

Structural and enzymatic characterization of human recombinant GDP-D-mannose-4,6-dehydratase

Angela Bisso, Laura Sturla, Davide Zanardi, Antonio De Flora, Michela Tonetti*

Department of Experimental Medicine, Section of Biochemistry, University of Genova, Viale Benedetto XV, 1, 16132 Genova, Italy

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Abstract GDP-D-mannose-4,6-dehydratase (GMD) is the key enzyme in the 'de novo' pathway of GDP-L-fucose biosynthesis. The reported cDNA sequences for human GMD predict three forms of different length, whose 'in vivo' occurrence and molecular properties are completely undefined. Here, we report the expression in *Escherichia coli* and the properties of each native recombinant GMD form. Only the 42 kDa long GMD (L-GMD) and the 40.2 kDa (M-GMD) forms were recovered as soluble functional proteins, while the 38.7 kDa form, short GMD (S-GMD), lacking an N-terminal domain critical for dinucleotide binding, was inactive and formed a precipitate. Both L-GMD and M-GMD are homodimers and contain 1 mol of tightly bound NADP⁺. Their kinetic properties (K_m , K_{cat}) are apparently identical and both forms are non-competitively feedback-inhibited by GDP-L-fucose to a similar extent. M-GMD is the predominant enzyme form expressed in several human cell lines. These data seem to suggest that modulation of the 'de novo' pathway of GDP-L-fucose biosynthesis involves mechanisms other than differential 'in vivo' expression of GMD forms.

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Key words: GDP-D-mannose-4,6-dehydratase; L-fucose; NADP⁺; Human lymphocyte

1. Introduction

GDP-D-mannose-4,6-dehydratase (GMD) (EC 4.2.1.47) is the first enzyme involved in the 'de novo' pathway of GDP-L-fucose biosynthesis, converting GDP-D-mannose to GDP-4-keto-6-deoxy-D-mannose. This pathway, initially described by Ginsburg in *Aerobacter aerogenes* [1,2], was then also identified in animals and plants [3–8]. GDP-L-fucose is the substrate for fucosyltransferases which catalyze the insertion of the deoxyhexose in a number of glycoconjugates in the Golgi apparatus [9]. L-fucose-containing oligosaccharides, in particular antigens of the Lewis system, such as Le_x and SLe_x have been demonstrated to be the physiological ligands for selectins and to play a critical role in the processes of rolling and extravasation of leukocytes beyond the endothelial barrier that start the inflammation [10–12].

Recently, the cDNA for GMD has been cloned from hu-

man HL-60 cells [13]. The cDNA sequence predicts a protein with two possible initiation methionines, corresponding to 42 kDa, long GMD (L-GMD) and to 40.2 kDa, medium GMD (M-GMD), respectively, and showing a 60% identity with the *Escherichia coli* enzyme. The human protein, starting at the second methionine (M-GMD), was expressed in *E. coli* as a fusion protein containing a His-leader sequence and was partially characterized [13]. An identical cDNA sequence was observed for GMD independently cloned from human lymphocytes in our laboratory [14]. A second cDNA sequence, encoding a shorter human GMD protein of approximately 38.7 kDa, short GMD (S-GMD), and lacking the N-terminal fragment, was proposed by another research group [15]. They isolated a cDNA from a human fetal brain library that was able to restore L-fucose production in the Lec-13 CHO clone, in which GMD activity is defective.

Since GMD represents the limiting step in the metabolic pathway for the 'de novo' production of GDP-L-fucose, we undertook a study aimed at to better characterize the structural and kinetic properties of this enzyme in human cells. As at least three slightly different proteins can derive from the cDNA sequences reported so far, all of them were expressed as glutathione transferase (GST) fusion proteins in *E. coli* and the properties of each native protein obtained after proteolytic removal of the GST tag were then investigated. Furthermore, the patterns of expression of the three GMD forms were analyzed in a panel of human cell lines.

2. Materials and methods

2.1. Protein expression and purification

The three putative forms of GMD, whose N-terminal sequences are reported in Fig. 1B, were expressed in *E. coli* as fusion proteins with GST, using pGEX-6P1 vector (Pharmacia, Milan, Italy). Total RNA was obtained from human blood lymphocytes using Trizol reagent (Life Technologies Italia, Milan, Italy) and was retrotranscribed using oligo-dT and MuMV-RT (Promega, Madison, WI, USA) as described [16]. The resulting cDNA was amplified using gene specific primers¹, which were obtained by TibMolBiol (Genova, Italy). PCR conditions were as previously described [14]. After purification, the products of amplification were ligated in the pGEX-6-P1 vector. A competent *E. coli* K803 strain was used for transformation.

Conditions for the expression of L- and M-GMD were as reported previously [14,17]. Due to the formation of inclusion bodies, which could not be prevented using different induction conditions, bacterial cells expressing the shortest GMD form (S-GMD) were lysed using sarkosyl, following the published protocol [18]. The fusion proteins were purified by affinity chromatography using GSH-Sepharose (Pharmacia), following the supplier's instructions. Cleavage of the

*Corresponding author. Fax: (39) (010) 354415.
E-mail: tonetti@unige.it

Abbreviations: GMD, GDP-D-mannose-4,6-dehydratase; L-GMD, long GMD (42 kDa); M-GMD, medium GMD (40.2 kDa); S-GMD, short GMD (38.7 kDa); GST, glutathione transferase; DTT, dithiothreitol; BSA, bovine serum albumin; BS³, bis(sulfosuccinimidyl)suberate; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

¹ Primers were designed using the sequences deposited in the GenBank database under GenBank accession number AF042377 [13] for L-GMD and M-GMD and under GenBank accession number AF040260 [15] for S-GMD.

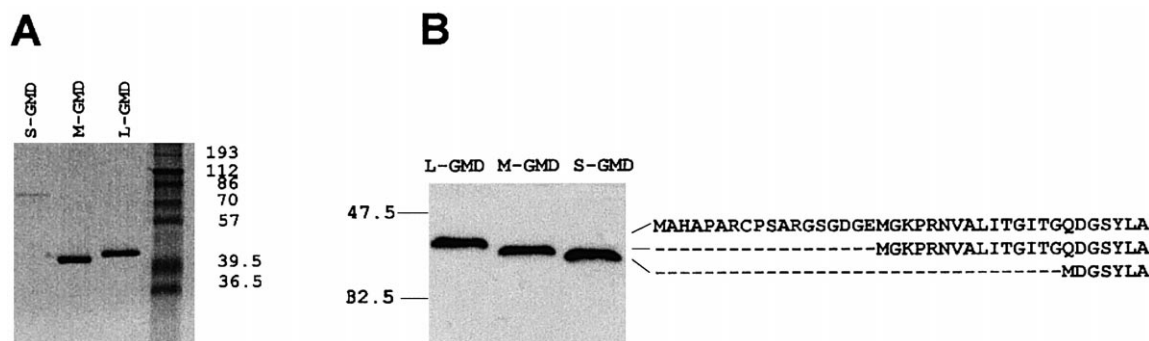


Fig. 1. (A) SDS-PAGE of the three recombinant forms of GMD, obtained after purification from bacterial lysates (Section 2.1). 5 μ g of L-GMD and M-GMD was loaded. Conversely, after proteolytic cleavage, S-GMD immediately formed a precipitate and could not recover in significant amounts as soluble protein. The corresponding eluate from GSH-Sepharose, analyzed by Coomassie brilliant blue R-250 staining, gave only one band of approximately 70 kDa. This might correspond to the *E. coli* chaperonine protein dnaK, a well known contaminant of recombinant proteins, which is involved in the degradation of abnormal proteins [22]. Prestained molecular markers (Sigma): 193 kDa, α -macroglobulin; 112 kDa, β -galactosidase; 86 kDa, fructose 6-phosphate kinase; 70 kDa, pyruvate kinase; 57 kDa, fumarase; 39.5 kDa, lactate dehydrogenase; 36 kDa, triosephosphate isomerase. (B) Western blot analysis and amino acid sequence of the three forms of recombinant GMD. Approximately 50 ng of proteins was loaded for L-GMD and C-GMD. For S-GMD, the eluate from GSH-Sepharose was directly loaded on the gel. Samples were subjected to SDS-PAGE on a 12% acrylamide gel and to blotting on a nitrocellulose membrane. Detection was achieved using a rabbit polyclonal anti-bacterial GMD antibody [14] and by the ECL method (Section 2.2). Prestained molecular markers (New England Biolabs): 175 kDa, MBP- β -galactosidase; 83 kDa, MBP-paramyosin; 62 kDa, glutamate dehydrogenase; 47.5 kDa, aldolase; 32.5 kDa, triosephosphate isomerase.

native forms of GMD from the matrix-bound fusion proteins was achieved using Prescission Protease (Pharmacia) upon incubation for 16 h at 4°C in 50 mM Tris-HCl buffer, pH 7.5, containing 1 mM Na₂EDTA and 1 mM dithiothreitol (DTT).

2.2. Structural characterization and determination of apoenzyme-bound NAD⁺/NADP⁺

The molecular weight and the purity of the recombinant proteins were analyzed by 12% discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [19] and by Western blotting. After blotting, the membrane was incubated with a rabbit polyclonal antibody raised against bacterial GMD [14], which, due to the sequence identity of the proteins between the two species (more than 60%), is cross-reactive with human GMD. Detection was performed, using a peroxidase-labelled secondary antibody, with the ECL system (Amersham, Milan, Italy). Protein was estimated by the Bradford method [20], using bovine serum albumin (BSA) as standard. The oligomeric structure of the protein was investigated by gel filtration and by crosslinking experiments, using bis(sulfosuccinimidyl)suberate (BS³) (Pierce, Rockford, IL, USA). Gel filtration was performed as described [21]. For BS³ experiments, the proteins were extensively dialyzed against phosphate-buffered saline (PBS) containing 1 mM DTT. GMD (100 μ g/ml) was then incubated in PBS with increasing concentrations of BS³, to define the optimal reaction conditions, for 1 h at 4°C, either in the presence or absence of 1 mg/ml BSA. The reaction was then stopped by incubating for 30 min at room temperature in the presence of Tris-HCl, pH 8.0, at a 50 mM final concentration and the samples were analyzed by Western blotting.

The presence of tightly protein-bound dinucleotide cofactors was investigated by a highly sensitive enzymatic cycling assay [17]. To avoid interference due to presence of DTT in the cleavage buffer, the purified proteins were extensively dialyzed against 100 mM sodium phosphate buffer, pH 8.0, prior to the assay.

2.3. Determination of kinetic properties

The enzymatic activity of the purified proteins was determined by high performance liquid chromatography and thin layer chromatography analyses as described [21], using as substrate GDP-D-[U-¹⁴C]mannose (Amersham) at a final specific activity of 23×10^{10} Bq/mol. Unlabelled GDP-D-mannose, NAD⁺, NADP⁺, NADPH and the compounds tested for inhibition studies (GDP- β -L-fucose, GDP- α -L-fucose, GDP-D-glucose, GMP, GDP, GTP, β -L-fucose-1-P and L-fucose) were from Sigma Chemicals (St. Louis, MO, USA). Incubations were performed in 100 mM sodium phosphate buffer, pH 8.0, containing 2 mg/ml BSA. K_m and V_{max} values of the enzymes were calculated by double-reciprocal plot treatment of kinetic data using linear regression, on at least three different preparations of the pro-

teins. All kinetic experiments were carried out immediately after purification of the GMD forms.

2.4. Cell culture conditions

Cell lines HepG2, Hct-8, MCF7, SKNSH (obtained from ATCC: Rockville, MD, USA) were grown in DMEM, supplemented with 10% FCS and 4 mM glutamine. The Jurkat cell line (ATCC) was maintained in RPMI 1640 supplemented with 10% FCS and 2 mM glutamine. To minimize proteolysis, Western blot analysis was performed following addition of boiling 2 \times SDS-PAGE loading buffer (2 \times buffer: 125 mM Tris-HCl, pH 6.5, 200 mM DTT, 4% SDS, 20% glycerol and 0.02% bromophenol blue) directly to culture flasks, previously washed twice with ice-cold PBS. Western blot conditions were as reported above (Section 2.2).

3. Results

3.1. Expression of multiple recombinant forms of human GMD

Following expression and lysis by standard procedures, purification by affinity chromatography and cleavage by Prescission protease, both L-GMD and M-GMD forms were obtained as homogeneous proteins of the expected molecular weight, as shown by SDS-PAGE and Western blot analysis (Fig. 1A and B). Recovery ranged from 0.6 to 1 mg of pure protein per l of bacterial culture. Conversely, since S-GMD was recovered only in inclusion bodies despite several different conditions of growth and induction of bacterial cells tested to avoid its precipitation, cell lysis was performed using sarkosyl. This procedure allowed us to solubilize GST-fused S-GMD, which could then be purified by GSH-Sepharose. However, immediately after cleavage by Prescission protease, the resulting 38.7 kDa protein formed a precipitate and could not be quantitatively recovered as soluble and functional protein (Fig. 1A), although small amounts of S-GMD were observed by Western blot experiments (Fig. 1B). Purification of M-GMD, also performed using the sarkosyl method, allowed us to recover a soluble and catalytically functional protein. This suggests that precipitation of S-GMD is not due to an effect exerted by sarkosyl per se, but rather results from an incorrect folding during protein production by bacterial cells.

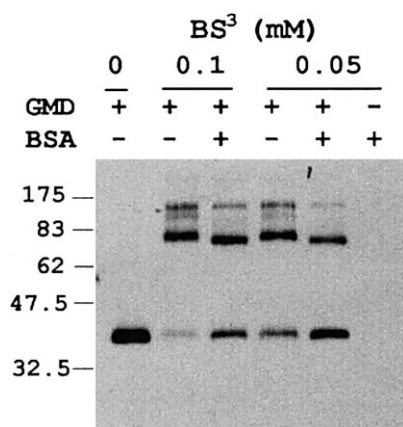


Fig. 2. Western blot analysis of M-GMD after treatment with two different concentrations of BS³, either in the presence or absence of BSA. 50 ng of protein was loaded in each lane and subjected to SDS-PAGE on a 12% acrylamide gel. Detection was achieved using an anti-GMD antibody by the ECL method (Section 2.2). Markers were from New England Biolabs (see Fig. 1B).

3.2. Subunit structure and content of apoenzyme-bound NADP⁺

In order to investigate their subunit structure, both L-GMD and M-GMD were subjected to size exclusion chromatography and treatment with the chemical crosslinker BS³, followed by Western blot analysis. Size exclusion analysis revealed that M-GMD eluted as a single peak corresponding to a homodimeric form of approximately 80 kDa (not shown). In order to confirm the oligomeric structure of M-GMD, we treated this protein with increasing concentrations of BS³, either alone or in the presence of an excess BSA, as a competitor to avoid non-specific intramolecular crosslinking (Fig. 2). Reaction of M-GMD alone with BS³ reproducibly led to the appearance of additional immunoreactive bands, with apparent M_r values consistent mainly for dimers and for a small amount of tetramers and to the concurrent decrease of the monomeric form. In the presence of BSA, BS³ treatment generated a similar pattern, but the extent of disappearance of the monomer was considerably less pronounced and the band corresponding to the dimeric form exhibited a slightly increased mobility, which might be explained by assuming a lower extent of intramolecular crosslinking of M-GMD dimers by BS³. Strictly comparable results from gel permeation and BS³ crosslinking experiments were also obtained for the L-GMD form (not shown).

Determination of GMD-bound coenzymes was performed both on extensively dialyzed, native GMD forms and, following their heat denaturation, on the corresponding supernatants (Table 1). No NAD⁺ could be detected in either condition. Conversely, NADP⁺ was found to be present in comparable amounts in the L-GMD and M-GMD forms.

While low contents of NADP⁺, accounting for 0.14–0.2 mol/mol protein monomer, were detectable in the two native GMD forms, higher dinucleotide levels were measured following their heat denaturation (Table 1). These data indicate that 1 mol of NADP⁺ is tightly bound to either GMD homodimeric form, since it is dialysis-resistant, is not easily accessible for the cycling reaction and can be released after denaturation of the protein only.

3.3. Kinetic properties of L-GMD and M-GMD

The kinetic properties of L-GMD and M-GMD are reported in Table 1. Both forms displayed comparable values of K_m and V_{max} , which were consistent in several preparations of the recombinant proteins and their activity was not affected by the addition of divalent cations (not shown). The K_m obtained for the recombinant proteins was comparable to that previously determined in cell lysates [14] and also with GMD purified from porcine thyroid [6]. No effect whatsoever on the enzymatic activity was observed upon addition of NAD⁺ or NADP⁺ and of their reduced forms. GMD showed a pH optimum around 7.5 and also exhibited maximal stability at pH 7.5–8.0 (not shown). Both forms, L-GMD and M-GMD, proved to be quite unstable, with a 50% loss of activity in 24–36 h at 4°C, which could not be prevented by addition of reducing agents. Similarly, a significant decrease of specific activity was observed upon freezing, even in the presence of cryoprotective agents such as glycerol.

Table 2 reports the potency of various inhibitors of GMD activity. GDP-L-fucose, and in particular the β -anomer, was the most active inhibitor, in agreement with the previously reported data [13,17], while the other guanine derivatives were much less effective. Interestingly, no effect was observed with free L-fucose or its phosphorylated form, thus indicating that the presence of the guanylic moiety is essential for recognition by the enzyme. No significant differences were observed between L-GMD and M-GMD as far as the inhibiting effects are concerned. Double-reciprocal plot analysis of the kinetic data indicated that GDP-L-fucose behaves as a classical non-competitive inhibitor, with a K_i of $11.25 \pm 1.7 \mu\text{M}$, while a competitive type of inhibition was observed for GDP and GDP-D-glucose (not shown).

3.4. Cell distribution of L-GMD and M-GMD

Fig. 3 shows the results of Western blot experiments performed on lysates from several human cell lines. In all samples, an immunoreactive band was observed with an apparent M_r of 40 kDa, corresponding to the expected mass for M-GMD, which seems therefore to be the predominant form of the native enzyme. Additional distinct immunoreactive bands were present. In particular, a second band showing an electrophoretic mobility comparable to standard L-GMD was observed in the MCF-7 cell line, suggesting that also the form

Table 1
NADP⁺ content and kinetic properties of L-GMD and M-GMD

	NADP ⁺ ^a (mol/mol of protein)		K_m (μM)	V_{max} ($\mu\text{mol/h/mg}$)	K_{cat} (s^{-1})	K_{cat}/K_m ($\text{M}^{-1} \text{s}^{-1}$)
	Native	Denatured				
L-GMD	0.20 ± 0.07	0.56 ± 0.05	3.64 ± 1.2	37.9 ± 2.6	0.8	2.2×10^5
M-GMD	0.14 ± 0.05	0.47 ± 0.14	3.50 ± 0.7	40.0 ± 6.5		

^aNADP⁺ was determined on both native and heat-denatured GMDs and after heat denaturation by an enzymatic cycling assay, after extensive dialysis of the purified recombinant proteins. Data are expressed as mol of dinucleotide per mol of monomeric protein.

Table 2
Effects of inhibitors on M-GMD enzymatic activity

	IC_{50} (mM)
GDP- β -L-fucose	0.024 ± 0.003
GDP- α -L-fucose	0.100 ± 0.017
GDP-D-glucose	0.530 ± 0.060
GDP	0.640 ± 0.100
GMP	0.700 ± 0.080
GTP	0.840 ± 0.170
L-Fucose	> 2
L-Fucose-1-P	> 2
ATP	> 10

M-GMD activity was determined using 50 μ M GDP-D-mannose in the presence of different concentrations of inhibitors. Results are expressed as mean \pm S.D. of the concentration required to give 50% inhibition and were obtained from at least three independent experiments. Closely comparable results were also obtained for L-GMD.

starting in correspondence of the first methionine is possibly produced in these cells. Other bands showing a faster electrophoretic mobility, in particular one of approximately 35 kDa, were also observed (mostly in HepG2 cells), which could not be related to the previously proposed [15], shortest 38.7 kDa form of GMD. Since precautions were taken to prevent artifactual proteolysis during cell lysis (Section 2.4), these forms may represent 'in vivo' occurring proteolytic products, whose role is presently unknown.

4. Discussion

GMD is a key enzyme in the 'de novo' biosynthesis of GDP-L-fucose and, accordingly, also plays a fundamental role in the control of the production of fucosylated oligosaccharides. Purification of this enzyme from mammalian sources, in particular from human tissues, proved to be difficult because of its intrinsic instability (Tonetti, M., unpublished results) and this prevented a detailed study of its enzymatic and structural properties. The recent cloning of the cDNA for human GMD has enabled us to produce the recombinant protein [13,14]. However, three different protein species could be expected from the proposed cDNA sequences and it was completely unknown which form is really expressed in human cells or whether a tissue or cell specificity is present.

Analysis of the sequence flanking the two possible initiating AUG codons did not indicate the presence of an ideal Kozak consensus sequence for either L-GMD and M-GMD, but a suboptimal context for translational initiation is present in both forms (including a purine in position -3 and a G in position +4) [23]. Western blot analyses on human cell lysates indicated that M-GMD represents the predominant form of the enzyme, while a band corresponding to the L-GMD form was found in significant amounts in only one of the cell lines tested (MCF-7, see Fig. 3). Analysis of the structural and kinetic properties of the two purified enzyme forms showed an identical behavior for L-GMD and M-GMD, but it is not yet clear whether the two forms display identical or different properties also in the intracellular environment, where post-translational control mechanisms could be involved.

The third form which has been proposed [15] and which displays a 5'-untranslated region completely different from the previously proposed sequence [13], corresponding to S-GMD, lacks part of the N-terminal region compared to L-GMD and M-GMD. This domain is extremely well-con-

served, not only among the GMDs from different organisms, but also in most enzymes involved in the metabolism of nucleoside diphosphate sugars [24], and it is essential for the binding of the dinucleotide cofactor [25,26]. Therefore, its absence and the consequent failure to bind NADP⁺ possibly playing a structural role may prevent a correct folding of the nascent S-GMD form and thus explain its lack of expression both as a soluble and functional recombinant protein in *E. coli* and also as S-GMD in any of the cell lines that we have analyzed in this study.

Both M-GMD and L-GMD are homodimers, while bacterial GMD [17] and the enzyme purified from porcine thyroid [6] proved to be homoheptamers, and each dimer binds one molecule of NADP⁺ tightly. The presence of bound cofactor playing a key role in catalysis was also suggested by the fact that supplementation with NAD(P)⁺ had no effect on the enzymatic activity. This finding is consistent with that described by Broschat et al. [6] on purified porcine GMD. It is, however, in contrast with what has previously been reported for recombinant human GMD expressed in *E. coli*, having N-terminal histidine-containing leader sequences, which required the addition of exogenous NADP⁺ and also of NADPH for activity [13]. The reason for this difference is not yet understood, but it is possible that the N-terminal tag, which was not removed in the earlier study, might interfere with the tight binding of NADP⁺. A defective binding of the dinucleotide to this recombinant protein is also strongly suggested by the significant differences observed in its kinetic properties, with a 20-fold higher K_m and a lower specific activity [13] compared to our data.

GDP- β -L-fucose is a strong feedback negative modulator of GMD acting as a non-competitive inhibitor, while the other nucleotides exhibit a competitive behavior. A non-competitive mechanism of inhibition by GDP-L-fucose, together with the low K_i value, suggests that this nucleotide sugar may represent one of the most important mechanisms involved in the 'in vivo' control of the GDP-L-fucose biosynthetic pathway. The lack of any significant inhibitory effect exerted by free L-fucose and L-fucose-1-P indicates that the guanine moiety is essential for the binding of the inhibitor. This finding seems to be relevant in the perspective to develop molecules able to modulate activity of the GDP-L-fucose biosynthetic pathway for therapeutic applications. In fact, molecules able to interfere with the production of fucosylated glycoconjugates could be useful as anti-inflammatory or anti-allograft rejection

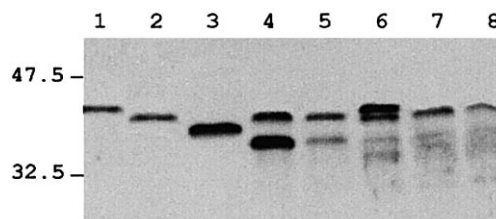


Fig. 3. Western blot analysis of human cell lines, performed using an anti-bacterial GMD polyclonal antibody (Section 2.2). 50 ng of standard GMD forms and 50 μ g of total cell proteins was loaded in each lane. In order to minimize possible proteolytic degradation following cell lysis, cell cultures were directly lysed with boiling SDS-PAGE loading buffer (Section 2.4). Lane 1: L-GMD, lane 2: M-GMD, lane 3: S-GMD, lane 4: HepG2, lane 5: Hct-8, lane 6: MCF-7, lane 7: Jurkat, lane 8: SKNSH. Markers were from New England Biolabs (Fig. 1B). For explanations, see text.

drugs, by preventing the selectin-mediated interaction between leukocytes and endothelial cells which is preluded to extravasation in tissues.

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