.9) was partially sequenced, and the amino acid sequences obtained could be aligned with 67 residues of the rat H-FABP (16). An immunopurified FABP from lactating rat mammary gland showed no differences to H-FABP from rat heart on the basis of molecular masses of the protein and of tryptic peptides (17). The cDNA for the FABP from mammary gland of the pregnant mouse (Ref. 18; revised in GenBankTM U02883) was identical with the exon sequences of the H-FABP gene isolated from a mouse genomic library (19). Virgin mouse mammary gland expressed a 14.5-kDa protein with 100% identity to A-FABP over two stretches of 57 amino acids between positions 25 and 90 (20). From pregnant and lactating mouse mammary gland, however, a protein was isolated and sequenced (20) that over a 98-amino acid stretch was only 97% identical to the H-FABP amino acid sequence deduced from the cDNA published by Tweedie and Edwards (21).

In our laboratory numerous attempts to clone the MDGI cDNA from bovine mammary gland failed, and doubts arose. 

*This work was supported by Deutsche Forschungsgemeinschaft Grant SFB 310/A4 and by the Fonds der Chemischen Industrie. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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¶ The abbreviations used are: MDGI, mammary derived growth inhibitor; FABP, fatty acid-binding protein; A-FABP, adipocyte-type FABP; H-FABP, heart-type FABP; IEF, isoelectric focusing; PCR, polymerase chain reaction; RACE-PCR, rapid amplification of cDNA ends by PCR; BSA, bovine serum albumin; PBS, phosphate-buffered saline.
about the true nature of the structure originally published by Bühmer et al. (2). In this report we show that H-FABP and A-FABP are simultaneously expressed in lactating bovine mammary gland, and on the basis of this observation we provide evidence that the published structure can be traced back to a mixture of H-FABP and contaminating A-FABP and propose that the MDGI function is exerted by the well characterized H-FABP.

EXPERIMENTAL PROCEDURES

Materials—First-strand synthesis kit, SureClone™ ligation kit, U-trap境内 dNTP set, dATP, terminal deoxynucleotidyl transferase, Taq DNA polymerase, oligo(dT) spin columns, and ampholytes were purchased from Pharmacia (Freiburg, Germany). Taq polymerase was from Perkin-Elmer (Foster City, CA). 5′-end labeling was from Perkin-Elmer (Foster City, CA). Taq polymerase was from Perkin-Elmer (Foster City, CA). 5′-end labeling was from Perkin-Elmer (Foster City, CA). Taq polymerase was from Perkin-Elmer (Foster City, CA). 5′-end labeling was from Perkin-Elmer (Foster City, CA).

Isolation of mRNA and cDNA Synthesis—Total RNA was isolated either from lactating bovine mammary gland tissue by the guanidine thiocyanate/oosomal chloride method as described by Chirgwin et al. (22) or from subcutaneous adipose tissue according to Lohrmann and Sachi (23). To obtain poly(A)+RNA, total RNA was passed through oligo(dT) spin columns according to the protocol supplied by the manufacturer.

First-strand cDNA was synthesized from 0.7 μg of poly(A)+RNA mammary gland RNA using the universal primer (5′-CTGGCACGCTGATCGGTAATTC-3′; 34 pmol). The reaction mixture (15 μl) was diluted immediately to 1 ml with TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 7.5) and stored at −70°C.

Rapid Amplification of cDNA Ends (RACE-PCR)—For rapid amplification of cDNA ends of bovine A-FABP the methodology of Frohman et al. (24) was employed. Primers for RACE-PCR were synthesized using the Gene Assembler Plus (Pharmacia).

3′-End Amplification of cDNAs—The amplification was performed using the specific primer (5′-CTGGCACGCTGATCGGTAATTC-3′; 23 pmol) identical to the 3′-end of the universal gene-derived primer from the first-strand synthesis of cDNA and the degenerated gene-specific sense primer (5′-GGNACCTGGAGGCTGNTTCTC-3′; 80 pmol). The PCR mixture (10 μl of cDNA, 0.2 mM each dNTP, 10 μl of 10× PCR buffer, 23 pmol of specific primer, and 80 pmol of degenerated gene-specific sense primer in a total volume of 100 μl, overlaid with mineral oil) was denatured for 5 min at 94°C and cooled to 72°C. After the addition of 2.5 units of Taq polymerase, PCR was performed for 30 cycles (1 min at 94°C, 1 min at 72°C, 1 min at 72°C) followed by a final extension at 72°C for 10 min using a TRIO-Thermalblock (Biometra, Gottingen, Germany).

5′-End Amplification of cDNAs—For reverse transcription, 0.7 μg of poly(A)+rich mammary gland RNA and 1.2 pmol of gene-specific antisense primer 1 (5′-AAAAAATAATATATGTTTGG-3′) were used. After reaction the mixture was diluted with 2 ml of 0.1 X TE-buffer, and excess primer was removed by using a CentriPrep 100 spin filter. The sample was then concentrated to 50 μl using a vacuum concentrator. For labeling, 1 μl of terminal deoxynucleotidyl transferase (16 units/μl), 4 μl of 1X dATP, 2 μl of 10× buffer, and 13 μl of the solution were mixed and incubated for 10 min at 37°C, heated for 15 min at 65°C, and then diluted with 0.5 ml of water. The compounds for the subsequent PCR reaction were 5 μl of template, 0.2 μl each dNTP, 10 μl of 10× PCR buffer, 10 pmol of universal primer, 23 pmol of specific primer, and 23 pmol of nested gene-specific antisense primer 2 (5′-CTGGTATTTAGTGACACCAAC-3′), in a total volume of 100 μl.

The Taq polymerase (2.5 units) was added after denaturation for 5 min at 94°C and cooling to 72°C. In this case, the PCR program was 2 min at 50°C, 40 min at 72°C, 35 × 1 min at 94°C, 1 min at 58°C, 1 min at 72°C, and 10 min at 72°C.

PCR Amplification—Amplification of the complete coding region from the cDNA used in 3′-RACE was done with two specific primers derived from the nucleotide sequence obtained after 3′-end and 5′-end amplification. The upstream primer (5′-CACCATATGGTTGAGCTT-TTG-3′) contained an NdeI and the downstream primer (5′-GGATCTT-TAGTCTCTCTCATAAAC-3′) a BamHI restriction site for subsequent cloning into an expression vector. The same protocol was also applied to cDNA from adipose tissue that was reverse-transcribed using the downstream primer.

Cloning and Sequencing of DNA—The RACE products and the PCR products from mammary gland and adipose tissue were purified by electrophoresis on 1% agarose gels (25). The RACE products were done into the blunted, dephosphorylated pUC-vector (SureClone™ Ligation Kit), whereas the PCR products of the complete coding regions were cloned into the pCR™-MI-vector (TA-Cloning Kit) following the recommendations of the manufacturer. Competent bacteria were prepared and transformed with plasmids as described elsewhere (25).

The NdeI/BamHI fragment of an A-FABP done derived from the mammary gland, which contained the complete coding region and which was sequenced on both strands with Sequenase as described by the manufacturer, was subcloned into the expression vector pET-3a (26) to yield pET-A-FABP. For comparison, the coding region derived from adipose tissue was sequenced using the digoxigenin system and direct blotting onto a membrane (MWG, Ebersberg, Germany).

In Vitro Transcription—The pET vectors containing the coding region of bovine A-FABP, bovine H-FABP, bovine A-FABP (Ref. 10), and artificial bovine MDGI cDNA (Ref. 10), were used in vitro transcription using the suppliers recommendations. Transcripts were capped by including 0.5 mM of the cap analogue Pτ7-methyl-5′-guanosine)- Pγ(5′-guanosine-3′-triphosphate) in the transcription reaction.

In Vitro Translation—Cell-free translation was carried out in rabbit reticulocyte lysate containing [35S]methionine (1000 Ci/mmol) as described by the manufacturer except for the addition of 20 units of RNasin. Typically, 3–10% of the transcripts were translated in a 25-μl translation assay, after which the reaction mix was immediately frozen and stored at −70°C until further use. N-terminal acetylation was inhibited by a preincubation with citrate synthase and oxaloacetic acid (27).

Immunoprecipitation—IgG-precipitated was carried out as described by Darley-Utman et al. (28) with protein A-Sepharose CL-4B and rabbit anti-bovine H-FABP antibodies affinity-purified on a bovine H-FABP-Sepharose column, which later turned out to be cross-reactive with bovine A-FABP.

Purification of FABP from Bovine Heart and Mammary Gland—FABP from bovine heart was isolated according to the protocol of Nielsen and Spener (30). For the isolation of FABP from mammary gland, the tissue was homogenized by using an ultraturrax in PBS buffer containing 0.3 mM phenylmethylsulfonyl fluoride. The FABP was purified from the 100,000 × g supernatant by affinity chromatography (rabbit anti-H-FABP antibodies covalently bound to protein A-Sepharose CL-4B) as described by Nielsen and Spener (30).

Cell-free Analysis—Prior to electrophoresis, immunoprecipitates for IEF were suspended in a lysis buffer containing 2% Triton X-100, 100 mM dithiothreitol, 2% amylolde (pH range 7–9), and 9 mM urea at 50°C for 10 min. In vitro translation samples (1 μl) were diluted with 9 μl of lysis buffer and then subjected to IEF. FABP isolated from bovine mammary gland and bovine heart (5 μl) was mixed with 5 μl of lysis buffer and 5 μg of urea and then applied to IEF.

Electrophoretic Focusing was performed under denaturing conditions as described earlier (31). Two-dimensional gel electrophoresis, which was also performed under denaturing conditions, and Western blotting were done as described by Nielsen and Spener (30). Proteins were detected by Coomasie staining or specifically immunodecorated after Western blotting using affinity-purified rabbit anti-H-FABP antibodies. For fluorography, 1E-4 gels were soaked in Amplify™ solution. The dried gels were exposed to x-ray films at −70°C for 2–4 days.

Monospecific Antibodies—Antibodies were raised in New Zealand White rabbits. Monospecific anti-bovine H-FABP antibodies were prepared by double-affinity chromatography. As affinity matrix, 5 mg of recombinant H-FABP (32) and recombinant A-FABP, respectively, were bound to CH-Sepharose Phosphate 4B (Pharmacia). First, antiserum raised against bovine H-FABP was passed over the H-FABP column, and specific antibodies were eluted with a buffer containing 10 mM citrate, 20 mM sodium phosphate (pH 2.8). This antibody fraction was then rechromatographed on the A-FABP column. The flow-through contained monospecific anti-bovine H-FABP antibodies as confirmed by
Western blotting. For Western blotting proteins were resolved by 13.5% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocel-
lulose membrane (Schleicher & Schuell, Dassel). The membrane was
incubated either with the double affinity-purified anti-bovine H-FABP
antibodies (5 μg/ml) or with bovine A-FABP antiserum (1:200) in 0.5%
BSA/PBS, 0.05% Tween followed by protein A-peroxidase (1:2500) and
stained with 4-chloro-1-naphthol. Recombinant bovine A-FABP for gen-
eration of A-FABP antiserum and as standard in Western blotting was
obtained by transforming E. coli BL21(DE3)pLysS with pET-BA-FABP.
Expression, purification, and characterization of the recombinant A-
FABP will be described elsewhere.

Immunofluorescence Microscopy—Lactating mammary gland, ob-
tained from a local slaughterhouse, was extensively washed with ice-
cold PBS, fixed in formalin, paraffin-embedded, and hematoxylin/eosin-
stained. Slides (5 μm) were deparaffinized and then blocked for 30 min
with 3% BSA/PBS, washed three times with PBS, and incubated for 30
min with double affinity-purified anti-bovine H-FABP antibodies (5 μg/
ml) or anti-bovine A-FABP antiserum (1:200 dilution), each in 0.5% BSA/
PBS. After washing with 0.5% BSA/PBS (three times), sections were
incubated with Cy3-labeled goat anti rabbit IgG (1:300 in 0.5% BSA/
PBS) for 30 min. All steps were performed in a humidity chamber at
37°C. Sections were viewed and photographed under a Nikon DIA-
PHOT microscope (Tokyo, Japan). Nonspecific staining was assessed by
omission of the primary antibody or by using preimmune serum.

RESULTS

Absence of MDGI in Bovine Mammary Gland at the mRNA and Protein Levels—Previously, Böhm and co-workers (2) isolated a 14.5-kDa protein from the bovine mammary gland, which, according to its particular function, was named "mam-
mary derived growth inhibitor" (MDGI). This protein cross-
reacted with antibodies against H-FABP purified from bovine
heart (12) but differed from the latter at positions 12, 14, 40, 43,
93, and 127 and additionally lacked the C-terminal amino acid
sequence. 

The analysis of the immunoprecipitated, in vitro translated products was subjected to immuno-
precipitation with affinity-purified anti-H-FABP antibodies. The analysis of the immunoprecipitated, in vitro translated proteins revealed two bands (Fig. 1, lane 1) corresponding to the authentic p 4.9 H-FABP and p 5.1 H-FABP isoforms isolated from bovine heart (Fig. 1, lane 4). Among the immu-
noprecipitated proteins no protein with a p 4.9 corresponding to the in vitro translation product of artificial MDGI mRNA could be identified.

To investigate the situation in more detail in tissue, a lac-
tating bovine mammary gland was homogenized, and the protein fraction obtained from the 100,000 × g supernatant was used for further experiments. Analysis of the soluble protein fraction by two-dimensional gel electrophoresis resolved sev-
eral hundred proteins (Fig. 2A). The position of FABP was identified by Western blotting and immunostaining using affinity-purified anti-bovine H-FABP antibodies.

No protein with a p 4.9 corresponding to the in vitro expressed product of artificial MDGI cDNA was detected (Fig. 2B). Sur-
prisingly, in addition to the p 4.9 and p 5.1 of H-FABP, a yet unknown protein with a more acidic pl value became immunodecorated. We also isolated FABP from bovine mammary gland by immunofinity chromatography and analyzed it by urea IEF. Again the third FABP band with a more acidic isoelectric point was observed along with p 4.9 H-FABP and p 5.1 H-FABP, whereas no spot corresponding to artificial MDGI could be detected. Interestingly, analysis of the immunopurifi-
cation by trypsin digestion, the translation product was analyzed by IEF under denaturing conditions (Fig. 1, lane 2). To detect possibly present MDGI in bovine mammary gland, the poly(A)-rich RNAs of this tissue were translated in vitro and the translation products were subjected to immuno-
precipitation with affinity-purified anti-H-FABP antibodies. The analysis of the immunoprecipitated, in vitro translated proteins revealed two bands (Fig. 1, lane 1) corresponding to the authentic p 4.9 H-FABP and p 5.1 H-FABP isoforms isolated from bovine heart (Fig. 1, lane 4). Among the immu-

nological detection of FABP by Western blotting and immunostaining using anti-H-FABP antibodies.
reactive pl 4.7 protein needed further attention. Since the mammary gland contains also fat and since A-FABP has relatively high homology to H-FABP (63% in the rat), resulting in immunological cross-reactivity, we suspected that the additional protein observed in two-dimensional gel electrophoresis could be the bovine A-FABP. The following experiments were carried out to determine the bovine A-FABP nucleotide sequence and to establish the presence of A-FABP in the lactating bovine mammary gland.

Isolation of the Full-length cDNA Encoding Adipocyte FABP from Bovine Lactating Mammary Gland Using RACE-PCR—To obtain the 3'-end of the bovine A-FABP cDNA, purified mRNA from bovine mammary gland was reverse-transcribed. For 3'-end amplification of cDNA, a degenerated gene-specific sense primer was used together with the specific primer. The degenerated gene-specific primer was designed according to the published cDNA sequences of human A-FABP (33) and mouse A-FABP (34) but would also allow the amplification of a yet unidentified MDGI cDNA (Fig. 4). Care was taken that the two 3'-nucleotides of this primer did not match the cDNA sequence of bovine pI 5.1 H-FABP, which is abundantly expressed in differentiated bovine mammary gland (13). The primers for 5'-end amplification were designed according to the DNA sequence obtained after sequencing of the 3'-end amplified PCR product. Once the 3'- and 5'-ends had been identified, the complete coding region of the bovine A-FABP cDNA was amplified and sequenced. The deduced amino acid sequence of bovine A-FABP reveals 85 and 87% identity to human A-FABP and mouse A-FABP, respectively (Fig. 8). The two bases (boldface) at the 3'-end of the degenerated primer (VII) were not complementary to the cDNA of bovine H-FABP.

Recombinant A-FABP mRNA Encodes the Unknown Immunoreactive pl 4.7 FABP—We transferred the coding part of the A-FABP cDNA into the pET-3a vector containing the T7 promoter. The product of the in vitro transcription by T7 RNA polymerase and subsequent translation in a cell-free system was analyzed by urea IEF (Fig. 5, lane 1). The in vitro expressed protein co-migrated with the unknown immunoreactive pl 4.7 protein present in the FABP isolated from bovine mammary gland by immunoaffinity chromatography (Fig. 5, lane 5). Thus, the three bands observed in the urea IEF gel of immunopurified FABP from bovine mammary gland can be explained as the pl 4.9 and 5.1 isoforms of H-FABP and as A-FABP, respectively. This result points to the existence of A-FABP in addition to H-FABP in lactating bovine mammary gland.

The appearance of two additional variants with more basic pl values observed in urea IEF of in vitro expressed products of bovine A-FABP cDNA (Fig. 5, lane 1) is probably due to different N-terminal processing (A-FABP, Ac-Cys-Asp-Ala; variant 1, Ac-Asp-Ala; variant 2, Asp-Ala). By the addition of N-terminal acetylation inhibitors (27), the amount of variant 1 was decreased, whereas the band of variant 2 became stronger (Fig. 5, lane 2). By the same token, the N-terminal acetylation of in vitro expressed pl 5.1 H-FABP (Fig. 5, lane 3) could be partially suppressed as indicated by the appearance of an additional band with a more basic pl value (Fig. 5, lane 4).

Detection of A-FABP in Adipose Tissue and Lactating Mammary Gland—To demonstrate that the protein we had cloned from the lactating mammary gland and termed A-FABP according to its homology to other A-FABPs was indeed the adipocyte-type FABP we analyzed the expression of the corresponding protein in adipose and mammary gland tissue. Western blotting of cytosolic proteins with antiserum directed against the recombinant bovine A-FABP confirmed the presence of a protein of identical size (~14 kDa) in mammary gland as well as in adipose tissue (Fig. 6, lanes 1, 6, and 8). We then by PCR on cDNA from adipose tissue amplified a 400-base pair DNA fragment using the same primers as described above for the mammary gland. The amplified DNA was cloned and sequenced and represented bovine A-FABP cDNA (data not shown). The question arose whether the A-FABP cloned from the mammary gland originates from the adipocytes that may be present in mammary tissue or from the mammary cells. The staining of mammary gland and adipose cytosol with the anti-A-FABP antibodies in the Western blot (Fig. 6) already indicated that the amount of A-FABP was too large to be attributed...
to the relatively few adipocytes still present in the lactating mammary gland, in particular since we have tried to free the mammary gland tissue from all adhering adipose tissue. To prove this hypothesis we analyzed the cellular localization of both A-FABP and H-FABP by immunofluorescence. As a prerequisite, the specificity of the antibodies had to be demonstrated because it is known that anti-H-FABP antibodies often cross-react with A-FABP (see Fig. 2 and Ref. 35). Here we show that the A-FABP antiserum specifically recognized recombinant bovine A-FABP (Fig. 6, lane 7), whereas the same amount of recombinant bovine H-FABP was not stained (Fig. 6, lane 9). A monospecific antibody against bovine H-FABP which was obtained by double affinity purification did not cross-react with recombinant bovine A-FABP and detected no H-FABP in adipose tissue (Fig. 6, lanes 4 and 5) but, as expected, stained large amounts of H-FABP in heart and mammary gland (lanes 1 and 3).

Localization of A-FABP and H-FABP in the Mammary Gland—To analyze the expression of both FABP types on cellular level in the lactating mammary gland, these monospecific antibodies were used in immunofluorescence. The typical alveolar structure of the mammary gland is demonstrated in the hematoxylin/eosin stain (Fig. 7A). With monospecific anti-H-FABP antibodies a strong immunofluorescence was observed in the epithelial cells of the terminal acini (Fig. 7B). Reactivity of the myoepithelial cells surrounding the alveolar epithelium could not be judged with certainty. In contrast, the anti-A-FABP antiserum reacted with the myoepithelial cells, omitting the alveolar epithelium (Fig. 7C). Immunofluorescence of fibroblasts, connective tissue, and some smooth muscle cells around blood vessels was negligible with both antibodies compared with the epithelial cells.

DISCUSSION
Since DNA probe and immunological techniques cannot distinguish between the closely related MDGI and H-FABP, we applied an in vitro expression analysis to address the possible presence of both proteins in the mammary gland. Based on published amino acid sequences, the isoelectric points of MDGI and H-FABP should differ. From the in vitro translation experiments and from two-dimensional gel electrophoresis it became clear that the previously described sequence for MDGI (2) could not be correct. However, an as yet unidentified immunoreactive pl 4.7 protein was observed. For isolation of the cDNA of the unknown immunoreactive pl 4.7 FABP from lactating bovine mammary gland, a degenerated primer was used. The primer sequence was chosen according to the cDNA sequences of human A-FABP (33) and mouse A-FABP (34). By multiple alignment, the deduced amino acid sequence of the isolated cDNA was identified as bovine A-FABP (Fig. 8). The identity of the hitherto unknown bovine A-FABP with mouse A-FABP was 87% and with human A-FABP was 85%, in accordance with typical identities of FABPs of the same type but from different mammalian species of about 80–90% (4). The in vitro expressed protein of the bovine A-FABP cDNA comigrated with the unknown pl 4.7 FABP in urea IEF (see below), confirming that the unknown immunoreactive pl 4.7 FABP indeed represents A-FABP. This assignment was corroborated by cloning and sequencing of the same cDNA from bovine adipose tissue. Moreover, antibodies raised against recombinant bovine A-FABP in Western blotting recognized a 14-kDa protein in adipose and mammary gland tissue. The three FABPs observed in lactating bovine mammary gland were thus identified as pl 4.9.
H-FABP, pI 5.1 H-FABP, and A-FABP. We have earlier traced back the molecular basis for the two H-FABP isoforms, which were originally observed in bovine heart (29), to an Asn-Asp exchange at amino acid position 98. The published H-FABP cDNA (14) codes for Asn<sup>98</sup> and hence represents the pI 5.1 isoform (see also Fig. 1, lane 3). We presented arguments (31) that the isoforms stem from two different mRNAs rather than from posttranslational modification (deamidation).

The published amino acid sequence of MDGI deviated in only seven positions from that of bovine H-FABP (2, 11). Each of these seven amino acids was found at the same position in the sequence of bovine A-FABP. Consequently, the amino acid sequence published for MDGI represents a mix of H-FABP and A-FABP. Native IEF of FABP from bovine lactating mammary gland (Fig. 3B, lane 3) only showed the two bands corresponding to pI 4.9 H-FABP and pI 5.1 H-FABP. Apparently, A-FABP had comigrated with pI 4.9 H-FABP or pI 5.1 H-FABP and can be separated from H-FABP only in IEF under denaturing conditions (Fig. 3A, lane 1). In our hands it was not possible to separate A-FABP and H-FABP in an isolation procedure employing native conditions. This behavior likely led to isolation and sequencing of a mixture consisting of H-FABP and A-FABP by Böhm and co-workers (2).

A critical experiment was the confirmation of the identity of bovine A-FABP and the unknown immunoreactive pI 4.7 FABP by in vitro transcription/translation. The findings were somewhat obscured by the presence of more than one band in the case of in vitro expressed bovine A-FABP (Fig. 5, lane 1). Wold (36) formulated a general model for N-terminal processing of eukaryotic cytosolic proteins that incorporated the presence of an acetylated activity and an acetylaminoacyl-hydrolyzing activity as general components. N-terminal processing of class II actin (Met-Cys-Asp-) in rabbit reticulocyte lysate studied by Rubenstein and Martin (37) is consistent with this model. They found that methionine was removed as free amino acid, followed by acetylation of Cys-Asp-, and eventually the aspartate residue was acetylated after the removal of the acetylated cysteine, giving the final in vivo product. Analyzing the in vitro expressed products of bovine A-FABP cDNA encoding for Met-Cys-Asp- by urea IEF we observed a protein (Fig. 5, lane 1) corresponding to authentic A-FABP (Ac-Cys-Asp-; Ref. 38). Two additional variants were found (presumed N terminus of variant 1, Ac-Asp-Ala-; and of variant 2, Asp-Ala-), which were probably due to N-terminal processing as observed for class II actin (37, 39). Formation of fully processed class II actin (Ac-Asp-) in the rabbit reticulocyte lysate could be decreased by inclusion of acetylation inhibitors (39) as also observed by us for variant 1 of bovine A-FABP and pl 5.1 bovine H-FABP (Fig. 5). Although class II actin and A-FABP possess the same N-terminal sequence (Met-Cys-Asp-), their in vivo modifications apparently are different (A-FABP, Ac-Cys-Asp-; and class II actin, Ac-Asp-). It remains an open question whether this different N-terminal processing is due to specific recognition of an N-terminal sequence motif or whether N-terminal processing is tissue-specific.

Here we clearly demonstrate for the first time that A-FABP is expressed in cells of the lactating bovine mammary gland as well as H-FABP. Bansal and Medina (20) reported the expression of A-FABP in the virgin mouse mammary gland and the expression of H-FABP in the lactating mouse mammary gland. The differentiation-dependent expression of H-FABP in the rat mammary gland was earlier shown on mRNA (18) and protein level (17). The presence of A-FABP in virgin mammary gland was expected because virgin mammary gland contains considerable amounts of adipocytes. In contrast, in differentiated mammary gland 90% of the cells are epithelial cells. Since A-FABP could be clearly seen in the two-dimensional gel of mammary gland proteins (Fig. 2), in urea IEF of the immunopurified FABP (Fig. 3A, lane 1), and in Western blotting with the A-FABP-specific antiserum we believed that A-FABP was expressed in the mammary cells as well. We could then show by immunofluorescence with the monospecific antiserum that A-FABP was present in myoepithelial cells, whereas H-FABP was detected in mammary epithelial cells as reported earlier (35, 40). The cellular localization of both FABPs in the mammary gland will be addressed in more detail in a separate immunohistochemical study. Some reports in the literature describe the presence of two FABP types in one organ (heart-type and intestinal-type FABP in stomach (41), heart-type and liver-type FABP in kidney (42), heart-type and brain-type FABP in brain (43)), as well as in a single cell-type (intestinal-type and liver-type FABP in enterocytes of the intestine (44)).

What would be the reason for the presence of two types of FABP (A-FABP and H-FABP) in the same organ? Coexpression of FABP types is generally interpreted in terms of specialized functions in the metabolism of fatty acids. H-FABP is predominantly found in cells where fatty acids are used as energy source and is probably involved in β-oxidation (5–7). Its abundance in the mammary gland, which during lactation has an active triglyceride synthesis, remains to be understood. The role of adipocytes in vivo is the storage and mobilization of triglycerides. The highest concentration of A-FABP was found in adipocytes; therefore, A-FABP could be involved in triglyceride metabolism in the mammary gland as well.

| Source     | Protein Description                  | pI       |
|------------|-------------------------------------|----------|
| bovine H-FABP | MADFVGTWKLVD25FDDYMKSLGYPATQVGMNTKPTTI1TBVKEGTIIQTSTFKNTIESFLK | 5.1      |
| bovine MDGI  | MADFVGTWKLVD25FDDYMKSLGYPATQVGMNTKPTTLIESVMDTIIQSTFKNTIESFLK | 5.1      |
| bovine A-FABP | MADFVGTWKLVD25FDDYMKSLGYPATQVGMNTKPTTTLESNGGVTIKIESSTFKNTIESFLK | 4.9      |
| human A-FABP | MADFVGTWKLVD25FDDYMKSLGYPATQVGMNTKPTTTLESNGGVTIKIESSTFKNTIESFLK | 4.9      |
| mouse A-FABP | MDCAVGTWKLVD25FDDYMKSLGYPATQVGMNTKPTTTLESNGGVTIKIESSTFKNTIESFLK | 4.9      |

Fig. 8. Multiple alignment of bovine A-FABP and related proteins. Amino acids of bovine A-FABP that represent the differences between H-FABP and MDGI are in boldface type.
The results of this work clearly demonstrate that MDGI as a distinct protein does not exist. However, it has been shown earlier that its function can be exerted by H-FABP (10). H-FABP from bovine heart and bovine mammary gland, recombinant H-FABP, and recombinant MDGI derived from the artificial MDGI cDNA act as growth inhibitors on primary mouse mammary epithelial cells. In a mammary organ culture, growth inhibition was associated with functional differentiation.

On the contrary, intestinal-type FABP and liver-type FABP did not exhibit inhibitory activity in this assay (10). In a different approach Huynh et al. (40) increased the intracellular H-FABP content of MCF-7 human breast cancer cells by transfection by intestinal-type FABP and liver-type FABP may well be related to a low homology in the C-terminal region to H-FABP (10).

H-FABP (10). The C terminus of A-FABP has a modest similarity to H-FABP (Fig. 8). It remains to be established whether A-FABP can act as growth inhibitor as well. The lacking inhibition by intestinal-type FABP and liver-type FABP may well be related to a low homology in the C-terminal region to H-FABP.

Acknowledgments—The artificial MDGI cDNA was a gift from Dr. Arno Kromminga and Prof. Axel Lezius. Wethank Dr. Søren Nielsen for help with two-dimensional gel electrophoresis and Prof. Wolfgang Zschieche for helpful discussions regarding the immunohistochemical localization of FABPs in the mammary gland. Paraffin embedding and hematoxylin/eosin staining by Mechthild Humberg (Animal Facilities, University of Münster) are gratefully acknowledged.

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