

Gangliosides play an important role in the organization of CD82-enriched microdomains

Elena ODINTSOVA*¹, Terry D. BUTTERS†, Eugenio MONTI‡, Hein SPRONG§, Gerrit van MEER§ and Fedor BERDITCHEVSKI*

*Cancer Research U.K. Institute for Cancer Studies, The University of Birmingham, Edgbaston, Birmingham B15 2TT, U.K., †Department of Biochemistry, Glycobiology Institute, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K., ‡Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of Brescia, viale Europa 11, 25123 Brescia, Italy, and §Department of Membrane Enzymology, Bijvoet Center and Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Four-transmembrane-domain proteins of the tetraspanin superfamily are the organizers of specific microdomains at the membrane [TERMs (tetraspanin-enriched microdomains)] that incorporate various transmembrane receptors and modulate their activities. The structural aspects of the organization of TERM are poorly understood. In the present study, we investigated the role of gangliosides in the assembly and stability of TERM. We demonstrated that inhibition of the glycosphingolipid biosynthetic pathway with specific inhibitors of glucosylceramide synthase [NB-DGJ (*N*-butyldeoxygalactonojirimycin) and PPMP (*D*-*threo*-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol · HCl)] resulted in specific weakening of the interactions involving tetraspanin CD82. Furthermore, ectopic expression of the plasma-membrane-bound sialidase Neu3 in mammary epithelial cells also affected stability of the complexes containing CD82: its asso-

ciation with tetraspanin CD151 was decreased, but the association with EGFR [EGF (epidermal growth factor) receptor] was enhanced. The destabilization of the CD82-containing complexes upon ganglioside depletion correlated with the re-distribution of the proteins within plasma membrane. Importantly, depletion of gangliosides affected EGF-induced signalling only in the presence of CD82. Taken together, our results provide strong evidence that gangliosides play an important role in supporting the integrity of CD82-enriched microdomains. Furthermore, these results demonstrate that the association between different tetraspanins in TERM is controlled by distinct mechanisms and identify Neu3 as a first physiological regulator of the integrity of these microdomains.

Key words: CD82, epidermal growth factor receptor (EGFR), G_{D1a} ganglioside, microdomain, Neu3 sialidase, tetraspanin.

INTRODUCTION

The plasma membrane is a dynamic highly compartmentalized structure. Compartmentalization is important for the induction of signalling, for the assembly of adhesion complexes and for the generation of immune responses. Currently, there are a few models of membrane organization and they all agree on the fact that certain lipids and proteins within the plasma membrane are arranged in small aggregates (microdomains) that carry out signalling functions [1,2]. These microdomains enlarge or dissolve over time, depending on the cell's activities. A number of different types of microdomains have been described, including lipid rafts and caveolae [3], DRMs (detergent-resistant membranes), GEMs (glycosphingolipid-enriched microdomains) and glycosynapses [4]. With the exception of glycosynapses, they are all based on a classic model of 'lipid rafts'. The structure of lipid rafts in the intact plasma membrane is unknown, but an operational definition has been adopted according to which they are cholesterol-dependent and contain components that are isolated as detergent-insoluble membranes [5,6]. TERMs (tetraspanin-enriched microdomains) represent a novel type of molecular aggregate that are distinct from all these mentioned above. They could be operationally defined as the membrane complexes maintained after solubilization with mild detergents such as Brij96 or Brij98 [7,8]. Indeed, in contrast with classical lipid rafts, TERM are sensitive to Triton X-100 and do not contain glycosylphosphatidylinositol-linked proteins [8]. The principal components of TERM are tetraspanins, which constitute a large family of four-transmembrane-domain proteins. Although the exact biochemical function of

tetraspanins is not well defined, it has been established that these proteins play an important role in membrane compartmentalization and dynamics [8–11]. Tetraspanins interact with each other, thereby providing a structural platform for the recruitment of other transmembrane and cytoplasmic proteins into TERM [8,11,12]. A number of transmembrane receptors are known to associate with tetraspanin microdomains, including integrins [13], receptor tyrosine kinases and G-protein-coupled receptors [11,12]. Importantly, association of a particular receptor with TERM may lead either to the enhancement or to the attenuation of its activity [10,14]. The contribution of individual tetraspanins to the generation of co-stimulatory or inhibitory effects has not been studied in detail.

Gangliosides are essential structural components of the membranes. These glycosphingolipids are involved in the regulation of signalling through the growth factor and adhesion receptors [15–17]. Previous reports established various links between tetraspanins and gangliosides. It was shown that CD9 (and, possibly, CD82) and G_{M3} co-operatively down-regulate motility of tumour cells by attenuating signalling induced by laminin–integrin interactions [17–19]. The cross-talk between integrins and fibroblast growth factor receptor has also been influenced by the changes in gangliosides and/or tetraspanin expression levels [20]. Moreover, our previous results suggested that the activity of EGFR [EGF (epidermal growth factor) receptor] is attenuated by the tetraspanin CD82 through the modification of ganglioside composition at the membrane [21]. Specifically, we found that there was a correlation in the expression levels of CD82 and gangliosides in mammary epithelial cells [21]. However,

Abbreviations used: CT, cholera toxin B subunit; DRM, detergent-resistant membranes; EGF, epidermal growth factor; EGFR, EGF receptor; FCS, foetal calf serum; GEM, glycosphingolipid-enriched microdomain; HA, haemagglutinin; mAb, monoclonal antibody; M β CD, methyl- β -cyclodextrin; NB-DGJ, *N*-butyldeoxygalactonojirimycin; PPMP, *D*-*threo*-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol · HCl; SNA, *Sambucus nigra* (elderberry) agglutinin; TERM, tetraspanin-enriched microdomain.

¹ To whom correspondence should be addressed (email odintsoe@bham.ac.uk).

the underlying mechanism of the interactions of gangliosides and tetraspanins has not been identified. Most likely gangliosides provide stability and structural support for TERM. Gangliosides may also control localization of the transmembrane proteins to the segregated microdomains.

In the present study, we examined the importance of gangliosides for the assembly of TERM. By manipulating ganglioside content pharmacologically and enzymatically, we demonstrated that removal of gangliosides affects associations of CD82 with its partners, components of TERM. We also found that there is a clear specificity in the effect that ganglioside depletion had on the interactions involving different tetraspanins. Particularly, only associations of CD82 (but not CD9) with EGFR, $\alpha 3\beta 1$ integrin and other tetraspanins have been affected. Thus our results indicate that structural integrity of CD82-containing microdomains relies on the presence of gangliosides.

MATERIALS AND METHODS

Cell lines, antibodies and reagents

Human mammary epithelial cells HB2/ZEO and HB2/CD82 [21] were maintained in DMEM (Dulbecco's modified Eagle's medium; Gibco) supplemented with 10% (v/v) FCS (foetal calf serum), 10 $\mu\text{g/ml}$ cortisol and 10 $\mu\text{g/ml}$ insulin. Mouse melanoma cells GM95 and GM95/CG1cT-ER were grown in DMEM supplemented with 10% FCS. The anti-CD82 mAbs (monoclonal antibodies) γC11 and ιA4 were kindly provided by Dr H. Conjeaud (Institut Cochin, Paris, France). The anti-CD82 mAb M104 was kindly provided by Dr O. Yoshie (Shionogi Institute, Osaka, Japan). The anti-CD82 mAb TS82b and anti-CD9 mAb TS9 were kindly provided by Dr E. Rubinstein (INSERM U268, Villejuif, France). 5C11, anti-CD151 mAb, and C9BB, anti-CD9 mAb, were described elsewhere [22,23]. Dr L. Ashman (University of Newcastle, Newcastle, Australia) generously provided polyclonal anti-CD151 antibody and 11G1B4 anti-CD151 mAb. Dr F. Watt (Cancer Research UK London Research Institute, London, U.K.) kindly provided polyclonal antibody to $\alpha 3$ integrin subunit. Anti-EGFR mAb Ab-15 was purchased from Neomarkers; phosphospecific anti-EGFR polyclonal antibody was from Cell Signaling Technology. Anti-phosphotyrosine mAb (4G10) was purchased from Upstate Biotechnology. Monoclonal (H7) and polyclonal (Y-11) antibodies against HA (haemagglutinin) tag sequence were purchased from Autogen Bioclear. Dr R. Schnaar (The Johns Hopkins School of Medicine, Baltimore, MD, U.S.A.) kindly provided mouse mAbs to gangliosides G_{D1a} and G_{M1} . FITC-conjugated CT (cholera toxin B subunit) was purchased from Sigma. NB-DGJ (*N*-butyl-deoxygalactonojirimycin) was prepared as described in [24]. PPMP (D-threo-1-phenyl-2-hexadecanoylamino-3-morpholino-1-propanol-HCl) was purchased from Matreya. Other reagents were from Sigma or Fisher unless stated otherwise.

Transfections

GM95 and GM95/CG1cT-ER transfected with CD82 and CD151 tetraspanins were selected in zeocin-containing medium, and pools of drug-resistant clones were sorted with anti-CD82 mAb IA4 and anti-CD151 mAb 11G1B4. HB2 cells were transfected with sialidase Neu3 cDNA by using FuGENETM 6 (Roche) and selected (when necessary) in zeocin-containing medium.

Inhibition of ganglioside biosynthesis

For the inhibition of ganglioside biosynthesis, cells were incubated at 37°C with the appropriate concentration of the inhibitors, either NB-DGJ or PPMP, for 6–8 days. The inhibitor was added

to the media upon replating of cells. After the incubation, the cells were used for lipid extraction, immunoprecipitation, flow cytometry analysis, fractionation in sucrose density gradient, immunofluorescence staining or for the incubation with EGF. Control cells were grown in parallel without the inhibitor for the water-soluble NB-DGJ or in the presence of an equivalent amount of ethanol (0.1%) used for the solubilization of PPMP.

Exogenous administration of gangliosides

For exogenous administration of gangliosides G_{D1a} or G_{M3} , cells were plated 2 days before the experiment. Subsequently, G_{D1a} (Calbiochem), or G_{M3} (Matreya) was added to serum-free medium at a final concentration of 50 $\mu\text{g/ml}$. DMSO (25 $\mu\text{l/ml}$) was used in the control experiments. After the incubation (2–24 h), cells were washed with PBS and used in various assays.

Immunoprecipitation and Western blotting

Proteins were solubilized into the immunoprecipitation buffer containing 1% Brij98/PBS, 2 mM PMSF, 10 $\mu\text{g/ml}$ aprotinin and 10 $\mu\text{g/ml}$ leupeptin for 2 h at 4°C. The insoluble material was pelleted at 7000 g for 10 min. The cell lysates were then precleared by incubation for 2 h at 4°C with agarose beads conjugated with goat anti-mouse antibodies (mIgG-beads; Sigma). Immune complexes were collected using appropriate mAbs prebound to mIgG-beads and washed four times with the immunoprecipitation buffer. In some experiments we incubated an aliquot of the precleared lysate with biotinylated SNA [*Sambucus nigra* (elderberry) agglutinin] bark lectin (Vector Laboratories) that was bound to the immobilized Neutravidin (Pierce). The complexes were eluted from the beads with Laemmli sample buffer. Proteins were resolved by SDS/10–12% PAGE, transferred to a nitrocellulose membrane and developed with the appropriate antibody. Protein bands were visualized using horseradish-peroxidase-conjugated goat anti-mouse (or goat anti-rabbit) antibodies (Dako) and chemiluminescence reagent (PerkinElmer).

Flow cytometry

Cells were detached with EDTA solution, washed in PBS, diluted in 1% heat-inactivated BSA/PBS, and incubated with saturating concentrations of primary mouse mAbs for 1 h on ice. After the incubation, the cells were washed twice and then labelled with FITC-conjugated goat anti-mouse immunoglobulin (Sigma). Stained cells were analysed using Coulter Epics program (Becton Dickinson).

Fractionation in sucrose density gradient

Cells were scraped into 2 ml of PBS, centrifuged at 200 g for 3 min, and washed once with PBS. Sodium carbonate (pH 11.0) buffer (100 mM) supplemented with 10 $\mu\text{g/ml}$ aprotinin, 10 $\mu\text{g/ml}$ leupeptin and 2 mM PMSF was mixed with the pelleted cells. Lysates were homogenized using Dounce homogenizer (15 strokes) and a sonicator (4 × 20 s bursts, 1 min interval, power 10). The resulting homogenate was mixed with equal volume of 90% (w/v) sucrose prepared in 25 mM Mes buffer (pH 6.5), and overlaid with 2 vol. of 35% (w/v) sucrose and followed by 1 vol. of 5% sucrose (w/v; both in Mes). Samples were centrifuged at 55 000 g for 16–18 h at 4°C. Then, 400 μl aliquots were collected from the top of the tube. The pellet was dissolved in Laemmli buffer supplemented with protease inhibitors. Equal amounts of each fraction were mixed with 4 × Laemmli loading buffer and the distribution of proteins was analysed by SDS/PAGE followed by Western blotting. Protein concentration has been measured by Bio-Rad kit.

Cholesterol depletion

To remove cholesterol, cells were washed three times in PBS and incubated in serum-free medium containing 20 mM M β CD (methyl- β -cyclodextrin) for 1 h at 37°C. Levels of cholesterol in M β CD-treated cells were determined using Amplex Red Cholesterol Assay kit (Molecular Probes). After M β CD treatment the cells were used for the fractionation in sucrose density gradient as described above.

Ganglioside extraction and TLC

The gangliosides were extracted according to the small-scale isolation of gangliosides protocol [25], which is described briefly further. Cells were collected, washed and pelleted. A small cell pellet was adjusted to 0.6 ml with water and homogenized in Dounce homogenizer (15 strokes) on ice, and 1.6 ml of methanol was added. After vigorous agitation chloroform (0.8 ml) was added, and the capped mixture was vigorously agitated again at ambient temperature. The tube was centrifuged in a table-top centrifuge (3000 g and 10 min) at ambient temperature, the supernatant was transferred to a fresh tube, and the pellet was re-extracted with 2 ml of chloroform/methanol/water (4:8:3, by vol.). After centrifugation, the second supernatant was added to the first, the volume of the combined supernatants was measured, and water was added to bring the final chloroform/methanol/water proportion to 4:8:5.6. After vigorous agitation, the two phases were separated by centrifugation (1500 g, 10 min and ambient temperature) and the upper phase was transferred to a fresh tube. The lower phase was re-extracted with theoretical upper phase, and the combined upper phases were applied to a prewashed [1 ml of methanol and 2 ml of methanol/PBS (1:10, v/v)] Sep-Pak cartridge, which was subsequently washed with chloroform/methanol/water (2:43:55, by vol.) and methanol/water (1:1, v/v) prior to elution of gangliosides in methanol. The ganglioside-containing fraction was vacuum-dried and resuspended in a small volume of chloroform/methanol (2:1, v/v) for subsequent TLC analysis. TLC was performed on HPTLC (high-performance TLC) plates (Merck, Darmstadt, Germany) using a solvent system of chloroform/methanol/0.22% CaCl₂ (12:8:1, by vol.). The gangliosides were visualized with resorcinol reagent. The standard mixture of gangliosides was purchased from Matreya.

Immunofluorescence staining

Cells were grown on glass coverslips in complete media for 24–36 h. When required, the cells grown on coverslips were transiently transfected with Neu3 sialidase cDNA. Spread cells were fixed with 2% (w/v) paraformaldehyde/PBS for 10 min. When required the cells were permeabilized with 0.4% saponin/PBS. Staining with primary and fluorochrome-conjugated secondary antibodies was carried out as previously described [26]. Staining with FITC-conjugated CT was carried out on live cells at 4°C for 1 h. Staining was analysed using a Nikon Eclipse E600 microscope. Images were acquired using Nikon digital camera DXM1200 and subsequently processed using a Nikon ACT-1 image processing program.

RESULTS

Inhibition of glycosphingolipid biosynthetic pathway weakens protein–protein interactions within TERMS

To elucidate the relationship between gangliosides and TERMS we used two inhibitors of glucosylceramide synthase (a key enzyme in the glycosphingolipid biosynthetic pathway [27]), NB-

DGJ and PPMP. In the preliminary experiments, we established that after 6 days of incubation with 2 mM NB-DGJ the level of gangliosides in HB2/CD82 cells was below detection by TLC (Figure 1A, lane d). Notably, TLC analysis also demonstrated the absence of G_{M3} in HB2 cells (Figure 1A, lane b). Flow cytometry analysis indicated that in NB-DGJ-treated cells the surface expression of gangliosides had been reduced to 4% of G_{D1a} and 7% of G_{M1} (Figure 1B) in comparison with control cells. The cells were incubated for 6–8 days with 2 mM NB-DGJ in all subsequent experiments. Similarly, we found that using PPMP at 20 μ M for 7 days dramatically decreased the concentration of gangliosides. The efficacy of inhibition of ganglioside biosynthesis in each experiment was tested by measuring the surface expression level of gangliosides by flow cytometry. Interestingly, depletion of gangliosides led to an increase in the amount of CD82 (Figure 1B). We have previously demonstrated that there is a correlation between the expression levels of CD82 and gangliosides: increase in CD82 amounts led to a rise in G_{D1a} concentration [21].

Immunoprecipitation with the anti-CD82 antibody revealed significant changes in the associations of CD82 with other proteins under the conditions of ganglioside depletion (Figure 1C, compare lanes 1 and 3 or lanes 5 and 7; Figure 1D). There was less EGFR and CD9 (~35 and 25% decrease respectively) immunoprecipitated with the anti-CD82 antibody from the lysates prepared from the NB-DGJ-treated cells (Figure 1C, lanes 1 and 3, first panel from the top) in comparison with the controls, untreated cells. Association of CD82 with α 3 integrin subunit was also effected (~20% decrease) (Figure 1C, lanes 1 and 3, second panel from the top; Figure 1D). Notably, the association of CD82 with the tetraspanin CD151 was diminished by approx. 4-fold (Figure 1C, lanes 1 and 3, second panel from the bottom; Figure 1D). Western blotting with the anti-CD82 mAb indicated that the amounts of immunoprecipitated CD82 were comparable in the controls and NB-DGJ-treated cells (Figure 1C, lanes 1 and 3, lower panel). Depletion of gangliosides with PPMP also led to decreases in the association of CD82 with its interacting partners (Figure 1C, compare lanes 5 and 7 or lanes 6 and 8). To investigate how specific was the effect of ganglioside depletion, we also analysed interactions involving tetraspanin CD9. In contrast with CD82-containing complexes, there was no obvious effect of ganglioside depletion on the interactions involving CD9 (Figure 1C, compare lanes 2 and 4 or lanes 6 and 8; Figure 1D). We concluded from these results that gangliosides are important for the interactions within CD82-enriched microdomains.

Changes in ganglioside concentration on the cell surface caused by Neu3 sialidase expression had an impact on the stability of CD82-containing complexes

Inhibition of ganglioside biosynthesis does not allow evaluation of the contribution of individual gangliosides to the assembly of TERMS. To answer the question what ganglioside(s) is(are) important for the organization of these domains, we applied other methods for the manipulation of ganglioside composition in cells. We examined the effect of the overexpression of the plasma-membrane-bound Neu3 sialidase on the stability of protein complexes within tetraspanin microdomains. Sialidases are specific hydrolytic enzymes that remove sialic acid residues from gangliosides and sialated proteins [28]. Sialidase Neu3 is a cell surface enzyme that is characterized by its high substrate specificity towards gangliosides [29,30]. Previous studies showed that Neu3 selectively targets gangliosides G_{D1a} and G_{M3} at plasma membrane [31].

HB2/CD82 cells were transiently transfected with the HA-tagged form of sialidase Neu3. It has been shown previously

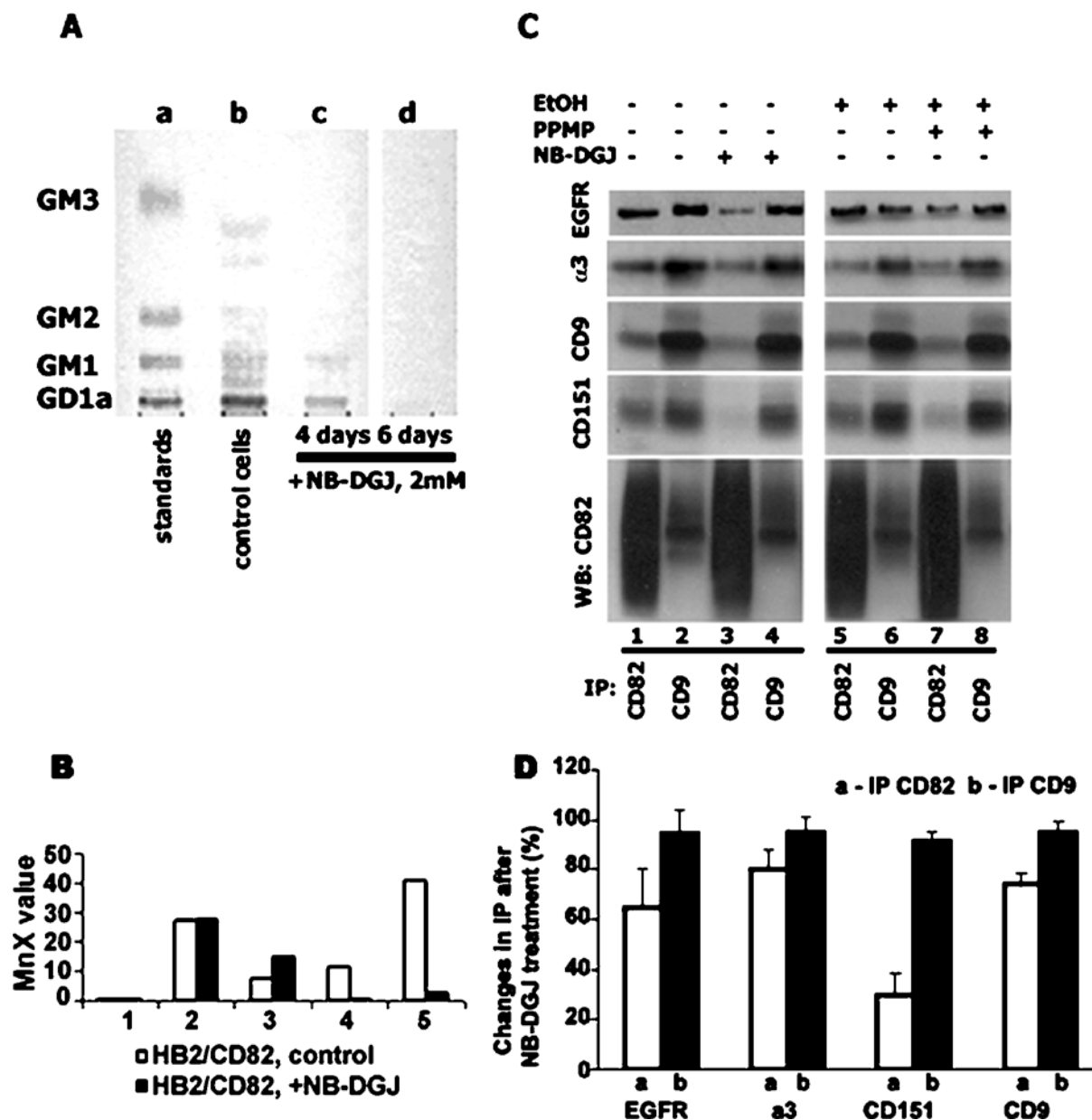


Figure 1 Effect of ganglioside depletion on the interactions in TERMs

HB2/CD82 cells were incubated with 2 mM NB-DGJ for 4–6 days or 20 μ M PPMP for 7 days; control cells were grown in parallel. (A) Lipids were extracted, purified, chromatographed on HPTLC (high-performance TLC) plates and revealed with resorcinol staining. The positions of co-chromatographed ganglioside standards are indicated. (B) Surface expression of gangliosides was analysed by flow cytometry using the Coulter Epics program. Results are presented as mean fluorescence intensity (MnX) and represent one of three experiments. The mAbs used were: mAb 1, negative control, 187.1; mAb 2, anti- α 3 integrin subunit, A3-IIF5; mAb 3, anti-CD82, M104; mAb 4, anti- G_{D1a} , G_{D1a-1} ; mAb 5, anti- G_{M1} , FITC-conjugated CT. (C) Cells were lysed in 1% Brij98 and the complexes were immunoprecipitated using specific mAbs: anti-CD82, γ C11; anti-CD9, TS9. The immunoprecipitated complexes were resolved by SDS/10% PAGE (for reduced samples) or SDS/12% PAGE (for non-reduced samples), transferred to a nitrocellulose membrane and probed with antibodies to α 3 integrin subunit (polyclonal sera), anti-EGFR (mAb Ab-15), anti-CD82 (mAb TS82b), anti-CD9 (mAb C9BB), or anti-CD151 (polyclonal sera). Results shown represent one of three independent experiments. WB, Western blot. (D) Quantification of the results of three immunoprecipitation (IP) experiments (treatment with NB-DGJ). Means of band intensities relative to that in the control samples (i.e. non-treated cells) are shown. Error bars indicate standard deviations. (a) IP with anti-CD82 mAb; (b) IP with anti-CD9 mAb.

that HA tag did not affect the activity of the enzyme [30]. Because HB2/CD82 cells do not have detectable G_{M3} , we expected that the expression of Neu3 in these cells would only affect the level of G_{D1a} . To validate the activity of Neu3 sialidase in transiently transfected HB2/CD82 cells we carried out immunofluorescence staining with the anti- G_{D1a} and anti-HA mAbs. Figure 2 illustrates that the cells transfected with Neu3 have significantly lower levels of G_{D1a} compared with the surrounding non-transfected

cells (compare Figures 2A and 2B). We also tested whether G_{M1} expression was affected by Neu3 sialidase. Staining with CT indicated that levels of G_{M1} were comparable in the Neu3-positive and Neu3-negative cells (compare Figures 2C and 2D). Interestingly, we consistently observed that incubation with CT affected distribution of HA-tagged Neu3 sialidase, making it more disperse. This may be due to the multivalent nature of CT. We did not examine G_{M3} levels, since we demonstrated earlier that

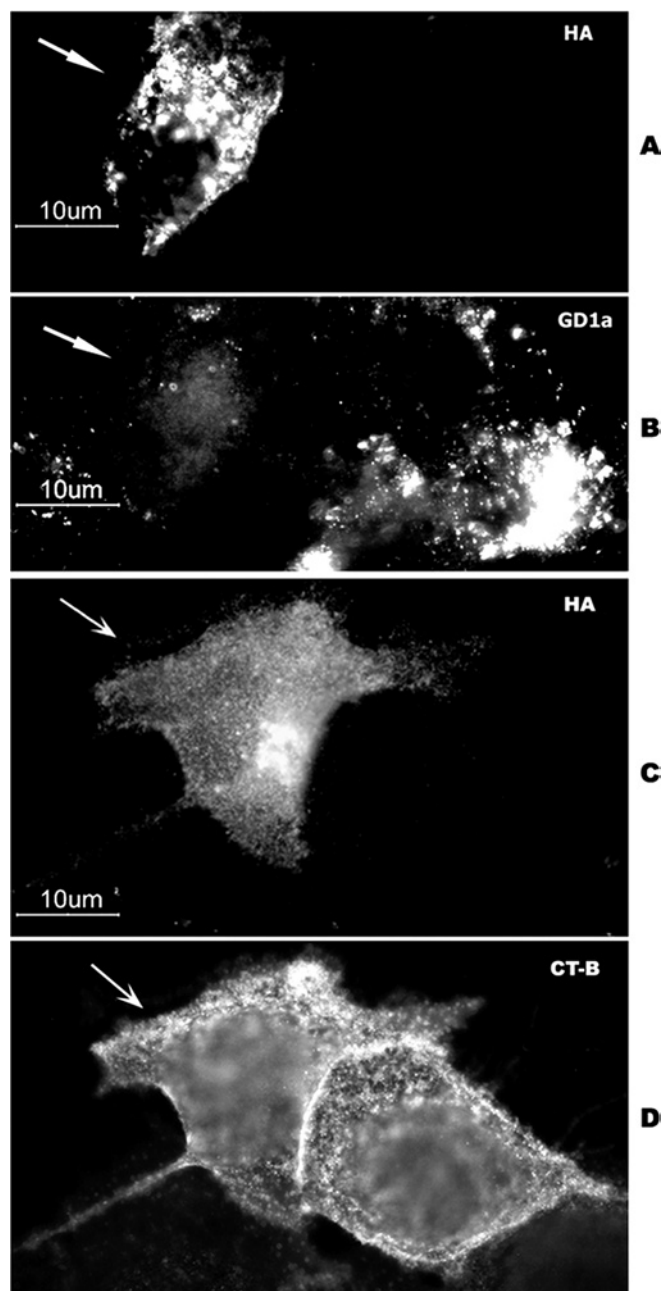


Figure 2 Immunofluorescence staining of HB2/CD82 cells transiently transfected with Neu3 sialidase

Cells grown on glass coverslips were transfected with sialidase Neu3 cDNA. **(A, B)** After 48 h the cells were fixed with paraformaldehyde and permeabilized with 0.4% saponin. Double staining immunofluorescence was carried out with the anti-HA (F-7) and anti- G_{D1a} (G_{D1a-1}) mAbs. Isotype-specific secondary antibodies conjugated to Alexa Fluor® 488 or Alexa Fluor® 594 fluorochromes were used for detection of the antigens. **(A)** Staining with the anti-HA antibody. **(B)** Staining of the same field with the anti- G_{D1a} antibody. Arrows indicate the cell expressing Neu3 sialidase. **(C, D)** After 48 h cells were incubated with FITC-conjugated CT for 1 h at 4°C. Cells were washed, fixed with 2% (w/v) paraformaldehyde for 10 min and permeabilized with 0.4% saponin. Subsequently, cells were stained with the anti-HA mAb (F-7). Alexa Fluor® 594-conjugated goat anti-mouse antibody was used to visualize cells expressing HA-tagged Neu3 (indicated with arrows). **(C)** Staining with the anti-HA mAb. **(D)** Staining of the same field with FITC-conjugated CT. Note that staining with the anti-HA mAb is more dispersed in the presence of CT, most likely due to the pre-incubation of cells with CT prior to fixation.

G_{M3} could not be detected in HB2 cells (Figure 1A, lane b). These results provided evidence that of the two main A-series gangliosides expressed on the surface of HB2 cells (i.e. G_{D1a}

and G_{M1}) expression of Neu3 sialidase specifically affected G_{D1a} levels.

Neu3 transfectants and the control cells were examined in the immunoprecipitation experiments with the anti-CD82, anti-CD9 and anti-HA-tag mAbs. Control cells and Neu3 transfectants expressed comparable levels of tetraspanins CD82, CD9 and CD151 (Figure 3A, lanes 9 and 10). Notably, anti-CD82 mAb co-immunoprecipitated significantly less of CD151 from the cells transfected with Neu3 than from the control cells (~50% decrease; Figure 3A, second panel from the top, lanes 1 and 5); the amounts of immunoprecipitated CD82 in both samples were comparable (Figure 3A, third panel from the top, lanes 1 and 5). In contrast, we observed that the expression of Neu3 increased the association of CD82 with EGFR (~40% increase over the control sample; Figure 3B, lanes 1 and 4). These results provide additional evidence that microdomains containing CD82 are sensitive to the modulation of ganglioside composition at the cell surface. Importantly, interactions of CD9 with other tetraspanins and EGFR were not affected by the expression of Neu3 sialidase (Figures 3A, lanes 3 and 7, and 3B, lanes 2 and 5). In additional experiments we found that ectopic expression of Neu3 had no effect on the ability of CD82 to bind SNA lectin (Figure 3C). These results indicated that Neu3 is inactive towards sialic acid residues on CD82 and exclude a possibility that the effect of the sialidase on the interactions involving CD82 was due to changes in glycosylation of the protein.

To assess the effect of the Neu3 overexpression on tetraspanin–tetraspanin associations in the absence of CD82, we examined the interactions within TERM, in HB2 cells, which have negligible amounts of CD82. As expected, transfection of Neu3 sialidase into HB2 cells decreased surface expression of ganglioside G_{D1a} present in these cells (Figure 4A), indicating that the enzyme was active. We used one of the clones transfected with Neu3 and one of the control clones transfected with the vector in further experiments. The anti-CD151 and anti-CD9 mAbs co-immunoprecipitated comparable amounts of CD9, CD151 and $\alpha 3$ integrin subunit from the lysates (Figure 4B, lanes 1 and 6, and lanes 3 and 8). In these experiments, we also examined interactions of the tetraspanin CD81 (Figure 4B, lanes 2 and 7). There were no apparent differences in the immunocomplexes co-immunoprecipitated by the anti-CD81 antibody from Neu3-transfected and control cells. Taken together, these results led us to the conclusion that, in mammary epithelial cells, only CD82-based interactions are sensitive to the modulations in ganglioside composition at the membrane. Furthermore, in the absence of CD82, changes in the concentration of ganglioside G_{D1a} had no effect on the tetraspanin–tetraspanin interactions.

Assembly of CD82-enriched microdomains is not affected by G_{M3} ganglioside

We showed that G_{M3} ganglioside could not be detected in significant quantities in HB2 cells (Figure 1A). However, there is evidence that this ganglioside may be important for the interactions involving some of the tetraspanins (e.g. CD9) [17–19]. To examine the contribution of G_{M3} to the stability of TERM, we analysed TERMS in GM95 and GM95/CGIcT-ER cells, two derivatives from MEB-4 mouse melanoma cell line [32,33]. While GM95 cells are glycosphingolipid-deficient, GM95/CGIcT-ER cells are transfected with ceramide glucosyltransferase and predominantly express ganglioside G_{M3} . Both cell lines were stably transfected to express human CD82 and CD151 proteins.

We used GM95/CD82 + CD151 and GM95/CGIcT-ER/CD82 + CD151 cells in the immunoprecipitation analysis. When we compared CD82 immunoprecipitates prepared from both

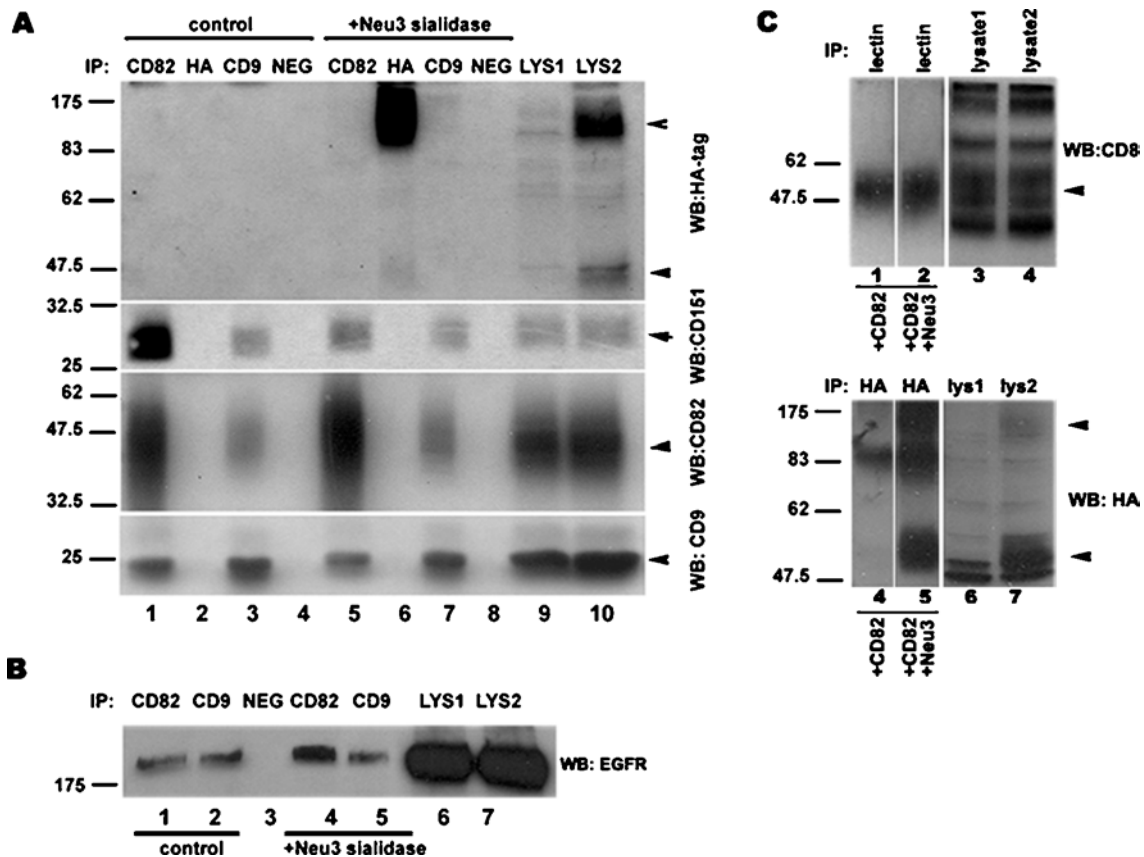


Figure 3 Expression of Neu3 sialidase affects the interactions involving CD82 but does not affect glycosylation pattern of the protein

(**A** and **B**) HB2/CD82 cells were transiently transfected with the HA-tagged form of sialidase Neu3 or with the control DNA. After 48 h cells were lysed in 1% Brij98 and analysed for antigen expression. Data of one of the two separate experiments are shown. Complexes were immunoprecipitated using specific mAbs: anti-HA-tag, F-7 (lanes 2 and 6); anti-CD82, γ C11 (lanes 1 and 5); anti-CD9, TS9 (lanes 3 and 7). The immunoprecipitated complexes were divided into three aliquots (20–40 μ l) and resolved by SDS/12% PAGE (non-reduced samples) for probing with the anti-HA-tag polyclonal antibody (Y11), anti-CD82 (mAb TS82b), anti-CD9 (mAb C9BB) or anti-CD151 (polyclonal sera), or in SDS/10% PAGE (reduced samples) for probing with the anti-EGFR (mAb Ab-15). Two bands at the top panel (**A**, lane 10) developed with the anti-HA antibody most likely represent the monomer and dimer of Neu3 sialidase. Lys1 (lane 9 in **A**, and lane 6 in **B**): lysate derived from the control cells; Lys2 (lane 10 in **A**, and lane 7 in **B**): lysate derived from the cells transfected with Neu3 sialidase. (**C**) HB2 cells were transiently transfected with the plasmid encoding CD82 alone or in combination with the plasmid encoding HA-tagged form of sialidase Neu3. After 48 h cells were lysed in 1% Brij98 and the proteins containing sialic acid moiety were precipitated using the immobilized SNA lectin. Proteins were resolved by SDS/12% PAGE, transferred to the nitrocellulose membrane and probed with the anti-CD82 mAb (TS82b). Top panel: lanes 1 and 2, lectin IP; lanes 3 and 4, lysates from the appropriate sample. Lower panel shows results of Western blotting with the anti-HA mAb (lanes 5 and 6). Molecular masses are given in kDa. WB, Western blot.

cell lines, there were no differences in the amounts of CD151 found in each of the samples (Figure 5, lanes 1 and 4, lower panel). Accordingly, the anti-CD151 mAb co-immunoprecipitated similar amounts of CD82 (Figure 5, lanes 2 and 5, upper panel). Both cell lines had comparable expression levels of transfected tetraspanins as detected by Western blotting (Figure 5, lanes 7 and 8) and flow cytometry (results not shown). We concluded from these experiments that CD82 interactions are not affected by G_{M3} ganglioside.

To examine the links between CD82 and gangliosides further, we incubated ganglioside-deficient cells GM95/CD82 + CD151 with exogenously added G_{D1a} or G_{M3} . In our experiments we used the non-toxic concentration of gangliosides (50 μ g/ml) that was determined in pilot experiments. Flow cytometry analysis confirmed incorporation of gangliosides into the plasma membrane of the cells (results not shown). Comparable amounts of CD151 and CD82 were co-immunoprecipitated by the anti-CD82 (Figure 6, lanes 1, 4 and 7) and anti-CD151 mAbs (Figure 6, lanes 2, 5 and 8). Similar results were obtained when we used HB2/CD82 cells (results not shown). These results showed that exogenous administration of gangliosides does not affect tetraspanin–tetraspanin interactions.

Inhibition of ganglioside biosynthesis has specific effect on the EGFR activity in CD82-expressing cells

To analyse biological consequences of changes in the ganglioside content in relation to the activity of CD82, we examined phosphorylation of EGFR in CD82-expressing cells depleted of gangliosides. We have shown previously that CD82 modulates activity of EGFR [14,21], possibly through gangliosides. We compared ligand-induced phosphorylation of EGFR in HB2/CD82 cells incubated with PPMP inhibitor and in the control cells (Figure 7). Cells were serum-starved and stimulated with EGF for 15 and 60 min. Western-blot analysis indicated that depletion of gangliosides in HB2/CD82 cells reduced tyrosine phosphorylation levels of EGFR (~2-fold reduction for both 15 and 60 min time points; Figure 7B, compare lanes 2 and 5, and lanes 3 and 6). On the other hand, depletion of gangliosides in cells lacking CD82 (HB2/ZEO) had a slight positive effect on the ligand-induced phosphorylation of EGFR (Figure 7A, compare lanes 2 and 5, and lanes 3 and 6). Differences in the response to ganglioside depletion in HB2/ZEO and HB2/CD82 cells provide further evidence for the co-operative way of modulation of EGFR activities by gangliosides and CD82.

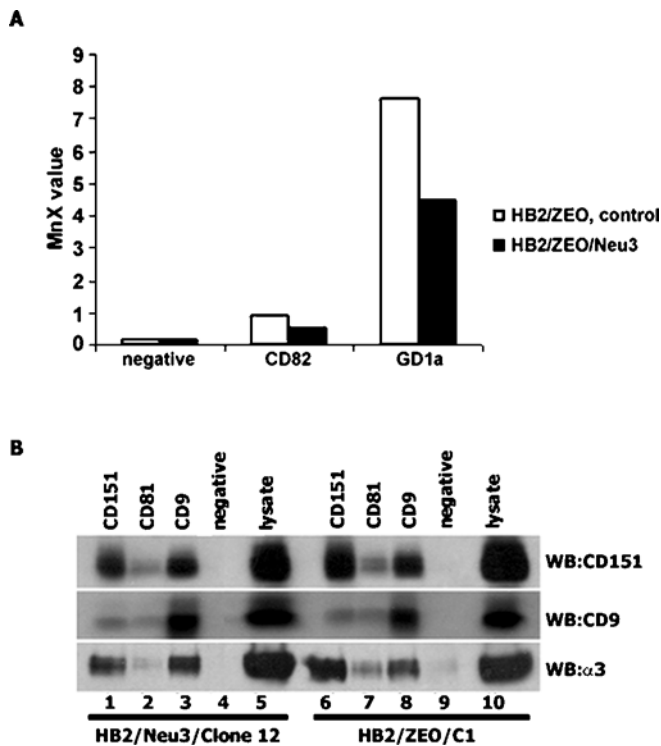


Figure 4 Expression of Neu3 sialidase does not affect interactions of tetraspanins in the absence of CD82

HB2 cells were transfected with the plasmid encoding the HA-tagged form of sialidase Neu3 or with the control DNA. Positive clones were tested by flow cytometry for decrease in G_{D1a} surface expression and by Western blotting for expression of HA-tagged sialidase. Experiments were repeated three times. **(A)** Surface expression level of G_{D1a} in cells expressing Neu3 was analysed by flow cytometry. The mAbs used were: negative control, 187.1; anti-CD82, M104; anti- G_{D1a} , IgG1. Results are presented as means of fluorescence intensity (MnX). **(B)** Cells were lysed in 1% Brij98 and the complexes were immunoprecipitated using specific mAbs: anti-CD151, 5C11 (lanes 1 and 6); anti-CD81, M38 (lanes 2 and 7); anti-CD9, TS9 (lanes 3 and 8); negative control, 187.1 (lanes 4 and 9). The immunoprecipitated complexes were resolved by SDS/10% PAGE (for reduced samples) or SDS/12% PAGE (for non-reduced samples), transferred to a nitrocellulose membrane and probed with the antibodies to α 3 integrin subunit (polyclonal sera), or anti-CD151 (polyclonal sera), or anti-CD9 (mAb C9BB). WB, Western blot.

Inhibition of ganglioside biosynthesis causes redistribution of CD82-associated proteins

We hypothesized that the effect of ganglioside depletion on the associations involving CD82 may be due to the alterations in membrane distribution of the proteins. Thus we examined how treatment of HB2/CD82 cells with NB-DGJ affected protein distribution within the membrane by fractionation of cellular lysates in continuous sucrose density gradient. For the preparation of lysates for fractionation, we used a modified detergent-free protocol that allowed concentration of most of the tetraspanin-containing complexes to the light membrane fractions of the gradient (Figure 8A, panels c in each frame). We avoided detergents in these experiments, as there is evidence that some detergents may induce artefactual formation of microdomains in the membranes [34]. The bulk of CD82 was found in fractions 2–4 together with significant amounts of EGFR, α 3 integrin subunit and CD151 tetraspanin (Figure 8A, panels c in each frame, lanes 2–4). Depletion of gangliosides had a dramatic effect on the membrane distribution of all tested proteins (Figure 8A, panels i in each frame). The most striking fact was the difference in the fractional distribution of CD82 and CD82-associated proteins. The bulk of EGFR, α 3 integrin subunit and CD151 were

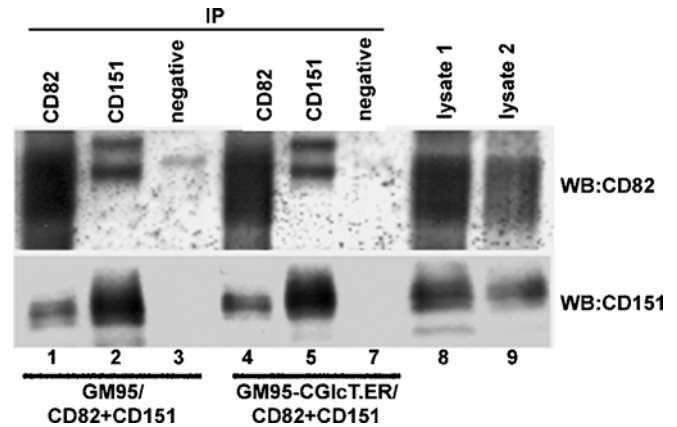


Figure 5 Ganglioside G_{M3} has no effect on the interactions of CD82

GM95/CD82 + CD151 and GM95/CGlcT-ER/CD82 + CD151 cells were lysed in 1% Brij98 and the complexes were immunoprecipitated using specific mAbs: anti-CD82, γ C11 (lanes 1 and 4); anti-CD151, 5C11 (lanes 2 and 5); and negative control, 187.1 (lanes 3 and 6). The immunoprecipitated complexes were resolved by SDS/12% PAGE, transferred to a nitrocellulose membrane and probed with the anti-CD82 (mAb TS82b) or anti-CD151 (polyclonal sera). Upper bands in CD151 immunoprecipitates revealed by the anti-CD82 mAb are non-specific. Lysate 1 (lane 7) corresponds to the lysate derived from GM95/CD82 + CD151 cells; lysate 2 (lane 8) corresponds to the lysate derived from GM95/CGlcT-ER/CD82 + CD151 cells. WB, Western blot.

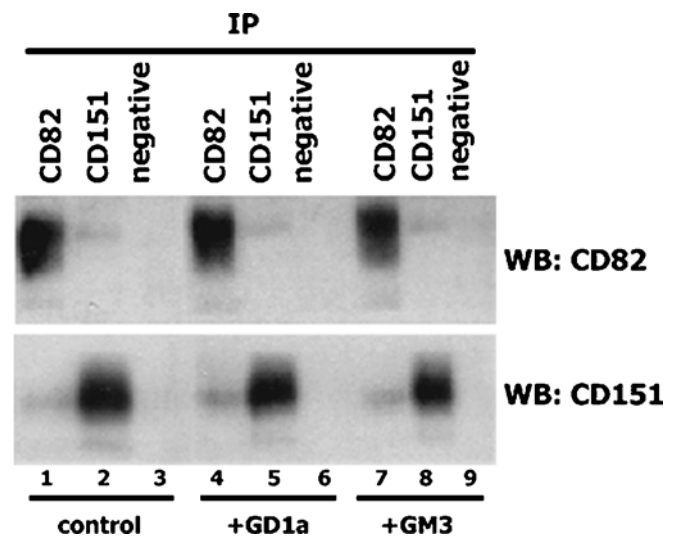


Figure 6 Exogenous administration of gangliosides does not affect stability of CD82 complexes

Ganglioside-deficient GM95/CD82 + CD151 cells were incubated with 50 μ g/ml G_{D1a} or G_{M3} or 25 μ l/ml DMSO in serum-free medium for 24 h at 37 $^{\circ}$ C. After the incubation, cells were subjected to immunoprecipitation (IP) analyses. The complexes were immunoprecipitated using specific mAbs: anti-CD82, γ C11 (lanes 1, 4 and 7); anti-CD151, 5C11 (lanes 2, 5 and 8); and negative control, 187.1 (lanes 3, 6 and 9). Proteins were resolved by SDS/12% PAGE, transferred to a nitrocellulose membrane and probed with the antibodies to CD82 (mAb TS82b) or CD151 (polyclonal sera). WB, Western blot.

shifted to the heavy fractions (fractions 7–10) of the gradient (Figure 8A, panels i in each frame). The distribution of CD82 has also shifted to the right, to heavier fractions, but this shift was not as pronounced as for the other proteins: most of the protein was floating in fractions 4–6 of the gradient. Thus CD82 and associated proteins were enriched in different fractions of the sucrose density gradient. Distribution of CD9 has been less affected by gangliosides depletion. It was localized mainly to the

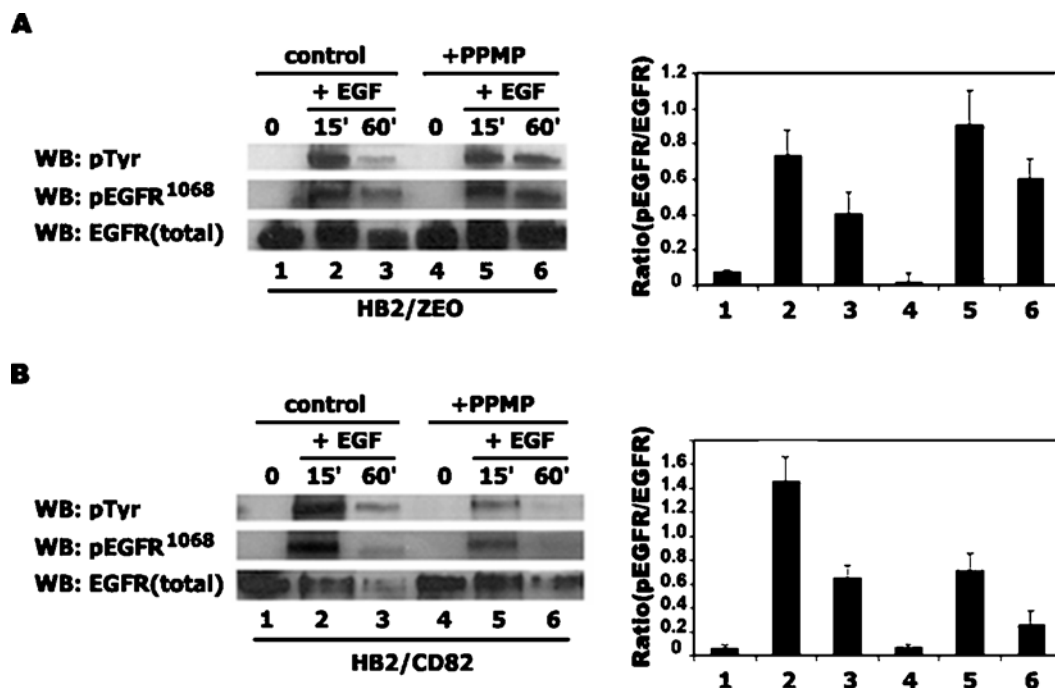


Figure 7 Inhibition of ganglioside biosynthesis has a specific effect on the EGFR activity in CD82-expressing cells

Cells (HB2/ZEO, **A**, or HB2/CD82, **B**) were incubated with 20 μ M PPMP or 0.1% ethanol (control) for 7 days and then serum-starved for 6 h and stimulated with EGF (100 μ g/ml) for 15 or 60 min. Lysates were prepared in 1 \times Laemmli buffer. Equal amounts of proteins were resolved by SDS/10% PAGE, transferred to a nitrocellulose membrane and probed with the anti-phosphotyrosine mAb (4G10, Upstate Biotechnology), or phosphospecific anti-EGFR polyclonal antibody (Tyr¹⁰⁶⁸), or anti-EGFR mAb (Ab-15). Data presented are results of one of three independent experiments. Quantification of three independent experiments was performed. Means of ratios of the densities of phosphorylated EGFR to the densities of total EGFR are shown. Error bars indicate standard deviations.

lighter fractions in control cells. Treatment with the inhibitor led to re-localization of the protein to the right, to heavier fractions. However, significant amount of CD9 could be still found in the light fractions. To examine the interactions involving CD82 in the microdomains floating in the light fractions of the sucrose density gradient, we carried out immunoprecipitation/Western blotting experiments with the anti-CD82 mAb on the pooled light gradient fractions (fraction 2–5). As shown in Figure 8(C), CD82–CD151 and CD82–CD9 complexes can be detected in these fractions. As expected, ganglioside depletion diminished the amount of CD82 recovered from the combined light fractions. Consequently, CD151 and CD9 were almost completely lost from the CD82 immunoprecipitate (Figure 8C, compare lanes c and i). The results of fractionation in sucrose density gradient indicated that alterations in glycosphingolipid contents affected associations of the proteins within CD82-containing complexes.

Redistribution of CD82 at the cell membrane was further illustrated by the immunofluorescence staining carried out on the cells depleted of gangliosides by incubation with NB-DGJ inhibitor. In non-permeabilized untreated cells, tetraspanin CD82 exhibited typical peripheral staining (Figure 9A). However, after incubation with NB-DGJ a significant proportion of CD82 relocalized to large dots randomly scattered on the cell surface (Figure 9B). These results indicated that the profound effect of gangliosides depletion on the stability of TERMs is likely to be a consequence of the re-distribution of proteins at the plasma membrane.

Cholesterol has an important role for the assembly and maintenance of various microdomains at the plasma membrane [35]. There are controversial data in the literature about the role of cholesterol in the assembly of TERMs [7,36]. To compare

effects of ganglioside and cholesterol depletion on the distribution of CD82-associated proteins we incubated HB2/CD82 cells with 20 mM M β CD for 1 h. Typically, after this treatment we removed approx. 40% of total cholesterol (as measured following cell lysis). Lysates after M β CD treatment were subjected to the fractionation in sucrose density gradient as described above. Cholesterol depletion led to the re-distribution of CD82-associated proteins in sucrose density gradient. The effect was more or less similar to the effect of ganglioside depletion (Figure 8B, compare panels c and d). Notably, the fractional distribution of CD82 itself was only marginally affected by the cholesterol depletion. Together, these results indicate that gangliosides and cholesterol have distinct roles in maintaining the structural integrity of the CD82-enriched microdomains.

DISCUSSION

Interactions between tetraspanins provide a structural platform for the assembly of TERM. Our results demonstrate for the first time that gangliosides can stabilize a subset of tetraspanin–tetraspanin interactions. This was shown by using both pharmacological and physiological (i.e. ectopic expression of Neu3) approaches. Notably, we found that only interactions involving CD82 (e.g. CD82–CD151, CD82–EGFR) are sensitive to changes in the ganglioside content, thus pointing to a diversity of the mechanisms that control the assembly and (or) maintenance of TERM. Extreme sensitivity of CD82-based interactions to gangliosides may also imply that there exist different types of TERM, which are specifically enriched with one (or two) tetraspanins (e.g. CD82-enriched TERM and CD9-enriched TERM). This idea is not

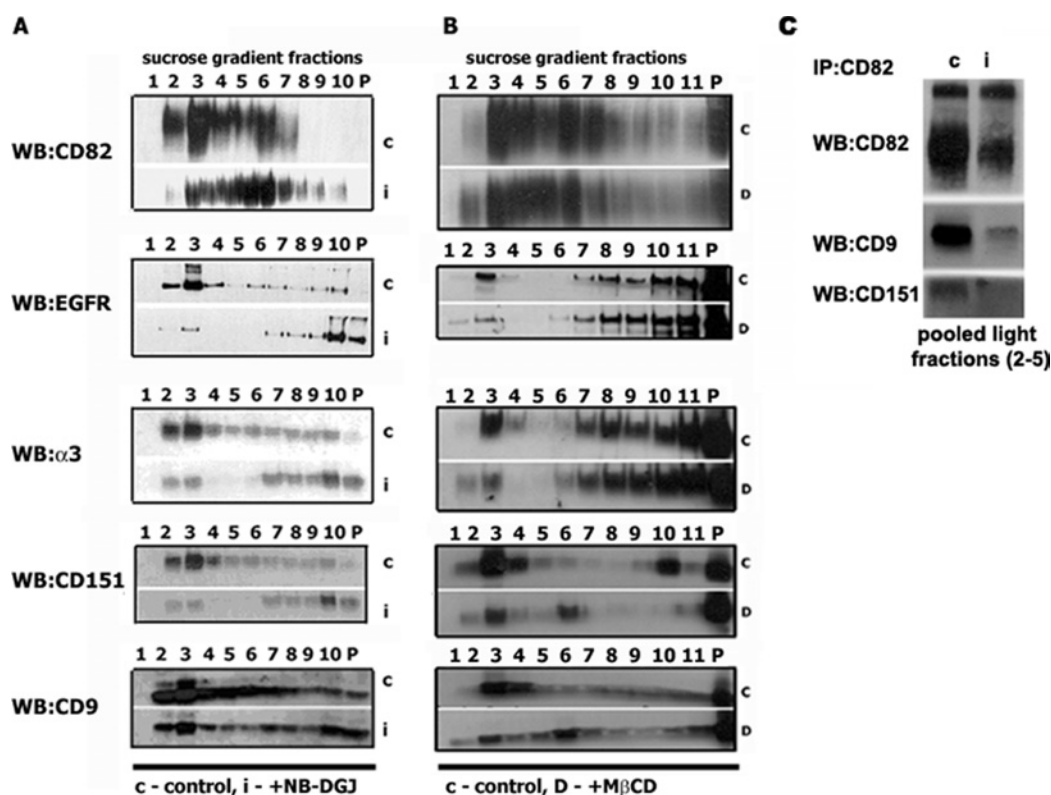


Figure 8 Effect of inhibition of ganglioside biosynthesis on the compartmentalization of membrane proteins

The lysates of HB2/CD82 cells treated with 2 mM NB-DGJ for 7 days (**A**) or 20 mM M β CD for 1 h (**B**) and control cells were prepared under the detergent-free conditions as described in the Materials and methods section. Lysates were fractionated by ultracentrifugation in continuous sucrose density gradient (5–45%). Fractions were collected from the top of the gradient. Equal volumes of each fraction were resolved by SDS/10% PAGE or SDS/12% PAGE. Distribution of proteins in the gradient fractions was assessed by Western blotting using specific antibodies: anti-CD82 (mAb TS82b), anti-EGFR (mAb Ab-15), anti- α 3 integrin subunit (polyclonal sera), anti-CD151 (polyclonal sera) and anti-CD9 (mAb C9BB). Results of one of two separate experiments are shown. c, Control cells; i, inhibitor-treated cells. Note that distributions of proteins in the gradients obtained from the control cells in the experiments shown in (**A**) and (**B**) are slightly different. This may be due to the differences in the culturing conditions of cells prior to lysis. Cholesterol depletion was carried out under serum-free conditions and, consequently, the control cells were kept under serum-free conditions (**B**). In the experiments depicted in (**A**), cells were grown in the serum-containing medium. P, pellet. (**C**) Light fractions (2–5) of sucrose density gradients obtained from the control untreated cells and cells depleted of gangliosides were pooled together, and the CD82-containing complexes were immunoprecipitated using mAb γ C11. Immunoprecipitated material was resolved by SDS/12% PAGE, transferred to a nitrocellulose membrane and probed with the anti-CD82 (TS82b) mAb, anti-CD9 (C9BB) mAb and anti-CD151 polyclonal antibody. WB, Western blot.

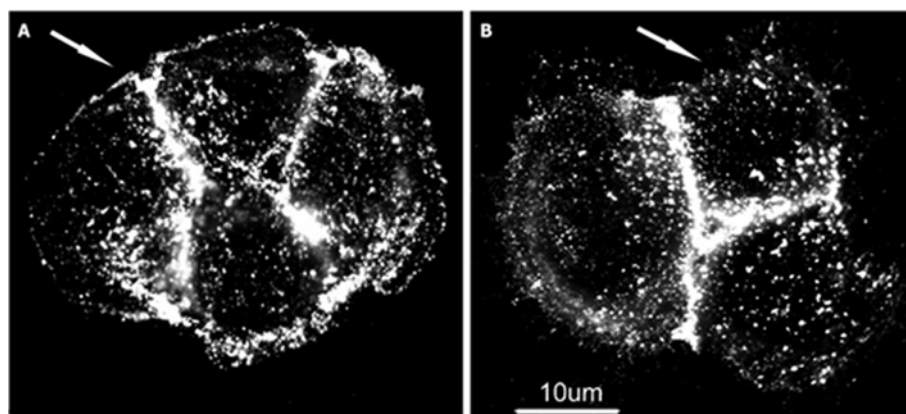


Figure 9 Effect of ganglioside depletion on the surface distribution of CD82

Cells incubated with NB-DGJ (7 days, 2 mM) and control cells were plated on to coverslips. After 24 h the cells were fixed with 2% paraformaldehyde and stained with primary anti-CD82 (IA4) mAb. Isotype-specific secondary antibodies conjugated to Alexa Fluor[®] 488 fluorochrome were used for the detection of the antigen. (**A**) Control cells. (**B**) Cells depleted of gangliosides. Arrows point to the peripheral area of the cells.

without a precedent: for example, urothelial tetraspanins UPIa and UPIb are specifically abundant in the asymmetric unit membranes, which do not seem to include any other tetraspanin proteins [37]. Further analysis will be necessary to verify this hypothesis.

In contrast with the results obtained with the inhibitor (and results with overexpression of Neu3), exogenous administration of purified gangliosides had no effect on the association of tetraspanins with each other. There may be at least two reasons

for this 'discrepancy'. It is well established that biosynthesis of complex gangliosides takes place in Golgi [28]. Furthermore, it is likely that many of the TERM-specific interactions are also initiated in this compartment [8,11]. Thus there may be a spatial and temporal co-ordination of these two processes in the Golgi complex. Although exogenously added gangliosides can be internalized and subsequently delivered to Golgi [28], they may be excluded from the 'TERM assembly line'. Alternatively, exogenous gangliosides may be incorporated into microdomains that are distinct from those containing the endogenous molecules. In this regard, it has been recently demonstrated that added G_{M1} accumulated mainly in non-DRM compartments, whereas the endogenous ganglioside was completely resistant to detergent extraction [38].

Our results indicate that there is a specific link between ganglioside G_{D1a} and tetraspanin CD82. Indeed, specific depletion of this ganglioside with Neu3 sialidase reveals that G_{D1a} has both positive (association of CD82 with CD151) and negative (interaction between CD82 and EGFR) roles in maintaining CD82-enriched microdomains. On the other hand, it is unlikely that G_{M3} , which is known to facilitate associations of CD9 with integrins [17,18], is involved in regulation of CD82-dependent interactions. Firstly, G_{M3} could not be detected in HB2 cells (perhaps, because it is quickly converted into G_{M2} and, subsequently, into G_{M1}). Thus the effect of Neu3 on the stability of CD82-enriched microdomains in HB2 cells is not mediated by this ganglioside. Secondly, our experiments with GM95 cells (Figure 5) also excluded the involvement of G_{M3} in the maintenance of CD82-enriched microdomains. The individual contribution of two other main gangliosides of A-series, G_{M2} and G_{M1} , is currently less clear. The fact that the association between CD82 and EGFR is potentiated in the G_{D1a} -depleted cells, but weakened in cells with decreased levels of all gangliosides, suggests that G_{M1} and/or G_{M2} may stabilize some of the interactions within CD82-enriched microdomains. Further experiments will be necessary to address this issue.

How does G_{D1a} affect the interactions of CD82 with other TERM-associated proteins? Hakomori and Handa [39] proposed a model in which gangliosides directly bind to a carbohydrate moiety in various tetraspanins and facilitate their interactions with other glycosylated proteins in tetraspanin-containing microdomains. Indeed, stability of the complexes involving CD82 was dependent on glycosylation of the protein [18,19]. Hence, one could envisage that binding of G_{D1a} (but not G_{M3}) to the large extracellular loop of CD82 (which has three N-linked glycosylation sites) either strengthens (e.g. CD151) or diminishes (e.g. EGFR) its interactions with other glycosylated proteins. Alternatively, it is possible that the effect of G_{D1a} on the interactions involving CD82 is not dependent on glycosylation of the protein. In this regard, we found that NB-DGJ still had a pronounced effect on the associations of glycosylation-deficient mutant of CD82 (E. Odintsova and F. Berditchevski, unpublished work). Finally, instead of (or in addition to) providing a direct physical link between the protein components, G_{D1a} may have a more general role in the spatial organization of the CD82-enriched microdomains. This could be achieved via the G_{D1a} -dependent recruitment of specific lipid or protein components that help stabilizing interactions involving CD82.

The ectopic expression of the membrane-bound sialidase Neu3 specifically affects the interactions involving CD82. Thus our results provide a first example of a physiological regulator of tetraspanin–tetraspanin interactions. Importantly, we found that Neu3 is not directly associated with TERM in transiently transfected epithelial cells (Figure 3A). Furthermore, recent results show that the enzyme is localized in the Triton X-100-resistant

microdomains (i.e. 'classical' GEM) where it interacts with caveolin [40,41]. Then how can one explain the effect of Neu3 on the interactions of the protein, which is excluded from the classical lipid rafts? One possibility is that Neu3, which is abundant at the plasma membrane, targets G_{D1a} and, consequently, the CD82-containing complexes *in trans* (i.e. on the surface of opposing cells) [31]. Alternatively, Neu3 may become involved during the assembly of the complexes while passing through Golgi *en route* to the plasma membrane.

We have previously shown that the CD82-dependent attenuation of signalling via EGFR is correlated with the increased level of G_{D1a} in the CD82 expressing cells [21]. Our current results indicate that the effect of CD82 on signalling via EGFR is even more complex than thought previously. Firstly, we established that only in CD82-expressing cells (HB2/CD82) depletion of gangliosides led to the attenuation of EGFR phosphorylation (Figure 7). On the other hand, inhibition of ganglioside biosynthesis caused re-distribution of the proteins and weakened interactions of CD82 with the receptor (Figures 1C and 8A). While the exact mechanisms remain to be established, one possibility is that alterations in glycosphingolipid composition of the membranes triggers activation of the protein(s) that regulate dephosphorylation of EGFR [e.g. tyrosine phosphatase(s)]. We propose that this (these) protein(s) can be recruited to the membrane by CD82. While presence of gangliosides in the CD82-enriched microdomains would prevent activation of this pathway, ganglioside depletion and subsequent re-distribution of the proteins lead to its activation. Accordingly, in CD82-negative cells (HB2/ZEO), this pathway would be inactive in either the presence or absence of gangliosides, because lack of CD82 precludes the recruitment of the regulatory proteins to the membrane.

It has been previously documented that CD9 is directly associated with G_{M3} [17], and depletion of G_{M3} in fibroblasts resulted in a minor increase in the association of CD9 with integrins [20]. Thus various gangliosides may have specific (and opposing) functions in stabilization of TERM. Notably, the pronounced effect of G_{D1a} on the stability of CD82-containing complexes was specific: interactions involving CD9 were not regulated by G_{D1a} . In this regard, we have noticed that HB2 cells do not accumulate G_{M3} (assayed in TLC and flow cytometry experiments), thereby suggesting that this ganglioside is rapidly converted into G_{M2} (and subsequently to G_{M1} and G_{D1a}) in Golgi. This may explain why depletion of gangliosides in these cells had no apparent effect on the interaction between CD9 and $\alpha 3\beta 1$ integrin (Figure 1C).

In summary, we have established that gangliosides are involved in stabilization of a wide range of interactions within CD82-enriched microdomains. Taken together, these and some of our previous results [42,43] indicate that multiple interactions within TERM are controlled at various levels and involve distinct mechanisms (e.g. direct protein–protein interactions and palmitoylation of tetraspanins) [42,44]. Our results also identify Neu3 sialidase as a physiological regulator of these interactions. Further examination of the links between tetraspanins and gangliosides may explain the diverse functions associated with tetraspanin microdomains.

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