Biochemical and Biophysical Research Communications 401 (2010) 396–400

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Echinophilic proteins stomatin, sorcin, and synexin locate outside ganglioside$_{M1}$ (GM1) patches in the erythrocyte membrane

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ARTICLE INFO

Article history:
Received 10 September 2010
Available online 19 September 2010

Keywords:
Ganglioside GM1
Membrane curvature
Erythrocyte
DRM
Fluorescence microscopy

ABSTRACT

The detergent (Triton X-100, 4 °C)-resistant membrane (DRM)-associated membrane proteins stomatin, sorcin, and synexin (anexin VII) exposed on the cytoplasmic side of membrane were investigated for their lateral distribution in relation to induced ganglioside$_{M1}$ (GM1) raft patches in flat (discocytic) and curved (echinocytic) human erythrocyte membrane. In discocytes, no accumulation of stomatin, sorcin, and synexin in cholera toxin subunit B (CTB) plus anti-CTB-induced GM1 patches was detected by fluorescence microscopy. In echinocytes, stomatin, sorcin, and synexin showed a similar curvature-dependent lateral distribution as GM1 patches by accumulating to spiculae induced by ionophore A23187 plus calcium. Stomatin was partly and synexin and sorcin were fully recruited to the spiculae. However, the DRM-associated proteins only partially co-localized with GM1 and were frequently distributed into different spiculae than GM1. The study indicates that stomatin, sorcin, and synexin are echinophilic membrane components that mainly locate outside GM1 rafts in the human erythrocyte membrane. Echinophilicity is suggested to contribute to the DRM association of a membrane component in general.

1. Introduction

The present study was undertaken to investigate how stomatin, sorcin, and synexin, detergent (Triton X-100, 4 °C)-resistant membrane (DRM)-associated proteins exposed on the cytoplasmic side of membrane [1,2] distribute laterally relative to glycosphingolipid ganglioside$_{M1}$ (GM1), a major raft marker [3,4], in the human erythrocyte membrane. While the insolubility of membrane components in Triton X-100 at 4 °C [5–7] was a key finding contributing to the development of the "membrane raft" concept [8,9], DRM composition may not perfectly reflect raft composition [10–16]. DRM fraction analysis is an indirect method. Following capping of GM1 [17] or cross-linking GM1 by fluorophore-labelled cholera toxin subunit B (CTB) plus anti-CTB to large domains (patches) [4], fluorescence microscopy (FLM) may be used to study GM1-enriched domains. While GM1 patches can be studied in flat membrane (discoid cells), their characteristics to accumulate into membrane spiculae (echinophilic cells), may additionally facilitate the identification of co-localizing membrane (raft) components. Our previous investigation indicated poor co-localization of outer-leaflet DRM-associated mobile glycosylphosphatidylinositol (GPI)-anchored CD59 protein with GM1 (raft) [18]. In the present study we examined the lateral distribution of stomatin, sorcin, and synexin, which are DRM associated proteins exposed on the cytoplasmic side of the membrane [1,2], and their co-localisation with induced GM1 patches in discoid and echinocytic cells.

2. Materials and methods

2.1. Chemicals

The lipid raft labelling kit (Cholera Toxin B Alexa 555 and anti-Cholera toxin, V-34404) and Alexa Fluor® 488 goat anti-mouse IgG (A-11029) were from Invitrogen, monoclonal antibody (mAb) against synexin (annexin VII, 610668) was from BD Biosciences Pharmingen, and mAb against sorcin (33-8000) from Zymed. The mAb against stomatin is described in Hiebl-Dirschmied et al. [19]. Ionophore A23187 (C-7522) and fish skin gelatine (Fsg, G-7041) were obtained from Sigma.

2.2. Isolation of erythrocytes

Blood was drawn from the authors and volunteers by venipuncture into heparinized tubes. The blood was washed three times with buffer (145 mM NaCl, 5 mM KCl, 4 mM Na_2HPO_4, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, pH 7.4). Erythrocytes

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doi:10.1016/j.bbrc.2010.09.065

were suspended in the buffer at 1.65×10^9 cells/ml, stored at +4 °C, and used within 5 h.

2.3. Staining

Erythrocytes (1.65×10^8 cells/ml, ~1.5% haematocrit) were incubated with Alexa 555 conjugated CTB (1/250, 20 min, RT). Following two washes, the cell suspension was incubated with anti-CTB (1/50, 20 min, RT) and washed twice. To obtain echinocytic cells, the erythrocytes were treated with A23187 (2 μM, 15 min, 37 °C). The cells were fixed with 5% paraformaldehyde (PFA) plus 0.01% glutaraldehyde (GA) (60 min, RT) and permeabilised in dry methanol on ice for 10 min. Following washing, the cells were settled on polylysine-treated (0.1 mg/ml, 10 min) cover glasses and washed. After blocking (1% Fsg, 30 min, RT) and washing, the cells were incubated with the primary antibody (1/20 for stomatin, synexin, 1/100 for sorcin, 60 min, RT). After washing, the cells were incubated with fluorophore-conjugated secondary antibody (1/200 Alexa Fluor® 488, 0.05% Fsg, 60 min, RT). Following washing, the cells were mounted on 80% glycerol. The cover slips were sealed with nail polish.

2.4. Microscopy

A large number of cells in several separate experimental samples was studied for GM1 and protein staining using a Leica DM RXA fluorescence microscope (100×/1.4 aperture immersion oil objective, 10× ocular). In double-staining experiments with GM1 patched cells, stained cells were compared with cells treated with the secondary antibody only. Images (single-section) were acquired with a Leica DC300F CCD-camera. Fluorescence data were not analysed in a strictly quantitative way. Inset figures show one and the same treatment. Erythrocytes were processed for scanning electron microscopy (SEM, Leo 1530 Gemini) according to a standard protocol as previously described [20,21]. For a control, erythrocytes were pre-treated with CTB and anti-CTB as above. Notably, GM1 patches were not detectable by SEM. Echinocytosis was induced by incubation with A23187 (2 μM, 15 min, 37 °C).

3. Results

3.1. Discoid erythrocytes

Using CTB plus anti-CTB as a tool to induce GM1 patches in the human erythrocyte membrane, as previously described [18], the lateral distribution of the DRM-associated [1,2] proteins stomatin, synexin, and sorcin in GM1 patched cells was examined. As indicated in Fig. 1A, C and E, in the membrane of discoid human erythrocytes, treatment with CTB plus anti-CTB induced the formation of distinct GM1 patches. No significant redistribution of stomatin (Fig. 1B), synexin (Fig. 1D), and sorcin (Fig. 1F) or accumulation

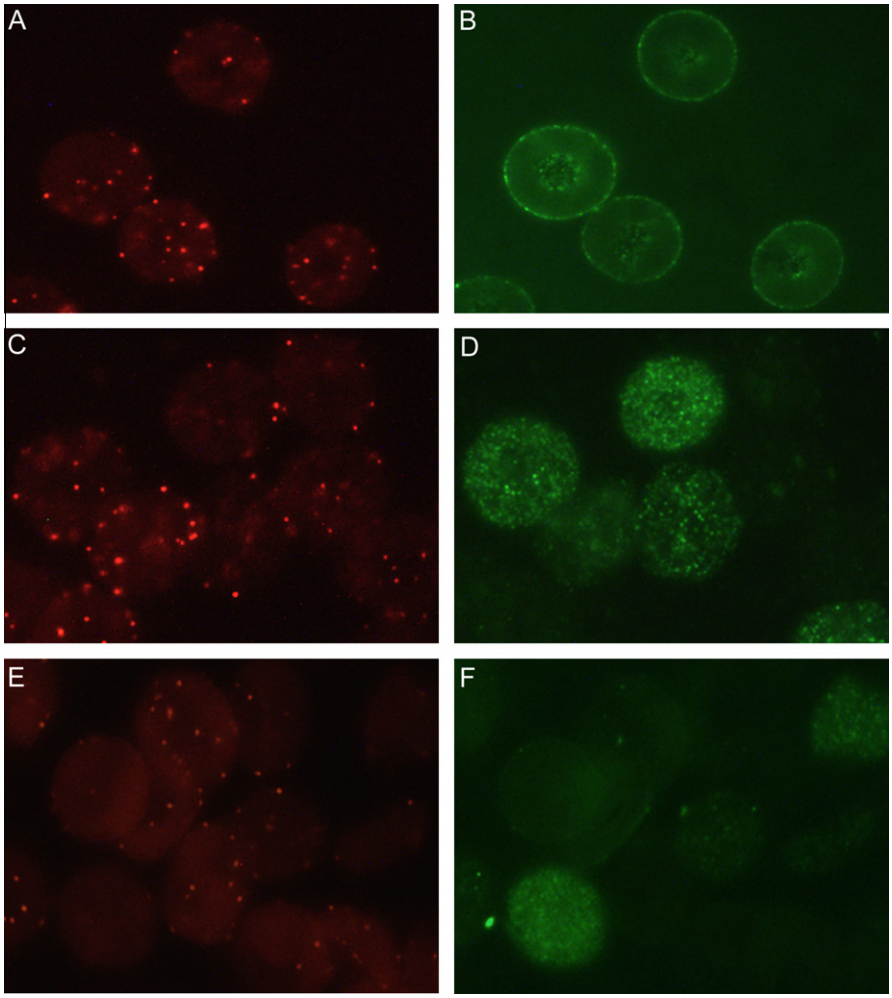

Fig. 1. Distribution of GM1 patches and proteins in discoid human erythrocytes. Fluorescence micrographs showing discoid human erythrocytes treated with CTB plus anti-CTB and stained for either stomatin, synexin, or sorcin. CTB (A, C, E) and stomatin (B), synexin (D), and sorcin (F) signals from the same corresponding fields are indicated. Cells were fixed with PFA (5%) and GA (0.01%).

to GM1 patches was detected. Stomatin remained evenly distributed around the membrane, showing a granular pattern, while synexin and sorcin showed a patchy midplane staining, indicating a primarily cytoplasmic localization (see [22]). Notably, synexin and sorcin are cytosolic proteins that require increased calcium concentrations for membrane binding [2].

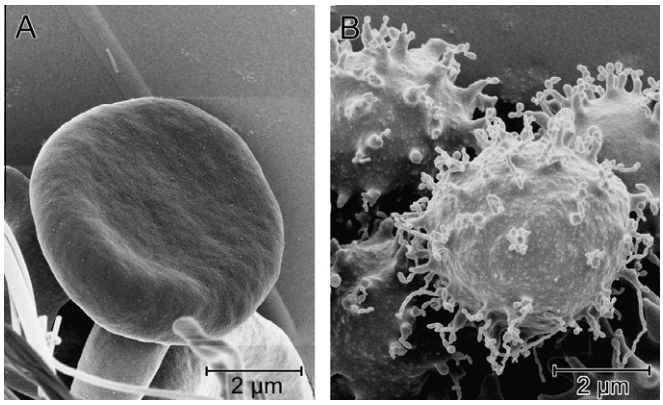

Fig. 2. Scanning electron micrograph showing discoid (A) and echinocytic (B) human erythrocytes. Erythrocytes were treated with ionophore A23187 (2 µM, 15 min, 37 °C) to induce echinocytosis. Cells were fixed with PFA (5%) and GA (0.01%) and pooled. For a control, cells were pre-treated with CTB plus anti-CTB.

3.2. Echinocytic (spiculated) erythrocytes

To indicate in detail the morphology of the echinocytic cells with protruding spiculae induced by ionophore A23187 plus calcium (in CTB plus anti-CTB pretreated cells), scanning electron microscope (SEM) micrographs are shown in Fig. 2. FLM showed that GM1 accumulated at the tips of the echinocytic spiculae (Fig. 3A, C and E). While stomatin stained all membrane (Fig. 3B), accumulated staining of whole spiculae (Fig. 3B, up inset) and at the spiculae tip (Fig. 3B, middle and down insets) was observed. Synexin (Fig. 3D) and sorcin (Fig. 3F) preferentially accumulated at the tips of spiculae, giving a spot-like staining. The staining pattern of stomatin, synexin, and sorcin in calcium-induced echinocytic cells is in accordance with a previous report [23]. Importantly, stomatin, synexin, and sorcin showed only a partial co-localization with GM1 patches in the spiculae and frequently accumulated in different spiculae from GM1 (Fig. 3B, D and F). Similar results were previously reported for CD59 [18]. It should be noted that in echinocytic cells, some exovesiculation may have occurred resulting in loss of material (staining) from the spiculae.

4. Discussion

The present study investigating the co-localisation of stomatin, synexin, and sorcin, which are monotopically exposed on the cytoplasmic side of the membrane (the latter two become

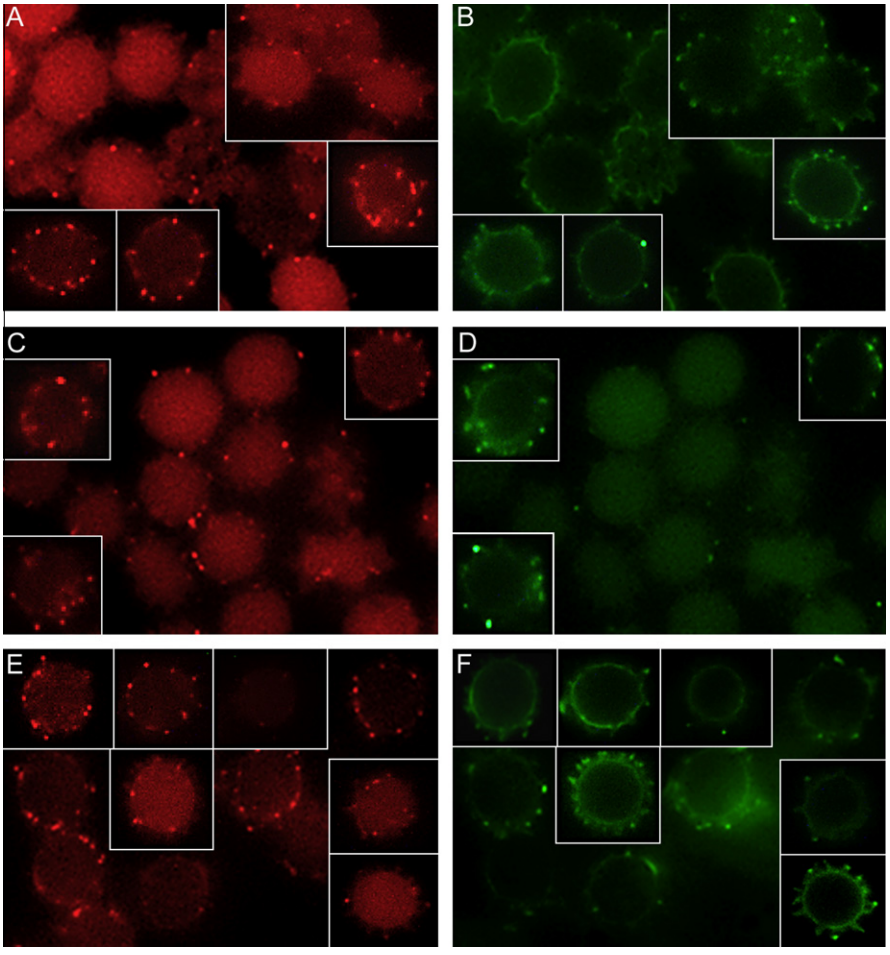

Fig. 3. Distribution of GM1 patches and proteins in echinocytic human erythrocytes. Fluorescence micrographs showing human erythrocytes pre-treated with CTB plus anti-CTB, treated with ionophore A23187 (2 µM, 15 min, 37 °C), and stained for either stomatin, synexin, or sorcin. CTB (A, C and E) and stomatin (B), synexin (D), and sorcin (F) signals from the same corresponding fields are indicated. Cells were fixed with PFA (5%) and GA (0.01%).

membrane associated upon increased intracellular calcium concentration), with GM1, indicates that membrane components found in DRM fractions are not necessarily stably associated with GM1 domains in either flat (discoid) or curved (echinocytic) erythrocyte membrane. This is in line with a previous study that showed no close positional correlation between the GPI-linked DRM-associated CD59 protein and GM1 [18]. Previous TEM and confocal fluorescence microscopy studies with mast cells [16] and lymphocytes [17], respectively, similarly suggested a more complex topographical organization of GM1-enriched domains than predicted by DRM fraction analysis.

These studies indicate that the relation between rafts and DRMs must be critically evaluated [24–26]. On the one hand it has to be considered that artefacts may arise during solubilisation and on the other hand it has to be taken into account that various types of membrane domains with respect to size and composition, which presumably coexist at the membrane, become merged in the DRM preparation [10–15].

Our results indicate that the resistance to Triton X-100 solubilisation is related to membrane curvature-dependent properties of the membrane components. In particular, Triton X-100 may not effectively solubilise echinophilic membrane components.

Membrane curvature is a driving mechanism for the lateral segregation of mobile membrane components and domains. Due to their echinophilic (positive spontaneous curvature [27]) molecular properties, GM1 patches formed in discoid cells (flat membrane) probably serve as nucleation centres for the formation of echinocytic spiculae. The formation of separate competitive echinophilic nucleation centres, which are sterically excluded from GM1 patches and contain proteins (stomatin) and/or carrier molecules (synexin, sorcin), could explain the commonly observed accumulation of stomatin, synexin, and sorcin in different spiculae from GM1 patches. Fig. 4 shows that it is energetically favourable that

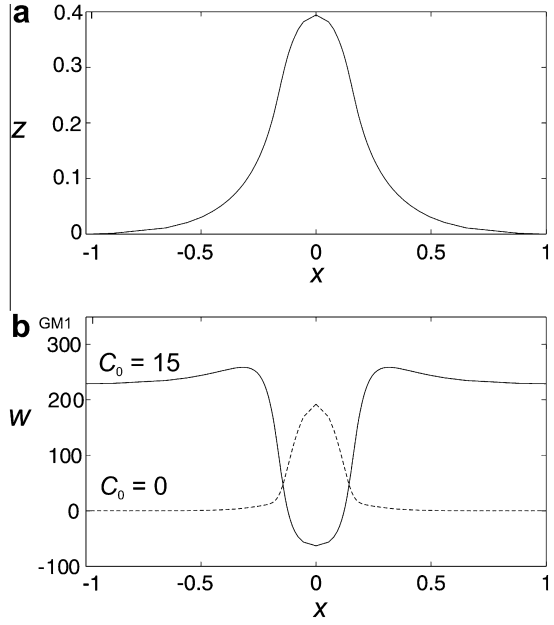

Fig. 4. The relative area density of the bending energy of the CTB plus anti-CTB-induced GM1 flexible domains $w_{GM1} = (C_1 + C_2 - C_0)^2 + (2k_{G,GM1}/k_{C,GM1})\, C_1\, C_2$ (panel B) in different regions of the echinocyte spiculum and its surroundings (panel A) calculated for two different values of spontaneous curvature GM1 domains (C_0): 0 and 15 (panel B). We assume the axisymmetric shape of spiculum as presented in the panel A. The coordinates x and z are given in the arbitrary units of R_0, while all values of the curvatures are given in units $1/R_0$. Here, $k_{C,GM1}$ and $k_{G,GM1}$ are the local and the Gaussian bending constants of the GM1 domain, while C_1 and C_2 are the two principal curvatures. The Gaussian bending constant of the GM1 domain is expected to be negative ($k_{C,GM1} > -k_{G,GM1}/2$) [43]; therefore we assume $2k_{G,GM1}/k_{C,GM1} \sim -1$ (see [44]).

GM1 patches are, due to their positive spontaneous curvature C_0 (as a consequence of GM1 conical shape) [28], located at the tips of echinocytic spiculae. Namely, due to the positive spontaneous curvature of GM1 patches, the bending energy of these domains in the outer-membrane leaflet is then lowest (Fig. 4B). Due to the conical shape of GM1 molecules, GM1 patches may have a locally decreased bending constant [29,30], which would make it still more favourable for GM1 patches to serve as nuclei for spiculae formation.

Stomatin is an integral membrane protein that is monotopically exposed on the cytoplasmic side of the membrane [31]. It is found to be palmitoylated [32], to form high-order homo-oligomers [33], and to be present in DRMs [1,34]. Overlapping sequence motifs close to the C-terminus of the protein are responsible for the high-order oligomer formation and DRM association [35]. In nucleated cells, stomatin is found both on the plasma membrane and perinuclear vesicles; in platelets it is mainly located on intracellular alpha-granules [36]. The reason for the echinophilic property of stomatin seen in its partial accumulation in echinocytic spiculae of calcium-induced erythrocytes (see also [23]) and its enrichment in released microvesicles [2] remains unclear. However, in a human amniotic cell line and in activated platelets and neutrophils, stomatin is also found in plasma membrane protrusions and released microvesicles [36–38]. It is conceivable that the change from its stomatophilic (at the outer membrane of intracellular vesicles) to its echinophilic characteristics (at the lumenal membrane of exovesicles) might be due to a change in its intrinsic molecular shape associated with altered posttranslational modifications, an altered oligomerisation status, or altered cytoskeletal interactions. GM1, but not stomatin, distributed to echinocytic spiculae induced by amphiphiles [23]. This finding indicates on the one hand that the echinophilic property of stomatin is not induced by amphiphiles but might depend on a calcium-specific regulation, and on the other hand that stomatin is located in an independent local membrane environment (domain) compared with GM1.

Synexin and sorcin are cytosolic proteins that are actors in vesicular trafficking and exocytosis [39]. They translocate to the membrane upon increasing cytosolic calcium concentrations and have been found to be associated with DRMs of calcium-induced exovesicles [2]. Synexin and sorcin particularly accumulate at the tips of echinocytic spiculae [23]. The similar location of these two proteins is not surprising since they are mutual binding partners [40]. Their high echinophilicity has specifically been indicated by the finding that both proteins were strongly enriched in the small (about 80 nm in diameter) and therefore highly curved calcium-induced nanovesicles [2,41]. Whether these strongly echinophilic properties of synexin and sorcin are due to their own calcium-dependent structural characteristics or due to interaction with an echinophilic integral membrane protein remains to be elucidated.

Our study was based on the anticipation that proteins associated with GM1 domains accumulate with GM1 into induced GM1 patches. As indicated, our study did not detect an accumulation of DRM-associated proteins in GM1 patches in discoid cells, suggesting that induced GM1 patches in flat membranes are poor in DRM-associated proteins. However, there are alternative explanations for our results. GM1 patches formed with CTB plus anti-CTB may be unfavourable milieus for proteins and lead to their dissociation from GM1 rafts. Such a mechanism could be critically temperature dependent or patch size dependent, whereby membrane components surrounding mono-/oligomeric GM1-cholesterol domains are sterically excluded upon GM1 patching. Furthermore, proteins accumulated in GM1 patches may be difficult to detect, one reason being their low abundance in patches. However, CD59, for example, is the most abundant human GPI-linked erythrocyte protein, present in ~30,000 copies/cell and accounting for

~75% of all red cell GPI proteins [42], and accumulated CD59, as well as other proteins, are well detected when accumulated in spiculae. Finally, staining and cross-linking of GM1 and proteins may alter their intrinsic molecular shape and interaction with other components and curvature-dependent distribution.

In conclusion, stomatin, sorcin, and synexin were shown to locate mainly outside induced GM1 patches in the human erythrocyte membrane.

Acknowledgments

This study was supported by grants from the Research Institute of Åbo Akademi University, Understödsföreningen Liv och Hälsa, the Tor, Joe and Pentti Borg Foundation, the Paulo Foundation, and ARRS grants J3-9219-0381 and P2-0232-1538. Gunilla Henriksson, Esa Nummelin, Thomas Bymark, and Jari Korhonen are gratefully acknowledged for technical assistance.

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