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PURIFICATION OF A MAMMARY-DERIVED GROWTH INHIBITOR (MDGI) RELATED POLYPEPTIDE EXPRESSED DURING PREGNANCY

Ralf Brandt* and Richard Grosse**

Max-Delbrück-Center for Molecular Medicine, O-1115 Berlin-Buch, Germany

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The present study was undertaken to screen immunochemically for MDGI-related proteins in the mammary gland. A new form, MDGI 2, not present in lactation could be detected in the bovine gland during pregnancy. It was further distinguished from MDGI by its lower molecular weight, its association with a complex binding to WGA, and by lacking immunoreactivity to an anti-MDGI antibody directed against the C-terminus of MDGI. MDGI 2 was purified by chromatography over DEAE-Sepharose, Bio-Gel P-30 in 1% acetic acid, Sephacryl S-200 in 6 M urea and Mono Q. Final purification included HPLC on TSK G-3000 SW and electroelution from SDS-gels. Cell-free translation of poly (A^+)mRNA from glands of pregnant animal yielded one form identical with MDGI. We assume that post-translational processing of MDGI is involved in its activities.

MDGI was first purified from lactating bovine mammary gland (1) and Milk Fat Globule Membranes (2) when we searched for proteins inhibiting growth of mammary epithelial cells (3). MDGI has been sequenced (1) and cDNAs encoding the mouse and bovine form were cloned (5,17). MDGI belongs to a family of proteins known to bind hydrophobic ligands, referred to as Fatty Acid Binding Proteins, FABPs (6-8). The most close relatives to MDGI are the FABPs from heart (9) and brain (10). Although the function of FABPs is still unknown, a role in long chain fatty acid transport is widely assumed (7,8). However, adipocyte FABP is also a target for insulin receptor tyrosine kinase (3) and liver FABPs bind products of the lipoxygenase (11) and cyclooxygenase (12) pathways as well

*,**Present addresses:*National Institutes of Health, NCI/DCBDC,Laboratory of Tumor Immunology and Biology, and**Fogarty International Center, Bethesda, Maryland 20892.

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as selenium (13), indicating other regulatory properties. For the adipocyte (14), liver (15) and intestinal (16) FABP, a differentiation associated expression was shown. We have reported about tissue and cell specific MDGI expression linked to lobuloalveolar differentiation in the mouse and bovine mammary gland (5,17). For MDGI, the existence of various forms expressed at different stages of development was suggested (1,5). In order to address this question more directly, we have now analyzed mammary gland tissue from first-pregnant cow for MDGI-related proteins. Here we report about a new form expressed during pregnancy, which apparently lacks C-terminal amino acids known to be important for biological activities of MDGI. This unexpected finding raises the question whether post-translational processing of MDGI is required for its function.

MATERIALS AND METHODS

Materials: DEAE Sepharose, Sephacryl S-200, Mono Q and TSK G 3000-SW columns were from Pharmacia (Sweden) and Bio Gel P-30 from BioRad (USA). Anti-rabbit-IgG conjugated to biotin and streptavidine-alkaline phosphatase were obtained from Amersham (England).

Methods

Purification procedure: In all purification steps MDGI was detected by Western-blotting protein fractions using anti-MDGI-antibodies (5,18). First steps of purification of the MDGI-related form were according to the published procedure (1,4), except that mammary glands from first pregnant (5-7 months) cows were used. Proteins in a 100 000 xg supernatant were precipitated with ammonium sulfate at 80 % saturation, dialyzed against 50 mM imidazole-buffer, pH 8.0, and applied to a DEAE-Sepharose column equilibrated in the same buffer. The column was either eluted with a linear NaCl-gradient (100-700 mM NaCl in 50 mM imidazole, pH 8.0) or stepwise with 60 mM and 500 mM NaCl. The fractions containing MDGI 2 were dialyzed against 1 % acetic acid and passed over a BioGel P-30 column equilibrated in 1 % acetic acid. Immunoreactive fractions were lyophilized and redissolved in 20 mM PBS in 8 M urea. Subsequent chromatography on Sephacryl S 200 (2.5 cm x 95 cm) was performed in the same buffer. Immunoreactive fractions eluted with 20 mM PBS/6 M urea (2 ml/min) were pooled, dialyzed against 100 mM TRIS-HCL, pH 8.5, containing 6 M urea and applied to a Mono Q column (HR10/10). Bound proteins were eluted with a linear gradient (0-700 mM NaCl in 100 mM Tris/HCl, containing 6 M urea). The pooled immunoreactive peak fractions were precipitated with 10 % trichloroacetic acid, redissolved in 20 mM PBS, containing 0.1 % SDS , and applied to a TSK G 3000-SW column (7.5 mm x 600 mm, 0.25 ml/min), equilibrated in the same buffer. MDGI 2 containing fractions were pooled and subjected to preparative SDS-PAGE for electroelution as described in detail before (2). The eluted protein was precipitated with aceton, redissolved in Laemmli-buffer and subjected to SDS-PAGE. Slab gels were stained with silver ions according to (19).

Anti-MDGI Antibodies: Generation of antibodies against the 14.5 kDa MDGI molecule or against partial amino acid sequences of MDGI has been described earlier (5,18). Peptide-directed antibodies, referred to as anti-p69/78-IgG and anti-p121/131-IgG, are directed against peptides comprising amino acids 69-78 and 121-131 of the bovine MDGI-sequence (1), respectively.

Western Blot Analysis and Dot Immunobinding Assay : These methods were used as outlined before (2,18). The concentrations of antibodies used were 5 μg/ml and

20-50 µg/ml for anti-MDGI-IgG and for anti-peptide IgGs, respectively. Blots were developed by biotinylated anti-rabbit IgG followed by the Streptavidin-alkaline phosphatase conjugate (Amersham) and visualized with NBT/BCIP (Sigma)

Preparation of poly (A^+)-RNA and cell free translation: Total RNA was extracted from mammary gland of first-pregnant cow by the guanidinium isothiocyanate method (20). Poly (A^+)-mRNA was prepared and translated in a reticulocyte lysate as described previously (18). 200 µl of translation mixture containing 10 µg poly(A^+)-mRNA, 200 µCi (^{35}S) methionine and 150 µl reticulocyte extract were incubated for 30 min at 25 °C. Aliquots of the ^{35}S-methionine labeled translation products containing 10^6 cpm TCA-precipitable material were immunoprecipitated exactly as described before (18).
SDS-PAGE.: Laemmli gels with a gradient of acrylamid ranging from 15-22.5 % were used (21).

RESULTS

Three polyclonal antibodies directed against the entire molecule (anti-MDGI-IgG), against residues 69-78 (anti-p69/78-IgG) and against the C-terminal sequence spanning the last eleven amino acids 121-131 (anti-p121/131-IgG) were used for immunochemical screening (Fig. 1). As expected from earlier studies, MDGI is present in the lactating gland and can be eluted from DEAE-Sepharose at about 60 mM NaCl (Fig. 1, lanes 1-3). As shown, both peptide directed antibodies recognize MDGI (Fig. 1, lanes 2,3). Eluates obtained at high ion concentrations do not contain MDGI (Fig. 1, lane 4). If DEAE-fractions derived from the developing mammary gland of pregnant cow were analyzed, we found MDGI-

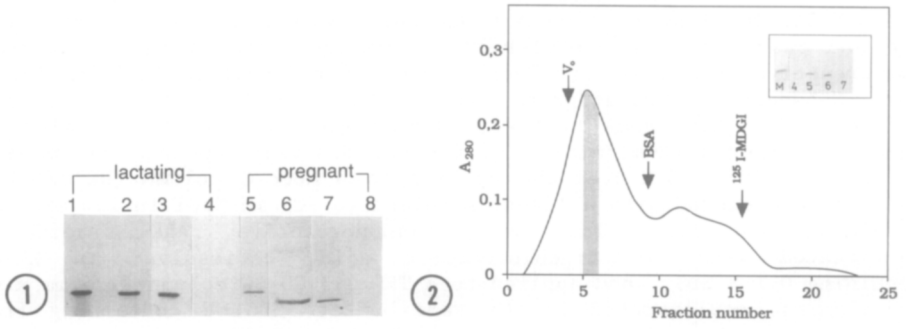

FIGURE 1. Immunochemical detection of MDGI 2 by Western-blotting DEAE-fractions obtained from 100 000 xg supernatant. Lanes 1,2 and 5, elution with 60 mM NaCl, in lanes 4 and 6-8 with 500 mM NaCl. Either lactating (lanes 1-4) or pregnant (lanes 5-8) mammary gland tissue was analyzed with anti-MDGI-IgG (lanes 1,4,5,6), with anti-p121/131-IgG (lanes 3,8), and with anti-p69/78-IgG (lane 2,7).

FIGURE 2. Separation of MDGI 2 containing fractions by gel chromatography. Proteins eluted with 500 mM NaCl from DEAE-Sepharose were applied to a Sephacryl S-200 column (1.5 cm x 95cm). Fractions (2 ml/min) were eluted in PBS, pH 7.4, and analyzed by Western-blotting with anti-MDGI-IgG (Insert). Filled bars show immunoreactive fractions.

immunoreactivity in the low salt concentration fraction (Fig 1, lane 5). This is in keeping with studies showing onset of MDGI synthesis during pregnancy (4,6,15). However, in contrast to the lactating gland, a second MDGI related form was detected in the mammary gland from pregnant cow (Fig. 1, lane 6). This form (referred to as MDGI 2) elutes at about 500 mM NaCl and was be distinguished from MDGI by its slightly lower apparent molecular mass (comp. lanes 5 and 6). More over, the new form does not cross-react with anti-p121/131-IgG (Fig. 1, lane 8). However, it is recognized by anti-p69/78-IgG (lane 7).

The relative contents of MDGI 2 and MDGI were compared by using a dot immunobinding assay. Assuming similar affinities of anti-MDGI-IgG to MDGI and MDGI 2 the relative yield of MDGI 2 in the mammary gland at pregnancy should be at least 10 fold lower than that of MDGI in lactation (not shown).

In order to further enrich MDGI 2, DEAE fractions were applied to a Sephacryl S-200 column under non-dissociating conditions (Fig. 2). As shown, immunoreactivity was found in the protein fractions 5 and 6 eluting with the void volume (proteins larger 70 kDa) (Insert, Fig. 2). Under same conditions, native MDGI elutes with fractions 15-20.

To address the question whether MDGI 2 could be associated with some higher molecular weight glycosylated complex, a high speed supernatant obtained from the mammary gland of pregnant animal was analyzed by WGA-chromatography and SDS-PAGE (Fig 3A). As shown, although both MDGI forms are present in the starting supernatant (Fig. 3A, lane S), only MDGI 2 was retarded by the

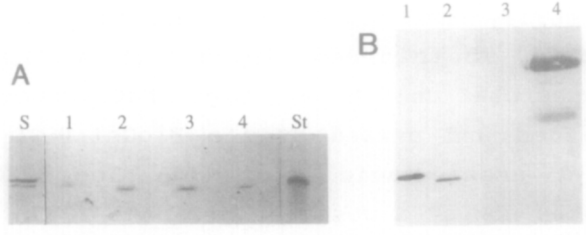

FIGURE 3. WGA-chromatography of a 100 000 xg supernatant from mammary gland of pregnant cow. A. Fractions of 1 ml eluted with N-acetylglucoseamin (lanes 1-4) were subjected to Western-blotting and analyzed with anti-MDGI-IgG. Starting supernatant (lane S) and MDGI standard (lane St) are shown in comparison to MDGI 2 containing fractions 2-4. B. Analysis of Western-blots of combined fractions 2+3. Blots of MDGI-standard (lane 1) and of fractions 2+3 (lane 2) were stained with anti-MDGI-IgG. 80 ng of MDGI standard (lane 3) and 20 μg of fractions 2+3 (lane 4) were loaded on a SDS-gel, transferred to nitrocellulose and incubated with concanavalin A conjugated to biotin as decribed under Methods.

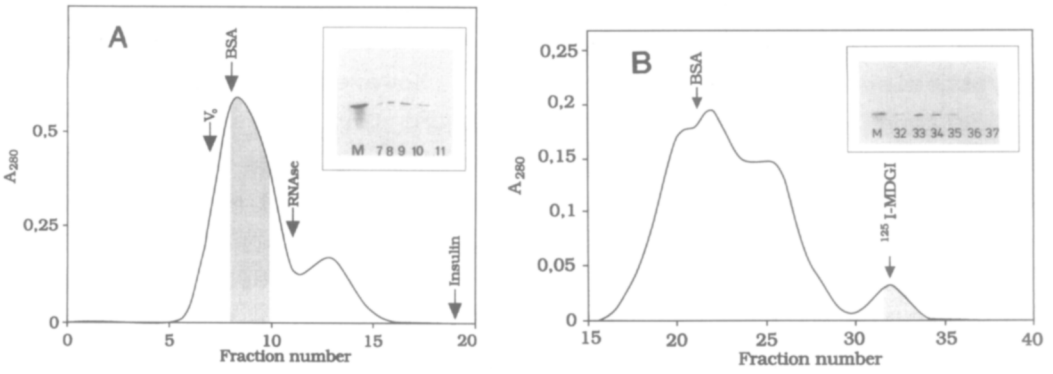

FIGURE 4. Gel-Chromatography of MDGI under dissociating conditions. A. Chromatographic separation of MDGI 2 containing fractions by BioGel P-30. The 500 mM fraction eluted from DEAE-sepharose was dissolved in 1 % acetic acid and passed over the column equilibrated in acetic acid. B. Fractions 7-10 were pooled, lyophilized, redissolved in 8 M urea and applied to a Sephacryl S-200 column (2.5 cm x 90 cm) equilibrated in 6 M urea in phosphate-buffer, pH 7.4. MDGI 2 was identified by Western-blotting eluates (Insert). Filled bars represent MDGI 2 containing fractions processed to further purification.

lectin and specifically eluted with N-acetylglucoseamin (Fig. 3A, lanes 1-4). We next tested whether MDGI 2 itself is glycosylated (Fig. 3B). To this end, fractions 2 and 3 of WGA-chromatography were combined, subjected to SDS-PAGE, electroblotted onto nitrocellulose and stained either by a concanavalin A-biotin-complex for glycosylation sides (Fig. 3B, lane 4) or by anti-MDGI-IgG for the protein (lane 2). As shown, MDGI 2 and MDGI (lane 3) are not glycosylated.

Summarizing this part of data a complex of MDGI 2 with some not yet identified glycosylated component eluting from Sepharose S-200 with the void volume and binding to WGA seems more likely than an aggregation of MDGI forms.

In order to purify MDGI 2, it was necessary to dissociate the presumed complex. To this end, immunoreactive fractions eluting from DEAE-Sepharose column at high NaCl-concentrations were pooled, acidified and passed over a BioGel P-30 column equilibrated in 1 % acetic acid (Fig. 4A). The MDGI 2 containing fractions eluted now at a position of about 20-30 kDa.

They were lyophilized, redissolved and passed again over the WGA-column. Under these conditions, MDGI 2 did not bind and was detected in the flow-through supporting our assumption about its association with some glycosylated component(s) (not shown).

Lyophilized BioGel P-30 fractions 7-10 were redissolved in presence of 8 M urea phosphate buffer, pH 7.4, by heating and

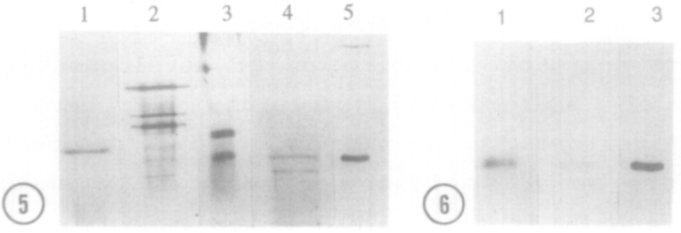

FIGURE 5. Purification of MDGI 2 by electroelution. Fractions obtained from chromatography over TSK G 3000-SW were pooled, subjected to SDS-PAGE, the MDGI 2 band was cut out, processed to electrotransfer and reanalyzed by SDS-PAGE (lane 4). Lane 5 represents the Western-Blot corresponding to lane 4. Lanes 1-3: SDS-gels of the MDGI standard, the Mono Q and of the TSK G 3000-SW fractions, respectively. Gels were stained with silver ions.

FIGURE 6. Immunoprecipitation of MDGI after cell-free translation. Poly (A^+) mRNA from the mammary gland of first-pregnant cow was isolated and translated in a reticulocyte lysate. ^{35}S-methionine labeled proteins were immunoprecipitated with anti-MDGI-IgG in the absence (lane 1), or presence of excess cold MDGI (lane 2). Coomassie stained MDGI standard is shown in lane 3.

applied to Sephacryl S-200 chromatography. MDGI 2 was now eluted with the 12-16 kDa fraction containing the ^{125}I-labeled MDGI standard (Fig. 4B). The MDGI 2 containing fraction was passed over Mono Q column, the fractions eluting at 120-150 mM NaCl were combined and applied for final purification to size exclusion HPLC (not shown). The immunoreactive fractions obtained after chromatography on Mono Q and TSK G 3000-SW were analyzed by SDS-PAGE and silver staining (Fig. 5, lanes 2 and 3, correspondingly). As the final fraction after size-exclusion chromatography still revealed heterogeneity, the band corresponding to MDGI 2 was cut out from the gel, subjected to electroelution and re-evaluated by SDS-PAGE (Fig. 5, lane 4). The electroeluted material was characterized by two bands, one unknown component and a second one which was confirmed by Western-blotting to be MDGI 2 (Fig. 5, lane 5).

Finally, in order to test whether glandular tissue taken from pregnant animal contains MDGI 2 mRNA, poly (A^+) mRNA was translated in vitro and the translation products were immunoprecipitated with the anti-MDGI-IgG in the absence (Fig. 6, lane 1) and presence of excess MDGI (Fig. 6,lane 2). As shown, only one form co-migrating with the MDGI-standard (Fig. 6,lane 3) could be detected. It seems therefore more likely that MDGI 2 is the product of some post-translational MDGI-modification than being translated by its own mRNA.

DISCUSSION

In order to approach the question of developmentally regulated synthesis of MDGI-related proteins, we have looked for immunoreactive forms in the mammary gland of pregnant animal.
The new form we first described here is by several criteria different from MDGI: First, MDGI 2 was detected in the mammary gland of pregnant cows and was absent in the lactating tissue where MDGI reaches highest level of expression (5,17,18). Second, compared to MDGI, it is rather a minor protein what prevented its sequencing in this study. Third, under non-dissociating conditions MDGI 2 is associated with proteins eluting in the void volume of Sepharose S-200 and binding to WGA. As neither MDGI nor MDGI 2 are glycosylated, other glycosylated polypeptides must be involved. Fourth, MDGI 2 is not recognized by an antibody raised against a peptide comprising the C-terminus of MDGI. It is therefore likely, that MDGI 2 is a slightly truncated version of MDGI or is missing C-terminally located epitopes of MDGI. Interestingly, the C-terminal peptide mimics MDGI activities (3,22).
Despite these differences, MDGI and MDGI 2 seem to be structurally related comparing molecular weight, chromatographic separation under dissociating conditions and reactivity to specific MDGI antibodies. We were not able to detect MDGI 2 mRNA in a cell-free translation assay. It is therefore tempting to assume that MDGI 2 is produced by post-translational proteolytic MDGI-processing during lobuloalveolar differentiation when expression of MDGI is known to begin (5,17). Indeed proteolytic cleavage of MDGI appears to be possible at position 126 where a potential cleavage side exists (2). Furthermore, as mentioned before, we have found that C-terminally derived synthetic MDGI peptides behave like MDGI in two respects: they inhibit growth of Ehrlich ascites tumor cells (1), and they prevent eicosanoid dependent sensitization of beta 2-adrenergic receptors (17). We currently testing the hypothesis whether MDGI 2 and an active peptide are proteolytically derived from MDGI during pregnancy and whether these events could be associated with growth inhibition of cells entering differentiation.

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