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Growth Inhibitory and Cytotoxic Polypeptides 1–6

A Polypeptide Growth Inhibitor Isolated From Lactating Bovine Mammary Gland (MDGI) is a Lipid-Carrying Protein

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Mammary-derived growth inhibitor (MDGI), a polypeptide growth inhibitor isolated from lactating bovine mammary tissue, previously shown to have extensive sequence homology with fatty acid-binding proteins, was demonstrated to meet the criteria of a fatty acid-binding protein. The protein was found to bind [^{3}H]palmitic acid in a saturable manner and to be complexed with endogeneous free fatty acids. [^{3}H]palmitic acid, when bound to the protein, was more rapidly taken up by the target cells (human mammary carcinoma cells [MaTu]) than was free [^{3}H]palmitic acid, suggesting a lipid carrier function for the inhibitor. It is suggested that the fatty acid-binding properties of MDGI may relate to its ability to inhibit cell growth in vitro and to regulate other cellular functions.

Key words: fatty acid-binding protein, mechanism of action

A new polypeptide growth inhibitor has recently been purified from lactating bovine mammy tissue, based on its inhibitory activity toward the proliferation of cultured Ehrlich ascites mammary carcinoma cells [1,2]. The protein, termed 'mammary-derived growth inhibitor' (MDGI) [3] has a molecular mass of 14.5 kD and an isoelectric point of 4.6–4.9. Based on the observation that an indistinguishable protein is associated with milk fat globule membranes [4], it was shown tht MDGI is produced by mammary epithelial cells at a level that is increased in the lactating vs. the nonlactating gland. MDGI is not only an inhibitor of cell growth but is also able to regulate lactate consumption and sensitivity to hydrophilic beta$_2$-agonists in cultures of neonatal rat heart cells (G. Wallukat et al., published observations).

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The amino acid sequence of MDGI was found to be homologous with the sequences of a number of proteins known or presumed to bind hydrophobic ligands, including a heart-derived fatty acid-binding protein (H-FABP), myelin protein P2, cellular retinoid binding proteins, and a differentiation-associated protein from 3T3-L1 adipocytes [3]. A study [5], prompted by the discovery of these sequence homologies revealed immunological cross reactivity not only between MDGI and H-FABP, but also between MDGI and a 13-kD fibroblast growth inhibitor (FGR-s) isolated from the conditioned medium of cultured 3T3 fibroblasts.

In recent work we have begun to investigate the possible hydrophobic ligand-binding properties of MDGI suggested by the similarity of the protein to H-FABP. This report describes some of our preliminary observations of the ability of MDGI to form complexes with fatty acid moieties and to promote the cellular uptake of ^{3}H-labeled palmitic acid. These properties of MDGI are discussed in the context of its ability to inhibit cellular growth in vitro.

MATERIALS AND METHODS

Growth Inhibitor Preparation

MDGI was purified and assayed for growth inhibitory activity using cultured Ehrlich ascites mammary carcinoma cells, as described [2,3]. Preparations to be analyzed for their content of endogeneous fatty acids or prostaglandins were stored under liquid nitrogen.

Cells and Reagents

Cells of the human mammary carcinoma cell line MaTu [6] were grown in minimal essential medium (MEM), supplemented with 50 μg/ml gentamycin and 10% calf serum. (9,10(n)[^{3}H]Palmitic acid (50 Ci/mmol) was purchased from Amersham, palmitic acid was from Sigma, and Lipidex 1000 was from Packard.

Binding of [3H]Palmitic Acid to MDGI

[^{3}H]Palmitic acid (10μCi) in 10μl of toluene was dried onto a glass ampoule under a stream of nitrogen and redissolved in 15μl of ethanol. MDGI (20μg) in 200μl of buffer (HBSS) (final concentration $\sim 7 \times 10^{-6}$ M) was then incubated overnight at 4°C with 5μl $\sim$ 3μCi, final concentration $\sim 3 \times 10^{-7}$ M) of the ethanolic [^{3}H]palmitic acid solution in the presence or absence of 1,000-fold excess of unlabeled palmitic acid (added in 5μl ethanol). The mixtures were then chromatographed on a 1 × 2.5-cm column of Lipidex 1000, previously equilibrated with HBSS at 4°C. For the recovery of protein-bound [^{3}H]palmitic acid, the column was eluted with HBSS. MDGI, together with MDGI-complexed [^{3}H]palmitic acid, was eluted in the void volume. [^{3}H]Palmitic acid bound to the column was then eluted with methanol. Fractions of 0.5 ml were collected, and radioactivity in aliquots of the fractions was measured by liquid scintillation counting (efficiency 35–40%).

Cellular Uptake of [^{3}H]Palmitic Acid

MaTu cells were grown to confluency in 35-mm (ID) dishes (Nunc). The cultures were washed three times with ice-cold HBSS and then incubated with either 20,000–40,000 cpm free [^{3}H]palmitic acid or MDGI-complexed [^{3}H]palmitic acid (obtained from the void volume fractions of the Lipidex column, described above) at

4°C in a total volume of 1.25 ml HBSS. In the case of MDGI-complexed tracer, the MDGI concentration ranged between 400 and 700 ng/ml (2.8×10^{-8}–4.8×10^{-8} M). For competition studies, a 2- to 80-fold excess of MDGI, not labeled with [^{3}H]palmitic acid, was included in the incubations.

Incubation was terminated by aspirating the medium and washing the cells three times with ice-cold HBSS. Then cells were scraped into a total volume of 1 ml HBSS, transferred to 1.5-ml microfuge tubes, and sedimented by centrifugation for 2 min at 4°C in an Eppendorf microfuge.

The supernatants were discarded, the pellets were dissolved by incubation in 0.2 ml 1% SDS/0.1 N NaOH for 30 min at 37°C and neutralized with hydrochloric acid, and radioactivity in the samples was measured with a toluene-based scintillation cocktail in a liquid scintillation counter (efficiency 35–40%). All determinations were performed in triplicate. Blanks without cells in the dishes yielded negligible radioactivity when processed according to the above method.

Chemical Analyses

Lipid extracts [7] of MDGI preparations were subjected to thin-layer chromatography to purify the fatty acid fraction. The fatty acids were then analyzed as their methyl esters by gas-liquid-chromatography, as described [8]. For analysis of endogeneous prostaglandins in the MDGI preparations, samples were subjected to conversion and radioimmunoassay procedures, as described previously [9].

RESULTS

The extensive sequence homology (84%) found between MDGI and H-FABP [3] suggested that MDGI might also display a fatty acid-binding capacity. As shown in Figure 1, when MDGI preequilibrated with [^{3}H]palmitic acid was passed over a Lipidex 1000 column [10], a substantial proportion (40–50%) of [^{3}H]palmitic acid

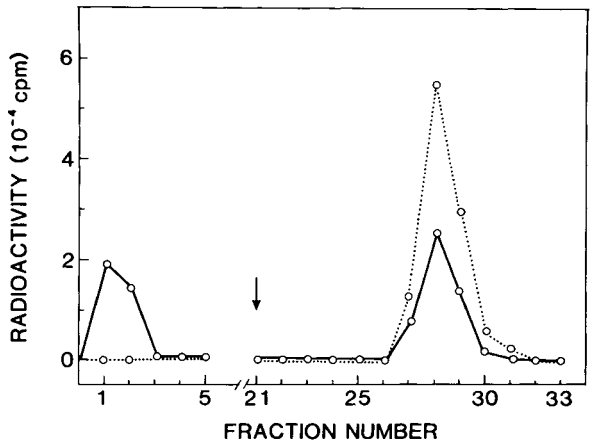

Fig. 1. [^{3}H]palmitic acid binding to MDGI. [^{3}H]palmitic acid (3 μCi) was incubated at 4°C for 16 h in the presence (. . .) or absence (—) of a 1,000-fold excess of unlabeled palmitic acid with 20 μg MDGI. The resulting mixtures were chromatographed at 4°C over a 1 × 2.5-cm Lipidex 1000 column. First, elution was performed with HBSS. The arrow indicates where the eluent was changed to methanol. Fractions of 0.5 ml were collected, and the radioactivity was measured in 50 μl aliquots.

was eluted in the void volume along with MDGI; nonprotein-bound [^{3}H]palmitate was recovered in the methanol-eluted fractions. In contrast, when MDGI was equilibrated with the same concentration of [^{3}H]palmitic acid (about 6×10^{-8} M) along with a 1,000-fold excess of unlabeled palmitate, passage of the mixture over the Lipidex column revealed an absence of radioactivity in the void volumne; all of the radioactivity was recovered in the methanol-eluted fractions. These data indicated that MDGI can form a complex with [^{3}H]palmitic acid that exhibits some degree of specificity and saturability, in that unlabeled palmitate can compete completely for the binding of [^{3}H]labeled palmitate. This binding is reversible, since prebound [^{3}H]palmitic acid can likewise be displaced by an excess of unlabeled palmitic acid (not shown).

To explore further the fatty acid-binding characteristics of MDGI, we analyzed a number of preparations for their content of both fatty acids (Table I) and prostanoids. We presume that these analyses reflect preformed complexes between MDGI and the fatty acid moieties formed either prior to or during the course of protein isolation. As outlined in Table I, lipid analysis revealed that appreciable amounts of long-chain fatty acids were associated with MDGI, with oleic acid and palmitic acid as the predominant ligands.

Using the complex formed between MDGI and (^{3}H)-labeled palmitate, we evaluated the ability of MDGI to promote the cellular uptake of fatty acid in comparison with the fatty acid uptake that can occur in the absence of MDGI (Fig. 2A). These experiments revealed that the uptake rate of the [^{3}H]palmitate precomplexed with MDGI was appreciably increased (by about 60%) over the rate of uptake of free [^{3}H]palmitic acid in the absence of MDGI. Since MDGI contains appreciable amounts of precomplexed unlabeled palmitate (Table I), the net rate of palmitate uptake promoted by MDGI would be even greater than that suggested by the data in Figure 2A.

Since MDGI appeared to promote the uptake of (^{3}H)-labeled palmitate, potentially via an MDGI binding site, we measured the ability of unlabeled MDGI to affect the cellular uptake of the MDGI-[^{3}H]palmitic acid complex (Fig. 2B). The concentration dependence of the observed inhibition by unlabeled MDGI for the uptake of [^{3}H]palmitate-labeled MDGI suggested the presence of a saturable carrier responsible for the MDGI-facilitated uptake of [^{3}H]palmitate (Fig. 2B).

DISCUSSION

The main finding of our study was the MDGI, a polypeptide growth inhibitor distinct from those described previously [3], exhibited fatty acid-binding activity.

TABLE I. Fatty Acid Content of MDGI

Fatty acid	Total fatty acid content (%) (mean ± SEM, n = 3)[a]
16:0	16.2 ± 4.5
18:0	9.4 ± 2.8
18:1	34.9 ± 11.2
18:2	7.5 ± 1.5
20:4	1.9 ± 0.6

[a]Total fatty acid content was 1.16 ± 0.58 nmol per nmol MDGI; minor fatty acids representing less than 3% of the total are not listed, except arachidonic acid (20:4).

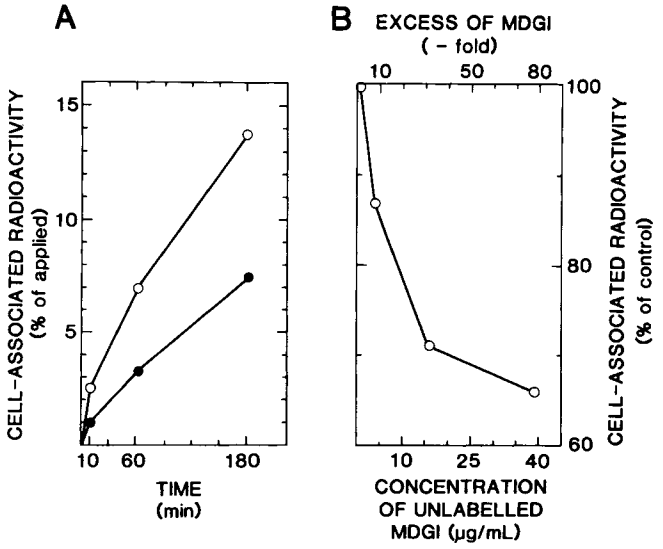

Fig. 2. MDGI-facilitated uptake of [^{3}H]palmitic acid by MaTu cells. **A:** MaTu cells were incubated with 21,000 cpm/ml [^{3}H]palmitic acid (final concentration ~ 0.5×10^{-9} M), either added directly from a ethanolic solution (●) or precomplexed to MDGI (○). The concentration of [^{3}H]palmitic acid-labeled MDGI was 500 ng/ml (~ 3.4×10^{-8} M). Cell-associated radioactivity was measured at the indicated intervals, as described in Materials and Methods. **B:** The effect of unlabeled MDGI on the uptake of [^{3}H]palmitic acid precomplexed to MDGI is shown. The experiment was performed as in A, except that the indicated amounts of unlabeled MDGI were added to the incubation mixture. Termination of incubation was performed after 60 min. Maximally, 5,100 cpm were taken up by the cells; this value was used as 100%.

This activity, along with the analytical data revealing the presence of near-stoichiometric amounts of fatty acid in the isolated protein and the sequence homology with H-FABP, suggests strongly that MDGI belongs to the family of fatty acid-binding proteins (or "Z"-proteins); such proteins are present in the cytosol of many cell types. The total fatty acid content to MDGI, as isolated (1–2 moles fatty acid per mole protein), and the fatty acid composition were similar to those observed for other FABP preparations [11,12]. The isolation of a fatty acid-binding protein from lactating mammary gland has recently been described by Whetstone et al. [12]. Although no structural information was provided by these authors, the physiochemical properties and chromatographic behaviour of the protein were in keeping with the known properties of MDGI [2,3,12]. It is possible that MDGI [2,3] and the recently isolated mammary FABP [12] represent the same protein isolated according to different functional criteria.

Another interesting finding of the present study was that MDGI was able to facilitate the cellular uptake of [^{3}H]palmitic acid when precomplexed to MDGI, compared with the uptake of free [^{3}H]palmitic acid. We cannot decide whether the cell-associated radioactivity measured in these studies represents intracellular or cell surface-associated radioactivity. So far, the term "cellular uptake" is only operational. The apparently saturable process has similarities to the interaction of the lipid carrier proteins serum albumin and alpha-fetoprotein with target cells [13–15]. Alpha-fetoprotein is thought to exhibit biological activity because of its ability to bind and/or regulate the cellular uptake of biologically active hydrophobic agents (for example,

estrogen) [15]. A similar mechanism underlying the growth-inhibitory activity of MDGI in vitro would be quite distinct from the receptor-mediated transmembrane signaling processes [16] triggered by growth-regulatory polypeptides like interferon, insulin, and epidermal growth factor. Although our data suggest a saturable mechanism for the cellular uptake of the MDGI-palmitate complex, this process need not necessarily involve a protein "acceptor" (or receptor of the class II type) like the one for low-density lipoprotein. For instance, it has been demonstrated thast a ligand precomplexed with H-FABP was readily delivered to artificial liposomes [17]; this process would occur without the participation of an acceptor. Thus the exact nature of the uptake process of an MDGI-bound ligand in intact cells remains to be determined.

Our preliminary study leaves many unanswered questions related to the specificity, reversibility, binding rate, affinity, and capacity of fatty acid binding activity of MDGI. Furthermore, it will be important to determine for the endogeneous constituents bound to MDGI the biological activities in the cellular assay systems employed so far in characterizing MDGI. Our ongoing work, aimed at answering these questions, should shed more light on the mechanisms whereby MDGI at nanomolar concentration can inhibit cell replication.

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