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A 13-Kilodalton Protein Purified from Milk Fat Globule Membranes Is Closely Related to a Mammary-Derived Growth Inhibitor

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ABSTRACT: With the use of specific antibodies against a previously purified [Boehmer, F.-D., Lehmann, W., Schmidt, H., Langen, P., & Grosse, R. (1984) *Exp. Cell Res. 150*, 466–477] and sequenced mammary-derived growth inhibitor (MDGI) [Boehmer, F.-D., Kraft, R., Otto, A., Wernstedt, C., Hellmann, U., Kurtz, A., Mueller, T., Rohde, K., Etzold, G., Lehmann, W., Langen, P., Heldin, C.-H., & Grosse, R. (1987) *J. Biol. Chem. 262*, 15137–15143], the localization and relative amount of immunoreactive 13-kilodalton (kDa) antigen in different fractions of bovine milk were determined. The highest amount of antigen was found to be associated with the milk fat globule membranes (MFGM). As revealed by a dot immunobinding assay, the amount of immunoreactive bovine and human MFGM-associated antigen increased dramatically with the onset of lactation after delivery. This finding corresponds to earlier data obtained for MDGI and indicates a relationship between the proliferative state of mammary epithelial cells and the amount of immunoreactive antigen. The 13-kDa antigen has been purified from MFGM to homogeneity by preparative sodium dodecyl sulfate–polyacrylamide gel electrophoresis and electroelution. The MFGM-derived 13-kDa polypeptide was found to be almost identical with MDGI as demonstrated by tryptic digestion and partial amino acid sequence analysis of tryptic fragments of both proteins. The results clearly show the presence of a membrane-bound MDGI-related 13-kDa protein, thus supporting the possible involvement of membrane-associated growth inhibitors in growth regulation of mammary epithelial cells.

Polypeptide growth factors have acquired an established place among growth modulators of the mammary gland during the last decade. For example, epidermal growth factor (EGF)[1] receptor level and transforming growth factor (TGF) action seem to be correlated with growth of breast cancer cells (Fitzpatrick et al., 1984; Sporn et al., 1986; Spitzer et al., 1987). Demonstration of numerous growth stimulatory factors in milk (Shing & Klagsbrun, 1984; Bano et al., 1985) also points to the mammary gland as an important source of growth factors. Polypeptide growth inhibitors (Wang & Hsu, 1986) are possibly of similar importance for the regulation of mammary epithelial cell proliferation (Dickson & Lippman, 1987). We have described previously a 13-kDa growth inhibitor

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[1] Abbreviations: DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EGF, epidermal growth factor; TGF, transforming growth factor; MDGI, mammary-derived growth inhibitor; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; TBS/Tween, Tris-buffered saline, pH 7.4, containing 0.2% Tween 20; MFGM, milk fat globule membrane(s); Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s); HPLC, high-performance liquid chromatography; RP, reverse phase; TFA, trifluoroacetic acid.

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(MDGI) purified from lactating bovine mammary gland (Boehmer et al., 1987a). MDGI inhibits proliferation of different cultured mammary epithelial cells at hormonelike concentrations of about 10^{-10}–10^{-11} M in a dose-dependent, nontoxic, and reversible manner (Boehmer et al., 1984, 1985). The inhibitory effect can be antagonized by different growth factors such as EGF or insulin (Boehmer et al., 1985). Determination of the entire amino acid sequence of MDGI revealed interesting homologies to retinoid binding and fatty acid binding proteins (Boehmer et al., 1987a). Specific antibodies raised against MDGI cross-react with FGR-s, a growth inhibitor purified from conditioned medium of fibroblasts (Boehmer et al., 1987b). The data suggest a family of structurally related polypeptides either carrying hydrophobic ligands or interacting with biological membranes or both. Our earlier data indicating growth inhibitory activity in MFGM (Herrmann & Grosse, 1986) prompted us to look in detail for MDGI-related membrane-associated antigens. The present data clearly show that MFGM contain a 13-kDa polypeptide almost identical with MDGI. This means that most probably MDGI is synthesized by mammary epithelial cells and might at least partially exist in a plasma membrane-associated state.

Materials and Methods

Chemicals. Acrylamide, Tween 20, and trypsin were from Serva (Heidelberg, FRG). Iodoacetamide was obtained from Ferak and Tris from Merck.

Calf serum was from Staatliches Institut fuer Immunpraeparate und Naehrmedien (Berlin, GDR).

Bis(acrylamide) (Reanal, Budapest) was recrystallized before use. All other reagents were of the highest purity available. Unless otherwise specified, all protein manipulations were carried out at 4 °C.

Preparation of MDGI and MFGM. Mammary-derived growth inhibitor (MDGI) was isolated exactly as outlined before (Boehmer et al., 1985, 1987a).

MFGM were prepared from the cream of fresh bovine or human milk according to Jarasch et al. (1977), omitting the final sucrose gradient centrifugation step. For disruption of MFGM, the washed cream was resuspended in water and churned with a knife homogenizer (E. Buehler, Tuebingen, FRG). Membranes were obtained after centrifugation at 100000*g* for 90 min. The lipid phase floating on top of the 100000*g* supernatant was saved for butanol extraction (see Results). The remaining supernatant is designated in the text as the 100000*g* supernatant of MFGM. The pellet was resuspended in 0.3 M sucrose containing 10 mM Tris-HCl buffer, pH 7.4, and 32 mM KCl. This suspension is designated as MFGM in the present paper. MFGM were characterized before by measuring marker enzyme activities and by demonstrating the absence of insulin and EGF receptor binding sites on MFGM (Herrmann et al., 1986; Spitzer & Grosse, 1987).

The soluble phase from bovine milk was obtained by centrifuging the milk first at 7000*g* for 20 min at room temperature. The cream was removed and the underlying aqueous phase centrifuged again at 100000*g* for 1 h at 2 °C. The 100000*g* supernatant is designated as the soluble phase of the milk ("whey").

Preparative Gel Electrophoresis and Electroelution. Separation of the MDGI-related 13-kDa polypeptide from other MFGM proteins was performed by SDS–PAGE in 15% polyacrylamide gels according to Nelson et al. (1973). To this end, 300 μg of MFGM protein was loaded on tube gels (6-mm diameter) of 8-cm length which were run at 5–10 mA/gel at room temperature overnight. In order to determine the relative position of the 13-kDa band, a reference gel was stained with Coomassie Blue according to Fairbanks et al. (1971). The unfixed gels were cut, and 4-mm slices corresponding to the position of a molecular mass of 13 kDa were pooled and directly applied to electrophoretic elution. Electroelution was performed in specially designed glass tubes (12 mm in diameter) through a layer of 7.5% polyacrylamide into a 0.2-mL reservoir separated from the anodic buffer tank by a dialysis membrane (Serva Visking). The elution buffer was 25 mM Tris-HCl, pH 8.0, and 0.1% SDS, and elution was performed at 40 V overnight at room temperature. The eluted protein was taken up with a syringe and the purity checked by SDS–PAGE according to the method of Laemmli (1970) using 15% polyacrylamide gels. Slab gels were stained with silver ions according to the method of Heukeshoven and Dernick (1985).

Anti-MDGI Antibodies. Raising and testing of specific mouse and rabbit antibodies against the 13-kDa inhibitor were described in detail before (Boehmer et al., 1985, 1987a). Both kinds of anti-MDGI antibodies were shown to precipitate specifically MDGI and to neutralize its biological activity under in vitro conditions.

Dot Immunobinding Assay. One microliter of the sample solution was dotted onto a piece of nitrocellulose and dried at room temperature. The nitrocellulose was then washed with TBS/Tween containing 20% calf serum and incubated with the same solution containing an appropriate dilution of the anti-MDGI antibody overnight at 4 °C. For control, dots were incubated with the corresponding preimmune or normal serum. Detection of the antigen–antibody complex was performed with sheep anti-mouse (or rabbit) immunoglobulin conjugated to horseradish peroxidase as described before (Boehmer et al., 1985).

Western Blot Analysis. Electrophoretic transfer of proteins from SDS gels to nitrocellulose was performed essentially as outlined (Burnette, 1981) and described before (Boehmer et al., 1985).

Tryptic Digestion and Amino Acid Sequence Analysis. For tryptic digestion, both the MFGM and MDGI were first reduced and alkylated and then subjected to SDS–PAGE and electroelution. Briefly, reduction with 5 mM dithiothreitol and alkylation with 50 mM iodoacetamide were performed for 20 min at room temperature according to the method of Blobel and Dobberstein (1975). Either 600 μg of MDGI or 6 mg of MFGM proteins was reduced in the presence of 5 mM DTT by boiling for 3 min and then alkylated. The reduced and alkylated proteins were then applied to 15% SDS gels as described under Preparative Gel Electrophoresis and Electroelution. After electrophoretic elution, the SDS was removed from the samples by ethanol precipitation. The precipitates were resuspended in 350 μL of a solution consisting of 0.1 M NH_4HCO_3, 1 mM $CaCl_2$, and trypsin using an enzyme to substrate ratio of 1:25 (w/w). Tryptic digestion was performed for 4 h at 37 °C. Then the pH was adjusted to 2–3; the mixture was concentrated to 70 μL and applied directly to a reversed-phase HPLC column as described previously (Boehmer et al., 1987a). Amino acid sequence analysis of the fractions was performed manually using the microsequence double-coupling method (Chang et al., 1978). The N-terminally blocked fraction (see Results) was first degraded with chymotrypsin (0.2 μg of chymotrypsin in 50 μL of 0.1 M *N*-ethylmorpholine acetate, pH 8.1) for 4 h at 37 °C, and the cleavage products were directly subjected to the Edman degradation. Utilizing 50% trifluoroacetic acid for the conversion, we could unambiguously differentiate Ile and Leu after two-dimensional polyamide thin-layer chromatography by the

appearance of the additional purple colored byproduct spot associated with Ile according to Von Bahr-Lindstroem et al. (1982).

Protein Determination. Protein concentration was determined by the method of Lowry et al. (1951) with bovine serum albumin as standard. MFGM were incubated in 1 N NaOH for 20 min at 60 °C before assaying.

Results

We have shown recently that an antiserum raised either in mice or in rabbits against MDGI specifically recognizes the 13-kDa polypeptide inhibitor (MDGI) and neutralizes its activity (Boehmer et al., 1985, 1987a). The same anti-MDGI antibodies were used to detect inhibitor-related antigens in MFGM (Figure 1). Different bovine milk fractions were characterized by SDS–PAGE (Figure 1a) and analyzed by means of the dot immunobinding assay (Figure 1b) in comparison to pure MDGI (Figure 1b, lane A). The relative highest immunoreactivity was found in the homogenized MFGM (Figure 1b, lane B), followed by the 100000*g* supernatant obtained from homogenized MFGM (Figure 1b, lane C). In contrast, under these conditions, the soluble phase containing the bulk of milk and serum proteins (Figure 1b, lane D) did not react with the antiserum. Some immunoreactivity was found, however, in the soluble phase after considerable enrichment of a respective fraction (not shown). The data seem to be supported qualitatively by the protein pattern displayed by the different fractions in SDS–PAGE, showing in case of MFGM a band which comigrates with MDGI (Figure 1a, lanes A and B). In order to answer the question whether the MDGI-related antigen found in the 100000*g* supernatant was originally membrane-associated and became released from the membranes due to the purification procedure or, alternatively, is associated with the lipid inside the membrane vesicles, a butanol extraction of the "floating fat globule" was performed (Figure 1c). It is obvious that the amount of cross-reactive antigen present in the aqueous phase of the butanol extract (Figure 1c, lane B) was much lower than in MFGM (Figure 1c, lane A) or in the 100000*g* supernatant (Figure 1b, lane C). This means that most probably the MDGI-related antigen is associated with the globule membranes and not with the milk lipid globule, both budded from the apical plasma membrane of mammary epithelial cells. Moreover, if the intact MFGM were analyzed omitting the disruption and 100000*g* centrifugation steps, no immunoreactivity was observed (Figure 1c, lane C). This finding suggests a localization of the MDGI-related antigen close to the inner surface of the membrane vesicles and is in agreement with the low yield of cross-reactive antigen in the whey.

The molecular composition of the MDGI-related antigen(s) in MFGM was characterized by Western blot analysis (Figure 2). Among the numerous proteins present in MFGM (Figure 1a, lanes B and C), only one polypeptide apparently migrating slightly slower than MDGI (Figure 2, lane D) was clearly detected by immunostaining of nitrocellulose strips after electrotransfer of bovine (Figure 2, lane B) or human (Figure 2, lane C) MFGM proteins. For a control, a 100000*g* supernatant obtained from a homogenate of lactating bovine gland (Figure 2, lane A) and purified MDGI (Figure 2, lane D) were included in the experiment. The soluble cross-reactive antigen purified from bovine MFGM by electroelution comigrated with MDGI (Figure 2, lane E), suggesting that the apparent slower mobility of the antigen in intact bovine membranes is caused by the presence of other membrane components. From the presently available data, it cannot be decided whether the same applies to the human MFGM antigen or whether the latter indeed has a somewhat higher molecular mass than MDGI.

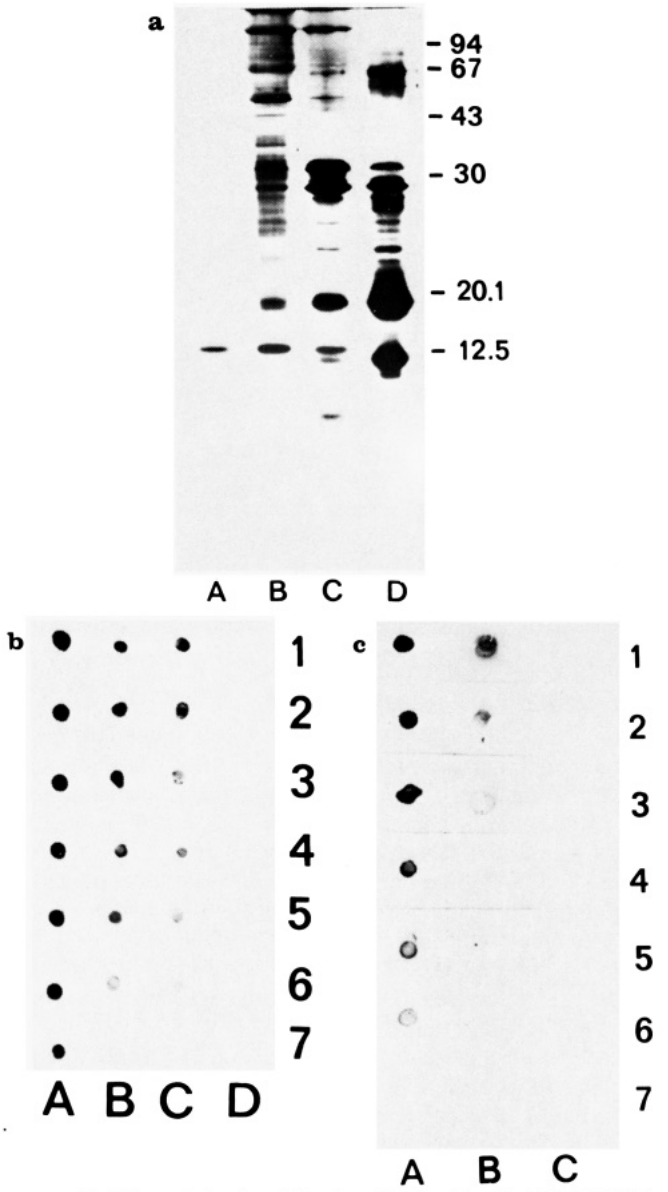

FIGURE 1: Characterization of bovine milk fractions by SDS–PAGE and dot immunobinding assay with an antiserum against MDGI. (a) SDS–PAGE of milk proteins. Polypeptides were detected by silver staining. Lane A, 1 μg of MDGI; land B, 30 μg of MFGM proteins; lane C, 30 μg of 100000*g* supernatant of MFGM preparation; lane D, 30 μg of soluble phase of milk. In lanes B–D, proteins were precipitated with acetone at −20 °C and then applied to gel electrophoresis. (b) Analysis of milk proteins by dot immunobinding assay. Lane A, MDGI; lane B, MFGM; lane C, 100000*g* supernatant of MFGM; lane D, soluble phase of milk. (c) Analysis of milk proteins by dot immunobinding assay. Lane A, MFGM; lane B, protein fraction obtained after butanol extraction of the lipid phase; lane C, MFGM proteins before the disruption and 100000*g* centrifugration step. For butanol extraction, 2 mL of the floating lipid phase recovered after centrifuging the homogenized MFGM at 100000*g* for 90 min was treated with 2 mL of 1-butanol and 1 mL of H_2O for 30 s. Then the mixture was centrifuged at 40000*g* for 30 min. The aqueous phase was applied to the immunodot analysis. SDS–PAGE of this phase showed an overall pattern similar to the 100000*g* supernatant (not shown). The following protein amounts were dotted onto nitrocellulose: 1, 500 ng; 2, 250 ng; 3, 125 ng; 4, 60 ng; 5, 30 ng; 6, 15 ng; 7, 7 ng. Immunostaining of proteins was performed with a 1:500 diluted rabbit anti-MDGI antiserum (1–7) or with the correspondingly diluted control serum which showed no reactivity (not shown). A sheep anti-rabbit immunoglobulin/peroxidase conjugate was used for detection.

Earlier, using an ELISA for detection of MDGI-related antigens, it was found that only samples from lactating bovine

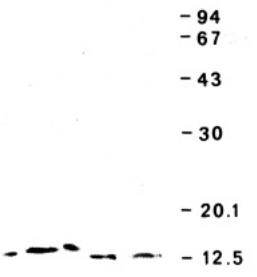

FIGURE 2: Western blotting analysis of MFGM proteins using a rabbit anti-MDGI antiserum. All samples were run in 15% SDS gels under reducing conditions. The gels were blotted to nitrocellulose. Immunostaining of transferred proteins was performed with the 1:250 diluted rabbit anti-MDGI antiserum. A goat anti-rabbit immunoglobulin/peroxidase conjugate was used for detection. Lanes A–C, 150 μg of protein was applied to electrophoresis and then blotted to nitrocellulose. Lane A, high-speed supernatant of a homogenate from bovine mammary gland (i.e., starting fraction of MDGI purification); lane B, MFGM from bovine milk; lane C, MFGM from human milk; lane D, 5 μg of MDGI; lane E, 5 μg of MFGM-derived antigen after preparative SDS–PAGE and electroelution.

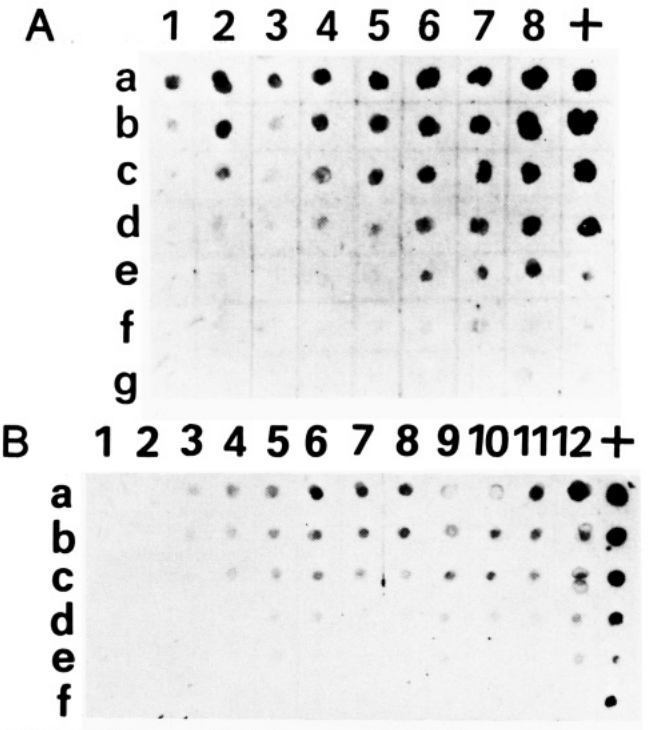

FIGURE 3: Dot immunobinding assay of MFGM from milk of a donor at different time intervals after delivery. MFGM were prepared from bovine (A) or from human (B) milk at different time intervals after delivery. Immunostaining was performed with a 1:100 diluted mouse anti-MDGI antiserum. A goat anti-mouse immunoglobulin/peroxidase conjugate was used for detection. (A) MFGM from bovine milk, hours after delivery: (1) 0.5; (2) 4; (3) 7.5; (4) 11; (5) 21; (6) 45; (7) 69; (8) 93 h. (+) shows MDGI as positive control. Protein amounts dotted onto nitrocelluose: (a) 100 ng; (b) 50 ng; (c) 25 ng; (d) 12 ng; (e) 6 ng; (f) 3 ng; (g) 1.5 ng. (B) MFGM from human milk, days after delivery: (1) 0.5; (2) 1; (3) 2; (4) 3; (5) 4; (6) 5; (7) 6; (8) 8; (9) 9; (10), 10; (11) 11; (12) 12 days. (+) shows MDGI as positive control. Protein amounts dotted onto nitrocellulose: (a) 200 ng; (b) 100 ng; (c) 50 ng; (d) 25 ng; (e) 12 ng; (f) 6 ng.

mammary gland gave a positive reaction, while samples from the gland of pregnant animals did not react (Boehmer et al., 1985). Further dot immunobinding assays were performed in order to test whether the relative amount of immunoreactive protein in MFGM also depends on the functional state of the mammary gland. The experiments revealed that the level of MDGI-related antigen increased dramatically with the onset of lactation after delivery both in human and in bovine MFGM (Figure 3A,B).

The structural relationship between MDGI and the MFGM-derived cross-reactive antigens was investigated directly by using tryptic digestion and partial sequence analysis of fragments of the 13-kDa polypeptide purified from bovine MFGM (Figure 4). Purification was achieved by running MFGM on preparative SDS gels followed by recovery from the gels of the band corresponding to a molecular mass of 13 kDa by electrophoretic elution. As shown in Figure 4a, lane B, the MFGM-derived 13-kDa polypeptide was homogeneous and exactly comigrated with MDGI when MDGI was applied both before (lane A) and after electroelution (lane C) to SDS–PAGE. Also, both MDGI and the MFGM-derived 13-kDa polypeptide revealed the same peptide maps after CNBr cleavage (not shown). When MDGI and the MFGM-derived 13-kDa polypeptide underwent tryptic digestion, both polypeptides were found to display an almost identical peptide map as demonstrated by the RP-HPLC runs of the tryptic fragments (Figure 4b). The peaks corresponding to peptides 1–5 were pooled, and a partial amino acid sequence analysis was performed. As shown in Table I, the amino acid sequences of peptides obtained from the MFGM-derived 13-kDa polypeptide are identical with those of MDGI. Therefore, most probably the MFGM-derived 13-kDa polypeptide consists of a polypeptide chain of almost identical structure as the mammary-derived growth inhibitor (MDGI).

Table I: Comparison of Amino Acid Sequences in Peptides Obtained from MDGI and the MFGM-Derived 13-kDa Polypeptide[a]

peak of elution pattern	sequence in MFGM-derived 13-kDa protein	position in sequence of MDGI[b]
1	Asn-Phe-Asp-Asp-Tyr	15–19
2[c]	Ser-Leu-Gly-Val-Gly-Phe-Ala-Thr-Arg Trp-Asn	22–30
3	Leu-Gly-Val-Glu-Phe-Asp	66–71
4	Leu-Ile-Leu-Thr-Leu	113–117
5	Val-Gly[d]	5–6

[a] The amino acid sequence of the tryptic fractions 3 and 4 was determined by the microsequence double-coupling method. For further details, see Materials and Methods. [b] The indicated positions mark the sequences in MDGI (Boehmer et al., 1987a) identical in both proteins. [c] Peak 2 is composed of two peptides. The main component is identical with seqeuence 22–30 in MDGI. For the minor component, the Edman degradation stops at Asn because of the well-known cyclization of Asn-Gly sequences. [d] Peak 5 in the case of MDGI representing the N-terminal tryptic fragment was N-terminally blocked in both proteins. Therefore, further cleavage was performed with chymotrypsin. Edman degration of the cleavage mixture yielded identical amino acids in both cases. For further details, see Materials and Methods.

Discussion

In this paper, a milk fat globule membrane-associated protein is described and shown to be closely related, most likely being identical with a previously described polypeptide growth inhibitor from bovine mammary gland (MDGI) (Boehmer et al., 1987a). MFGM originate from the apical membrane of the secretory cells of mammary epithelial tissue (Patton & Keenan, 1975). Therefore, a first conclusion is that MDGI isolated from bovine lactating mammary gland is an epithelial cell derived protein, providing confirmation of a previously

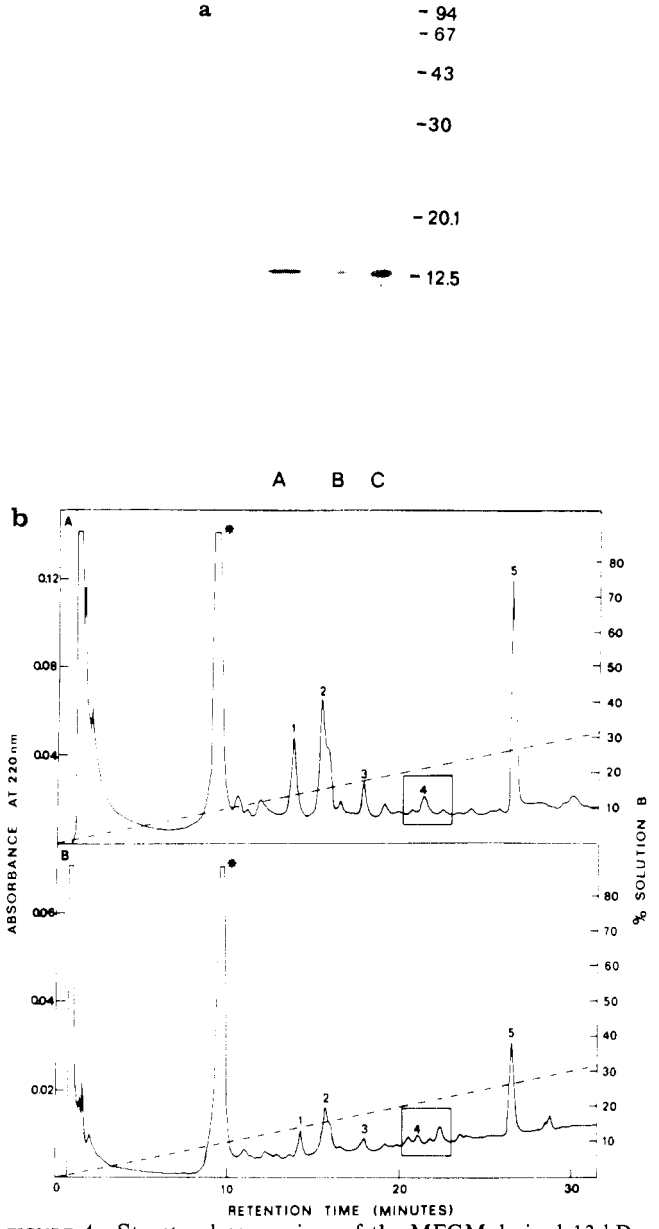

FIGURE 4: Structural comparison of the MFGM-derived 13-kDa polypeptide and MDGI by tryptic mapping. (a) Six micrograms of MDGI (lane A), 3 μg of the MFGM-derived 13-kDa polypeptide after electroelution (lane B), and 6 μg of MDGI after electroelution (lane C) were analyzed in 15% SDS gels under reducing conditions. Identical patterns were observed under nonreducing conditions (not shown). (b) Elution pattern after RP-HPLC of tryptic fragments obtained either from the MFGM-derived 13-kDa polypeptide after electroelution (A) or from MDGI which has, for reasons of comparison, undergone the identical electroelution procedure (B). Separation of tryptic peptides was obtained on a Vydac C4 (5 μm, 300 Å) column (75 × 4.6 mm) using a linear gradient of acetonitrile in 0.05% TFA (solution B). The flow rate was 1% buffer B per minute. Designation of peaks which were partially sequenced is arbitrary. The boxed area indicates fractions with an uncertain degree of coincidence. Therefore, peak 4 was additionally partially sequenced as shown in Table I. The peak marked with an asterisk is not composed of peptides, probably due to the isolation procedure.

hypothesized localization of the growth inhibitor in mammary gland tissue (Boehmer et al., 1985; Herrmann & Grosse, 1986).

Second, this protein seems to exist at least to a considerable extent in a plasma membrane associated state, pointing to an involvement in membrane-associated processes as transmembrane transport or receptor signal recognition. The experiments performed so far on intact MFGM with anti-MDGI antibodies suggest that the MDGI-related protein is bound to the inner surface of MFGM. Since it is found in the supernatant of homogenized MFGM and can be extracted easily (R. Brandt, unpublished results), it behaves as an extrinsic membrane protein. It should be noted that basolateral membranes purified from lactating bovine mammary gland (Spitzer & Grosse, 1987) also reacted with the anti-MDGI antiserum (M. Pepperle, unpublished results). Interestingly, for the fibroblast growth regulator (FGR-s) secreted or shed into the medium, it was suggested that membrane-bound forms of the molecule may exist (Steck et al., 1982). FGR-s is immunologically related to MDGI (Boehmer et al., 1987b). Therefore, a family of membrane-associated growth inhibitory polypeptides could exist, "stored" in and/or released from plasma membranes for exerting growth inhibition. In fact, the presence of MDGI in MFGM would explain the growth inhibitory effect of an aqueous extract of MFGM on proliferation of Ehrlich ascites mammary carcinoma cells (Herrmann & Grosse, 1986). Cessation of growth of cultured cells has been attributed partially to cell–cell contact. It was proposed that intercellular contacts generate intracellular "negative" signals for growth (Liebermann & Glaser, 1981). Our present findings support the idea of plasma membrane-associated growth inhibitory signals.

The MDGI concentration in mammary epithelial cells, as deduced from the presence in MFGM (this work) or from the reaction of immunoreactive antigen in glands from lactating or pregnant animals (Boehmer et al., 1985), seems to increase dramatically with the onset of lactation after delivery. This could be understood in a sense that a high concentration of growth inhibitor is associated with a state of low proliferation of mammary epithelial cells in the lactating gland. This would, in fact, be the reversed situation as observed with EGF in human milk (Shing & Klagsbrun, 1984) as well as with the number of EGF receptors in plasma membranes purified from lactating bovine mammary gland. The latter value was found significantly lower in membranes purified from lactating gland when compared to membranes from the gland of pregnant animals (Spitzer & Grosse, 1987).

The physiological significance of a growth inhibitor in milk, however, is difficult to understand. MDGI secretion into milk via MFGM might therefore be regarded as an abortive process. On the other hand, discussing the possible physiological significance of the presented findings, it should be taken into consideration that MDGI has been found to be structurally related to retinoid and fatty acid binding proteins (Boehmer et al., 1987a). These proteins are discussed to be capable of binding and transporting retinoic acid, fatty acids, or other hydrophobic compounds in an aqueous environment (Glatz et al., 1985). The hydrophobic ligands probably attached to MDGI have not yet been identified. The possible presence of MFGM-associated growth inhibitors might therefore have functional importance both for the transport of fatty acids or other hydrophobic ligands and also for the growth inhibitory potency of MDGI. For growth-stimulating factors, it is well-known that they are multifunctional both in vitro and in vivo (Sporn et al., 1986).

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Photoaffinity Labeling of Pituitary GnRH Receptors: Significance of the Position of Photolabel on the Ligand[†,‡]

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ABSTRACT: Photoreactive derivatives of GnRH and its analogues were prepared by incorporation of the 2-nitro-4(5)-azidophenylsulfenyl [2,4(5)-NAPS] group into amino acid residues at positions 1, 3, 6, or 8 of the decapeptide sequence. The modification of Trp[3] by the 2,4-NAPS group led to a complete loss of the luteinizing hormone (LH) releasing as well as LH-release-inhibiting activity of the peptide. The [D-Lys(2,4-NAPS)][6] analogue was a very potent agonist that, after covalent attachment by photoaffinity labeling, caused prolonged LH secretion at a submaximal rate. [Orn(2,4-NAPS)][8]-GnRH, a full agonist with a relative potency of 7% of GnRH, after photoaffinity labeling caused prolonged maximal LH release from cultured pituitary cells. In contrast, [Orn(2,5-NAPS)][8]-GnRH, although being equipotent with the 2,4-NAPS isomer in terms of LH releasing ability, was unable to cause prolonged LH release after photoaffinity labeling. Thus, [Orn(2,4-NAPS)][8]-GnRH is a very effective photolabeling ligand of the functionally significant pituitary GnRH receptor. Based on this compound, a pituitary peptidase resistant derivative, D-Phe[6],[Orn(2,4-NAPS)][8]-GnRH-(1–9)-ethylamide, was synthesized. This derivative showed high-affinity binding to pituitary membranes with a K_d comparable to those of other GnRH analogues. A radioiodinated form of this peptide was used for pituitary GnRH-receptor labeling. This derivative labeled 59- and 57-kDa proteins in rat and 58- and 56-kDa proteins in bovine pituitary membrane preparations, respectively. This peptide also labeled pituitary GnRH receptors in the solubilized state and therefore appears to be a suitable ligand for the isolation and further characterization of the receptor.

Receptors of peptide hormones can be identified and characterized and their role in mediating target cell response studied by photoaffinity labeling using suitable photoreactive ligands (Bayley & Knowles, 1977; Ramachandran et al., 1981; Eberle, 1983; Bayley, 1984). Successful application of photoaffinity labeling requires ligands with binding affinity close to the natural ligand. For direct receptor labeling it is of primary significance to select ligands whose photogenerated cross-linking groups are in optimal spatial arrangement and

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