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**GANGLIOSIDES PLAY AN IMPORTANT ROLE IN THE ORGANISATION OF
CD82-ENRICHED MICRODOMAINS.**

Elena Odintsova^{1*}, Terry D. Butters², Eugenio Monti³, Hein Sprong⁴, Gerrit van Meer⁴, and
Fedor Berdichevski¹.

¹Cancer Research UK Institute for Cancer Studies, The University of Birmingham, Edgbaston,
Birmingham B15 2TT, UK

² Glycobiology Institute, Department of Biochemistry University of Oxford, South Parks Road
Oxford OX1 3QU UK

³Department of Biomedical Sciences and Biotechnologies, School of Medicine, University of
Brescia, viale Europa 11, 25123 Brescia, Italy

⁴Department of Membrane Enzymology, Bijvoet Center and Institute of Biomembranes,
Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Address correspondence to: Elena Odintsova, Cancer Research UK Institute for Cancer
Studies, The University of Birmingham, Edgbaston, Birmingham B15 2TT, UK, Telephone:
44-121-414 7458; FAX: 44-121-414 4486; E-mail:odintsoe@bham.ac.uk

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Abbreviations used: **NB-DGJ** - N-butyldeoxygalactonojirimycin; **PPMP** - D-threo-1-Phenyl-2-hexadecanoylamino-3-morpholino-1-propanol•HCl; **TERM** – tetraspanin-enriched microdomain; **DRM** – detergent resistant membranes; **GEM** – glycosingolipid-enriched microdomain; **EGF** – epidermal growth factor; **EGFR** – epidermal growth factor receptor; **CT** – cholera toxin B subunit; **M β CD** - methyl- β -cyclodextrin; **TLC** – thin-layer chromatography.

Abstract

Four transmembrane domain proteins of the tetraspanin superfamily are the organisers of specific microdomains at the membrane (tetraspanin-enriched microdomains or TERM) that incorporate various transmembrane receptors and modulate their activities. The structural aspects of the organisation of TERM are poorly understood. Here 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 and PPMP) 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 association with tetraspanin CD151 was decreased but the association with EGFR 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 data 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.

Introduction

The plasma membrane is a dynamic highly compartmentalised structure. Compartmentalisation 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 organisation 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], detergent-resistant membranes (DRM), glycosphingolipid-enriched microdomains (GEM) 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]. Tetraspanin-enriched microdomains (or TERM) represent a novel type of molecular aggregates that are distinct from all the mentioned above. They could be operationally defined as the membrane complexes maintained after solubilisation with mild detergents such as Brij 96 or Brij 98 [7;8]. Indeed, in contrast to classical lipid rafts TERM are sensitive to Triton X-100 and do not contain GPI-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 compartmentalisation 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 GM3 cooperatively down-regulate motility of tumour cells by attenuating signalling induced by laminin/integrin interactions [17-19]. The cross-talk between integrins and FGFR has also been influenced by the changes in gangliosides and/or tetraspanin expression levels [20]. Moreover, our previous data suggested that the activity of EGFR 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, 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 localisation of the transmembrane proteins to the segregated microdomains.

In this report 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 data 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 DME medium (Gibco) supplemented with 10% foetal calf serum (FCS), 10 $\mu\text{g/ml}$ of hydrocortisone and 10 $\mu\text{g/ml}$ of insulin. Mouse melanoma cells GM95 and GM95/CGlcT-ER were grown in DME medium (Gibco) supplemented with 10% FCS. The anti-CD82 mAbs γC11 and IA4 were kindly provided by Dr.H.Conjeaud. The anti-CD82 mAb M104 was kindly provided by Dr.O.Yoshie. The anti-CD82 mAb TS82b and anti-CD9 mAb TS9 were kindly provided by Dr.E.Rubinstein. 5C11, anti-CD151 mAb, and C9BB, anti-CD9, mAb were described elsewhere [22;23]. Dr.L.Ashman generously provided polyclonal anti-CD151 antibody and 11G1B4 anti-CD151 mAb. Dr.F.Watt 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 Signalling. Anti-phosphotyrosine monoclonal antibody (4G10) was purchased from the Upstate Biotechnology. Monoclonal (H7) and polyclonal (Y-11) antibodies against HA-tag sequence were purchased from Autogen Bioclear. Dr.R.Schnaar kindly provided mouse mAbs to gangliosides GD1a and GM1. FITC-conjugated

cholera toxin B subunit (CT) was purchased from Sigma. NB-DGJ was prepared as described [24]. D-threo-1-Phenyl-2-hexadecanoylamino-3-morpholino-1-propanol•HCl (PPMP) was purchased from Matreya, Inc. Other reagents were from Sigma or Fishers unless stated otherwise.

Transfections. GM95 and GM95/CGlcT-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 FuGene6 (Roche) and selected (when necessary) in Zeocin-containing medium.

Inhibition of ganglioside biosynthesis. For the inhibition of ganglioside biosynthesis cells were incubated 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 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 equivalent amount of ethanol (0.1%) used for the solubilization of PPMP.

Exogenous administration of gangliosides. For exogenous administration of gangliosides GD1a or GM3 cells were plated 2 days before the experiment. Subsequently, GD1a (Calbiochem), or GM3 (Matreya) were added to serum-free medium at final concentration 50 µg/ml. DMSO (25µl/ml) was used in the control experiments. After the incubation (2-24 hours), cells were washed with PBS and used in various assays.

Immunoprecipitation and Western blotting. Proteins were solubilised into the immunoprecipitation buffer containing 1% Brij 98/PBS, 2 mM phenylmethylsulphonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin for 2 hours at 4⁰C. The insoluble material was pelleted at 7000 g for 10 minutes. The cell lysates were then precleared by incubation for 2 hours at 4⁰C 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 4 times with the immunoprecipitation buffer. In some experiments we incubated an aliquot of the pre-cleared lysate with biotinylated Sambucus Negra (Elderberry) Bark Lectin, SNA (Vector Laboratories), that was bound to the immobilized Neutraavidin (Pierce). The complexes were eluted from the beads with Laemmli sample buffer. Proteins were resolved in 10-12% SDS-PAGE, transferred to a nitrocellulose membrane and developed with the appropriate Ab. Protein bands were visualised using horseradish peroxidase-conjugated goat anti-mouse (or goat anti-rabbit) antibodies (Daco) 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 hour on ice. After the incubation, the cells were washed twice and then labelled with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin (Sigma). Stained cells were analysed using Coulter Epics programme (Becton Dickinson).

Fractionation in sucrose gradient. Cells were scraped into 2 ml of PBS, centrifuged at 1200 rpm for 3 minutes, washed once with PBS. 100 mM sodium carbonate (pH 11.0) buffer supplemented with 10 µg/ml aprotinin, 10 µg/ml leupeptin and 2 mM PMSF, was mixed with the pelleted cells. Lysates were homogenized using Dounce

homogeniser (15 strokes) and a sonicator (4x20 sec bursts, 1 min interval, power 10). The resulting homogenate was mixed with equal volume of 90% sucrose prepared in 25 mM MES buffer (pH 6.5), and overlaid with 2 volumes of 35% sucrose and followed by 1 volume of 5% sucrose (both in MES). Samples were centrifuged at 55 000 g for 16-18 hours at 4⁰C. 400 µl aliquots were collected from the top of the tube. The pellet has been dissolved in Laemmli buffer supplemented with protease inhibitors. Equal amounts of each fraction were mixed with 4X Laemmli loading buffer and the distribution of proteins was analysed by SDS-PAGE followed by Western blotting. Protein concentration has been measured by BioRad kit.

Cholesterol depletion. To remove cholesterol cells were washed three times in PBS and incubated in serum-free medium containing 20 mM methyl-β-cyclodextrin (MβCD) for 1 hour at 37⁰C. Levels of cholesterol in MβCD-treated cells were determined using Amplex Red Cholesterol Assay Kit (Molecular Probes). After MβCD treatment the cells were used for the fractionation in sucrose gradient as described above.

Ganglioside extraction and thin-layer chromatography. 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, homogenized in Dounce homogeniser (15 strokes) on ice, 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 (3000g, 10 min) at ambient temperature, the supernatant transferred to a fresh tube, and the pellet re-extracted with 2 ml of chloroform-methanol-water (4:8:3, v/v). 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 ratio to 4:8:5.6 (v/v). After vigorous agitation, the two phases were separated by centrifugation (1500g, 10 min, 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 pre-washed (1 ml of methanol and 2 ml of methanol:PBS, 1:10) Sep-Pak cartridge, which was subsequently washed with chloroform-methanol-water (2:43:55, v/v) 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. Thin layer chromatography was performed on HPTLC plates (Merck, Darmstadt, Germany) using a solvent system of chloroform:methanol: 0.22% CaCl₂ (60:40:5). The gangliosides were visualised with resorcinol reagent. The standard mixture of gangliosides was purchased from Matreya Inc. (PA, USA).

Immunofluorescence staining. Cells were grown on glass coverslips in complete media for 24-36 hours. When required the cells grown on coverslips were transiently transfected with Neu3 sialidase cDNA. Spread cells were fixed with 2% paraformaldehyde/PBS for 10 minutes. 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 cholera toxin B subunit (CT) was carried out on live cells at 4⁰C for 1 hour. Staining was analysed using Nikon Eclipse E600 microscope. Images were acquired using Nikon digital camera DXM1200 and subsequently processed using a Nikon ACT-1 image processing programme.

Results

Inhibition of glycosphingolipid biosynthetic pathway weakens protein-protein interactions within tetraspanin-enriched microdomains.

To elucidate the relationship between gangliosides and tetraspanin-enriched microdomains we used two inhibitors of glucosylceramide synthase (a key enzyme in the glycosphingolipid biosynthetic pathway [27]), N-butyldeoxygalactonojirimycin (NB-DGJ) and D-threo-1-Phenyl-2-hexadecanoylamino-3-morpholino-1-propanol•HCl (PPMP). In the preliminary experiments we established that after 6 days of incubation with 2mM of NB-DGJ the level of gangliosides in HB2/CD82 cells was beyond detection by TLC (Fig.1A, lane d). Notably, TLC analysis also demonstrated the absence of GM3 in HB2 cells (Fig.1A, lane b). Flow cytometry analysis indicated that in NB-DGJ-treated cells the surface expression of gangliosides has been reduced to 4% of GD1a and 7% of GM1 (Fig.1B) in comparison to control cells. The cells were incubated for 6-8 days with 2 mM of 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 the increase in the amount of CD82 (Fig.1B). We have previously demonstrated that there is a correlation between the expression levels of CD82 and gangliosides: increase in CD82 amounts led to the rise in GD1a 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 (Fig.1C, compare lanes 1 and 3 or lanes 5 and 7; Fig.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 (Fig.1C, lane 1 and 3, first panel from the top) in comparison to the controls, untreated cells. Association of CD82 with $\alpha 3$ integrin subunit was also effected (~20% decrease) (Fig.1C, lane 1 and 3, second panel from the top; Fig.1D). Notably, the association of CD82 with the tetraspanin CD151 was diminished by ~ 4 fold (Fig.1C, lane 1 and 3, second panel from the bottom; Fig.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 (Fig.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 (Fig.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 to CD82-containing complexes, there was no obvious effect of ganglioside depletion on the interactions involving CD9 (Fig.1C, compare lanes 2 and 4 or lanes 6 and 8; Fig.1D). We concluded from these data that gangliosides are important for the interactions within CD82-enriched microdomains.

Changes in gangliosides 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 TERM. To answer the question what ganglioside(s) is(are) important for the organisation 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 characterised by its high substrate specificity towards gangliosides [29;30]. Previous studies showed that Neu3 selectively target gangliosides GD1a and GM3 at plasma membrane [31].

HB2/CD82 cells were transiently transfected with the HA-tagged form of sialidase Neu3. It has been shown previously that HA-tag did not affect the activity of the enzyme [30]. Because HB2/CD82 cells do not have detectable GM3, we expected that the expression of Neu3 in these cells would only affect the level of GD1a. To validate the activity of Neu3 sialidase in transiently transfected HB2/CD82 cells we carried out immunofluorescence staining with the anti-GD1a and anti-HA mAbs. Figure 2 illustrates that the cells transfected with Neu3 have significantly lower levels of GD1a compared to the surrounding non-transfected cells (Fig.2, compare A and B). We also tested whether GM1 expression was affected by Neu3 sialidase. Staining with CT indicated that levels of GM1 were comparable in the Neu3-positive and Neu3-negative cells (Fig.2, compare C and D). 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 GM3 levels since we demonstrated earlier that GM3 could not be detected in HB2 cells (Fig.1A, lane b). These data provided evidence that of the two main A-series gangliosides expressed on the surface of HB2 cells (i.e. GD1a and GM1) expression of Neu3 sialidase specifically affected GD1a 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 (Fig.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, Fig.3A, second panel from the top, lanes 1 and 5); the amounts of immunoprecipitated CD82 in both samples were comparable (Fig.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, Fig.3B, lanes 1 and 4). These data 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 (Fig.3A, lanes 3 and 7, Fig.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 (Fig.3C). These data 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 GD1a present in these cells (Fig.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 (Fig.4B, lanes 1 and 6, and 3 and 8). In these experiments we also examined interactions of the tetraspanin CD81 (Fig.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 data 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 GD1a had no effect on the tetraspanin-tetraspanin interactions.

Assembly of CD82-enriched microdomains is not affected by GM3 ganglioside.

We showed that GM3 ganglioside could not be detected in significant quantities in HB2 cells (Fig.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 GM3 to the stability of TERM we analysed tetraspanin-enriched microdomains in GM95 and GM95-CGlcT.ER cells, two derivatives from MEB-4 mouse melanoma cell line [33;34]. While GM95 cells are glycosphingolipid-deficient, GM95-CGlcT.ER cells are transfected with ceramide glucosyl transferase and predominantly express ganglioside GM3. Both cell lines were stably transfected to express human CD82 and CD151 proteins.

We used GM95/CD82+CD151 and GM95-CGlcT.ER/CD82+CD151 cells in the immunoprecipitation analysis. When we compared CD82-immunoprecipitates prepared from both cell lines there were no differences in the amounts of CD151 found in each of the samples (Fig.5, lanes 1 and 4, lower panel). Accordingly, the anti-CD151 mAb co-immunoprecipitated similar amounts of CD82 (Fig.5, lanes 2 and 5, upper panel). Both cell lines had comparable expression levels of transfected tetraspanins as detected by Western blotting (Fig.5, lanes 7 and 8) and flow cytometry (data not shown). We concluded from these experiments that CD82 interactions are not affected by GM3 ganglioside.

To examine the links between CD82 and gangliosides further we incubated ganglioside-deficient cells GM95/CD82+CD151 with exogenously added GD1a or GM3. In our experiments we used the non-toxic concentration of gangliosides (50µg/ml) that was determined in pilot experiments. Flow cytometry analysis confirmed incorporation of gangliosides into the plasma membrane of the cells (data not shown). Comparable amounts of CD151 and CD82 were co-immunoprecipitated by the anti-CD82 (Fig.6, lanes 1, 4, 7) and anti-CD151 mAbs (Fig.6, lanes 2, 5, 8). Similar results were obtained when we used HB2/CD82 cells (data not shown). These data 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 EGF receptor in CD82-expressing cells depleted of gangliosides. We have shown before 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 (Fig.7). Cells were serum-starved and stimulated with EGF for 15 and 60 minutes. Western blotting analysis indicated that depletion of gangliosides in HB2/CD82 cells reduced tyrosine phosphorylation levels of EGFR (~ 2 fold reduction for both 15' and 60' time points; Fig.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 (Fig.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 cooperative way of modulation of EGFR activities by gangliosides and CD82.

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 effected protein distribution within the membrane by fractionation of cellular lysates in continuous sucrose gradient. For the preparation of lysates for fractionation we used a modified detergent-free protocol that allowed concentration of the majority of tetraspanin-containing complexes to the light membrane fractions of the gradient (Fig.8A, c panels in each frame). We avoided detergents in these experiments, as there is evidence that some detergents may induce artifactual formation of microdomains in the membranes [35]. The bulk of CD82 was found in fractions 2-4 together with significant amount of EGFR, $\alpha 3$ integrin subunit and CD151 tetraspanin (Fig.8A, c panels in each frames, lanes 2-4). Depletion of gangliosides had a dramatic effect on the membrane distribution of all tested proteins (Fig.8A, i panels in each frame). The most striking fact was the difference in the fractional distribution of CD82 and CD82-associated proteins. The bulk of EGFR, $\alpha 3$ integrin subunit and CD151 were shifted to the heavy fractions (7-10) of the gradient (Fig.8A, i panels 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 gradient. Distribution of CD9 has been less affected by gangliosides depletion. It was localised mainly to the lighter fractions in control cells. Treatment with the inhibitor led to re-

localisation 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 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 8C, 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 (Fig.8C, compare lanes **c** and **i**). The results of fractionation in sucrose 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-permeabilised untreated cells tetraspanin CD82 exhibited typical peripheral staining (Fig.9, A). However, after incubation with NB-DGJ a significant proportion of CD82 re-localised to large dots randomly scattered on the cell surface (Fig.9, B). These results indicated that the profound effect of gangliosides depletion on the stability of tetraspanin-enriched microdomains 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 [36]. There are controversial data in the literature about the role of cholesterol in the assembly of tetraspanin-enriched microdomains [7;37]. 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 hour. Typically, after this treatment we removed ~ 40% of total cholesterol (as measured following cell lysis). Lysates after M β CD treatment were subjected to the fractionation in sucrose gradient as described above. Cholesterol depletion led to the re-distribution of CD82-associated proteins in sucrose gradient. The effect was more or less similar to the effect of ganglioside depletion (Fig.8B, compare **c** and **d** panels). Notably, the fractional distribution of CD82 itself was only marginally affected by the cholesterol depletion. Together these data 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 stabilise 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, CD9-enriched TERM, etc.). This idea is not without a precedent: for example, urothelial tetraspanins UPIa and UPIb are specifically abundant in the asymmetric unit membranes (AUM), which do

not seem to include any other tetraspanin proteins [38]. Further analysis will be necessary to verify this hypothesis.

In contrast to the data 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 internalised 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 GM1 accumulated mainly in non-DRM compartments whereas the endogenous ganglioside was completely resistant to detergent extraction [39].

Our data indicate that there is a specific link between ganglioside GD1a and tetraspanin CD82. Indeed, specific depletion of this ganglioside with Neu3 sialidase reveals that GD1a has both positive (association of CD82 with CD151) and negative (interaction between CD82 and EGFR) role in maintaining CD82-enriched microdomains. On the other hand, it is unlikely, that GM3, which is known to facilitate associations of CD9 with integrins [17;18], is involved in regulation of CD82-dependent interactions. Firstly, GM3 could not be detected in HB2 cells (perhaps, because it is quickly converted to GM2 and, subsequently, to GM1). 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 (Fig.5) also excluded the involvement of GM3 in the maintenance of CD82-enriched microdomains. The individual contribution of two other main gangliosides of A-series, GM2 and GM1 is currently less clear. The fact that the association between CD82 and EGFR is potentiated in the GD1a-depleted cells, but weakened in cells with decreased levels of all gangliosides suggest that GM1 and/or GM2 may stabilise some of the interactions within CD82-enriched microdomains. Further experiments will be necessary to address this issue.

How does GD1a affect the interactions of CD82 with other TERM-associated proteins? Hakomori and collaborators 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 [40]. Indeed, stability of the complexes involving CD82 was dependent on glycosylation of the protein [18;19]. Hence, one could envisage that binding of GD1a (but not GM3) 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 GD1a 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 (our unpublished data). Finally, instead of (or in addition to) providing a direct physical link between the protein components GD1a may have a more general role in the spatial organisation of the CD82-enriched microdomains. This could be achieved via the GD1a-dependent recruitment of specific lipid or protein components that help stabilising 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 (Fig.3A). Furthermore, recent data show that the enzyme is localised in the Triton X-100-resistant microdomains (i.e. “classical” GEM) where it interacts with caveolin [41;42]. 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 GD1a, 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 EGF receptor is correlated with the increased level of GD1a in the CD82 expressing cells [21]. Our current data 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 (Fig.7). On the other hand, inhibition of ganglioside biosynthesis caused re-distribution of the proteins and weakened interactions of CD82 with the receptor (Fig.8A and Fig.1C). 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(-ese) 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 either in 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 GM3 [17], and depletion of GM3 in fibroblasts resulted in a minor increase in the association of CD9 with integrins [43]. Thus, various gangliosides may have specific (and opposing) functions in stabilization of TERM. Notably, the pronounced effect of GD1a on the stability of CD82-containing complexes was specific: interactions involving CD9 were not regulated by GD1a. In this regard, we have noticed that HB2 cells do not accumulate GM3 (assayed in TLC and flow cytometry experiments), thereby suggesting that this ganglioside is rapidly converted to GM2 (and subsequently to GM1 and GD1a) 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 (Fig.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 [44;45] indicate that multiple interactions within TERM are controlled at various levels and involve distinct mechanisms (e.g. direct protein-protein interactions, palmitoylation of tetraspanins) [44;46]. 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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FIGURE LEGENDS.

Figure 1. Effect of ganglioside depletion on the interactions in tetraspanin-enriched microdomains. 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 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 programme. Data presented as mean fluorescence intensity and represent one of three experiments. The mAbs used were: 1 - negative control, 187.1; 2 – anti- α 3 integrin subunit, A3-IIF5; 3 – anti-CD82, M104; 4 – anti-GD1a, GD1a-1; 5 – anti-GM1, FITC-conjugated cholera toxin B subunit. **C.** Cells were lysed in 1% Brij98 and the complexes were immunoprecipitated using specific monoclonal antibodies: anti-CD82, γ C11; anti-CD9, TS9. The immunoprecipitated complexes were resolved in 10% (for reduced samples) or in 12% (for non-reduced samples) SDS-PAGE, transferred to a nitrocellulose membrane and probed with Abs 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). Data shown represent one of three independent experiments. **D.** Quantification of the data of three immunoprecipitation experiments (treatment with NB-DGJ). Shown means of band intensities relative to that in the control samples (i.e. non-treated cells). Error bars indicate standard deviations. **a** – IP with anti-CD82 mAb, **b** – IP with anti-CD9 mAb.

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 hours the cells were fixed with paraformaldehyde, permeabilized with 0.4% saponin. Double staining immunofluorescence was carried out with the anti-HA (F-7) and anti-GD1a (GD1a-1) mAbs. Isotype specific secondary antibodies conjugated to Alexa 488 or Alexa 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-GD1a antibody. Arrows indicate the cell expressing Neu3 sialidase. **C-D.** After 48 hours cells were incubated with FITC-conjugated CT for 1 hour at 4⁰C. Cells were washed, fixed with 2% paraformaldehyde for 10 min and permeabilized with 0.4% saponin. Subsequently cells were stained with the anti-HA mAb (F-7). Alexa 594 – conjugated goat anti-mouse Ab was used to visualise 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.

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 hours cells were lysed in 1% Brij98 and analysed for antigen expression. Shown data of one of the two separate experiments. Complexes were immunoprecipitated using specific monoclonal antibodies: 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 equal aliquots and resolved in 12% SDS-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 10% SDS-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 Ab 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 hours cells were lysed in 1% Brij98 and the proteins containing sialic acid moiety were precipitated using the immobilised SNA lectin. Proteins were resolved in 12% SDS-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).

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 GD1a surface expression and by Western blotting for expression of HA-tagged sialidase. Experiments were repeated three times. **A**. Surface expression level of GD1a in cells expressing Neu3 was analysed by flow cytometry. The mAbs used were: negative control, 187.1; anti-CD82, M104; anti-GD1a, IgG1. Data presented as mean of fluorescence intensity. **B**. Cells were lysed in 1% Brij98 and the complexes were immunoprecipitated using specific monoclonal antibodies: 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 in 10% (for reduced samples) or in 12% (for non-reduced samples) SDS-PAGE, transferred to a nitrocellulose membrane and probed with the Abs to $\alpha 3$ integrin subunit (polyclonal sera), or anti-CD151 (polyclonal sera), or anti-CD9 (mAb C9BB).

Figure 5. Ganglioside GM3 has no affect 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 monoclonal antibodies: 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 in 12% SDS-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.

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 of GD1a or GM3 or 25 μ l/ml of DMSO in serum-free medium for 24 hours at 37⁰C. After the incubation, cells were subjected to immunoprecipitation analyses. The complexes were immunoprecipitated using specific monoclonal antibodies: anti-CD82, γ C11 (lanes 1, 4, 7); anti-CD151, 5C11 (lanes 2, 5, 8) and negative control, 187.1 (lanes 3, 6, 9). Proteins were resolved in 12% SDS-PAGE,

transferred to a nitrocellulose membrane and probed with the antibodies to CD82 (mAb TS82b) or CD151 (polyclonal sera).

Figure 7. Inhibition of ganglioside biosynthesis has 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, then serum starved for 6 hours and stimulated with EGF (100 η g/ml) for 15 or 60 minutes. Lysates were prepared in 1x Laemmli buffer. Equal amounts of proteins were resolved in 10% SDS-PAGE, transferred to a nitrocellulose membrane and probed with the anti-phosphotyrosine mAb (4G10, Upstate), or phosphospecific anti-EGFR polyclonal Ab (Tyr 1068), or anti-EGFR mAb (Ab-15). Data presented show results of one of three independent experiments. Quantification of three independent experiments was performed. Shown mean of ratios of the densities of phosphorylated EGFR to the densities of total EGFR. Error bars indicate standard deviations.

Figure 8. Effect of inhibition of ganglioside biosynthesis on the compartmentalisation of membrane proteins. The lysates of HB2/CD82 cells treated with 2 mM of NB-DGJ for 7 days (**A**) or 20 mM of M β CD for 1 hour (**B**) and control cells were prepared under the detergent-free conditions as described in the "Materials and Methods". Lysates were fractionated by ultracentrifugation in continuous sucrose gradient (5-45%). Fractions were collected from the top of the gradient. Equal volumes of each fraction were resolved in 10 or 12% SDS-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-

$\alpha 3$ integrin subunit (polyclonal sera), anti-CD151 (polyclonal sera), anti-CD9 (mAb C9BB). Shown results of one of the two separate experiments. **c** – control cells; **i** – inhibitor-treated cells. Note, distribution 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 (panel B). In the experiments depicted in panel **A** cells were grown in the serum-containing medium. **C**. Light fractions (2-5) of sucrose 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 in 12% SDS-PAGE, transferred to a nitrocellulose membrane and probed with the anti-CD82 (TS82b) mAb, anti-CD9 (C9BB) mAb and anti-CD151 polyclonal Ab.

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 cover slips. After 24 hours the cells were fixed with 2% paraformaldehyde and stained with primary anti-CD82 (IA4) mAb. Isotype specific secondary antibodies conjugated to Alexa 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.

Figure 1

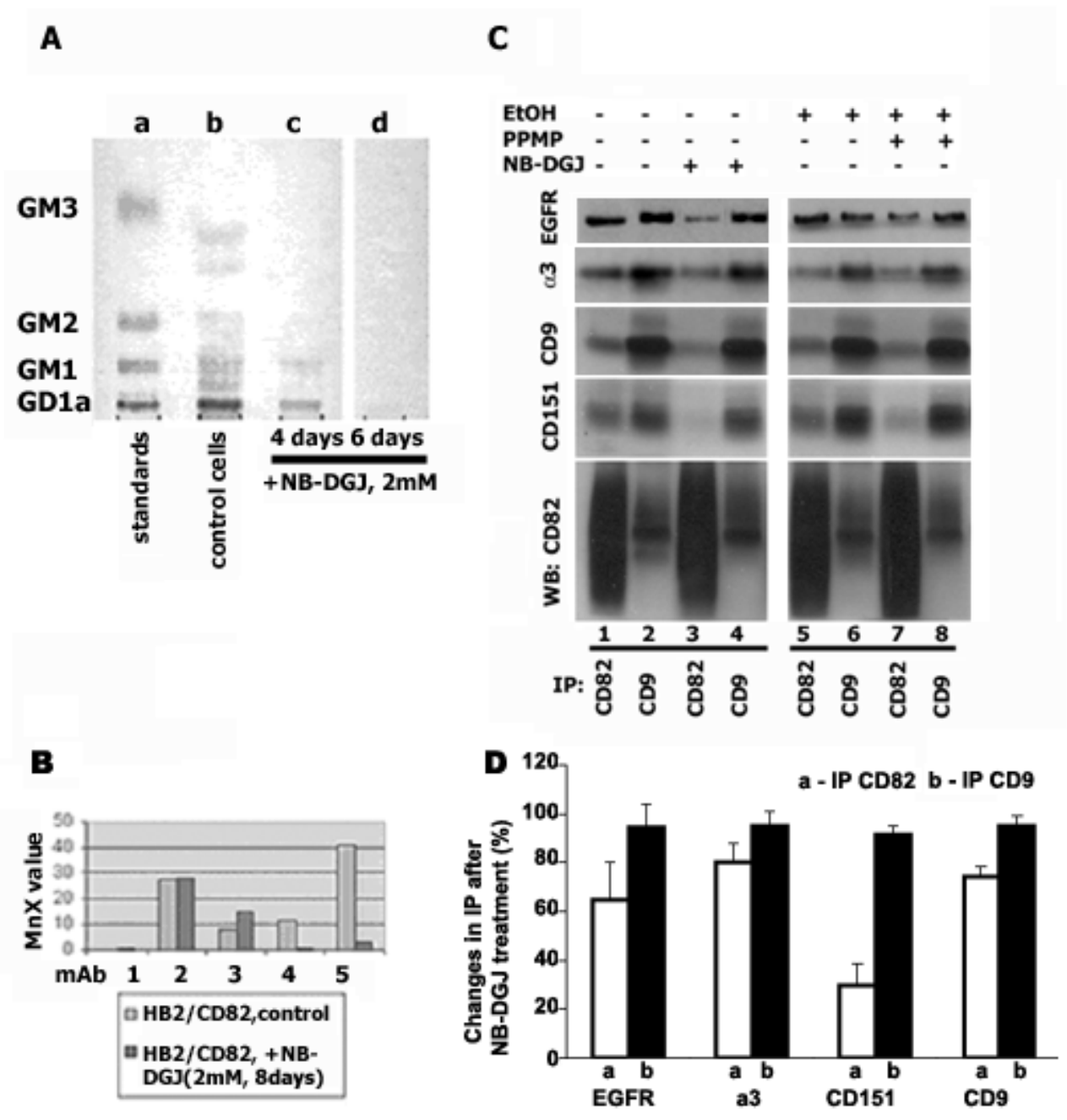


Figure 2.

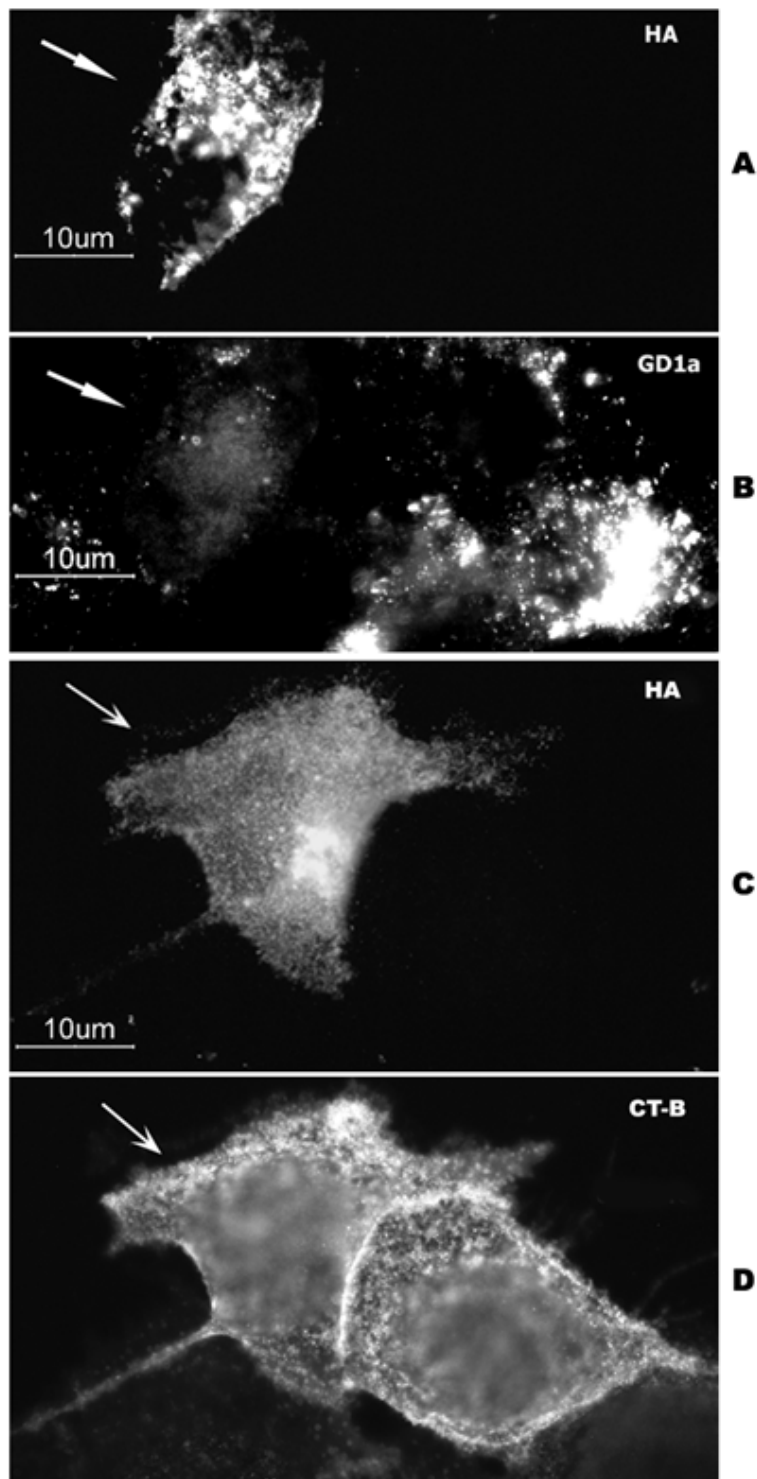


Figure 3.

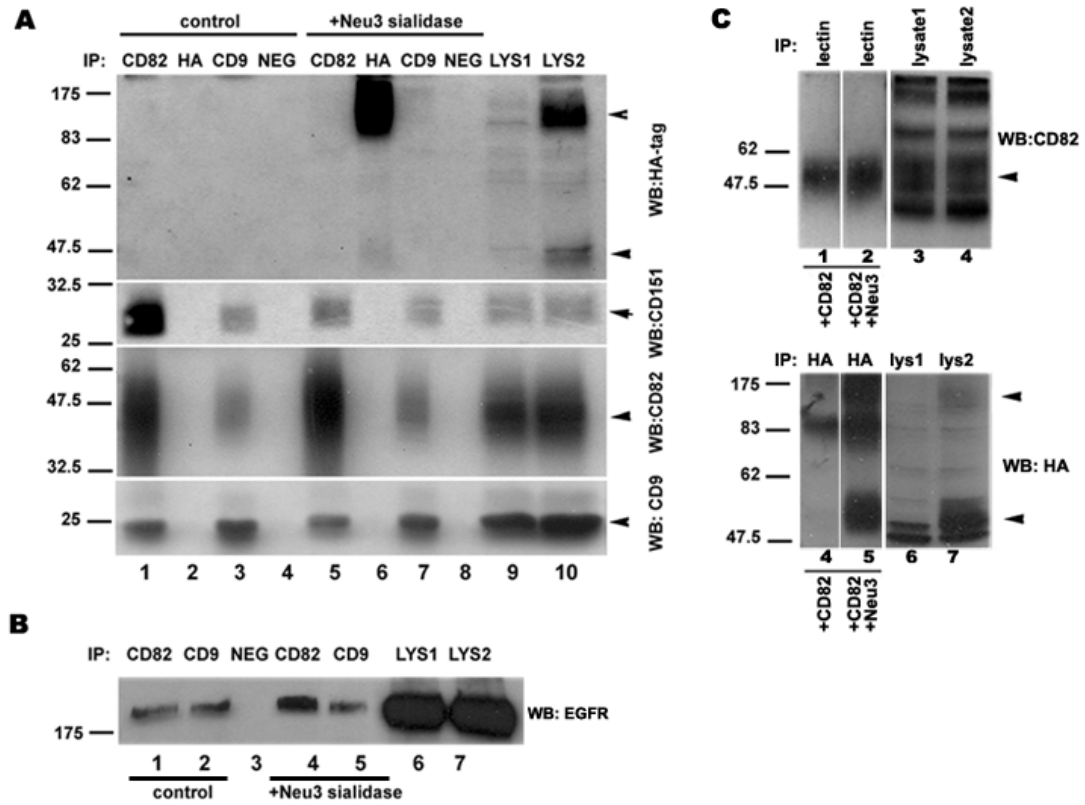
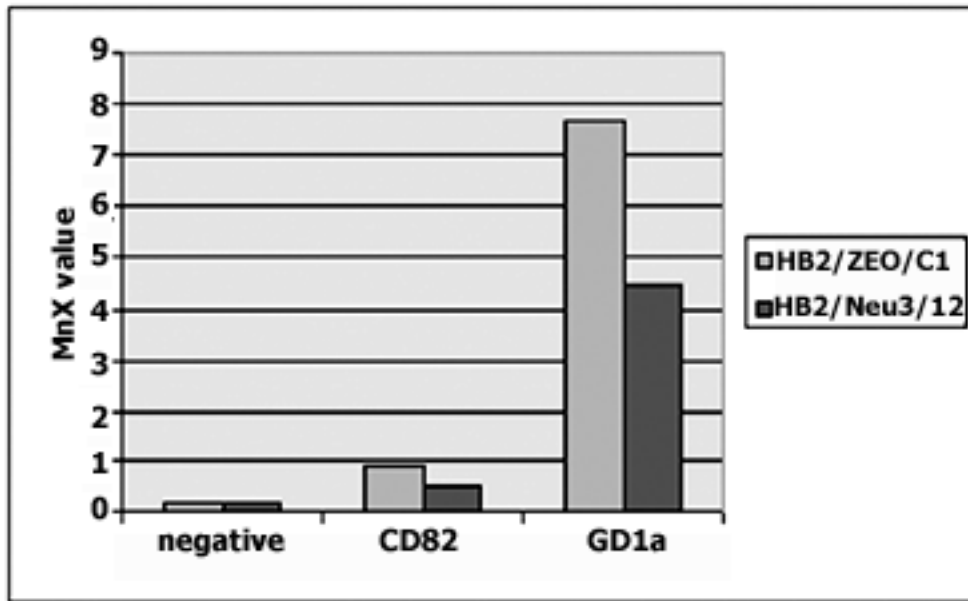


Figure 4.

A



B

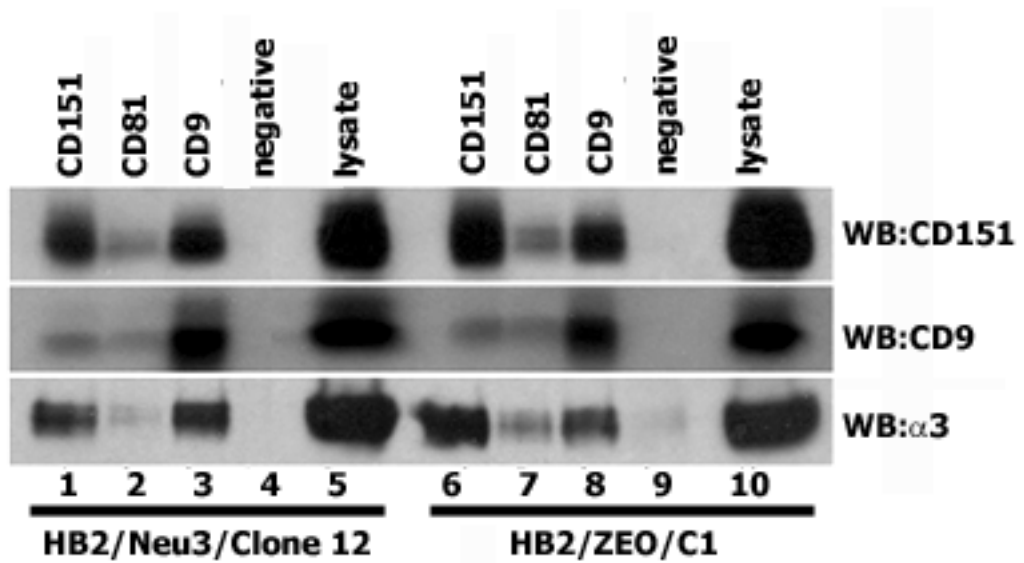


Figure 5.

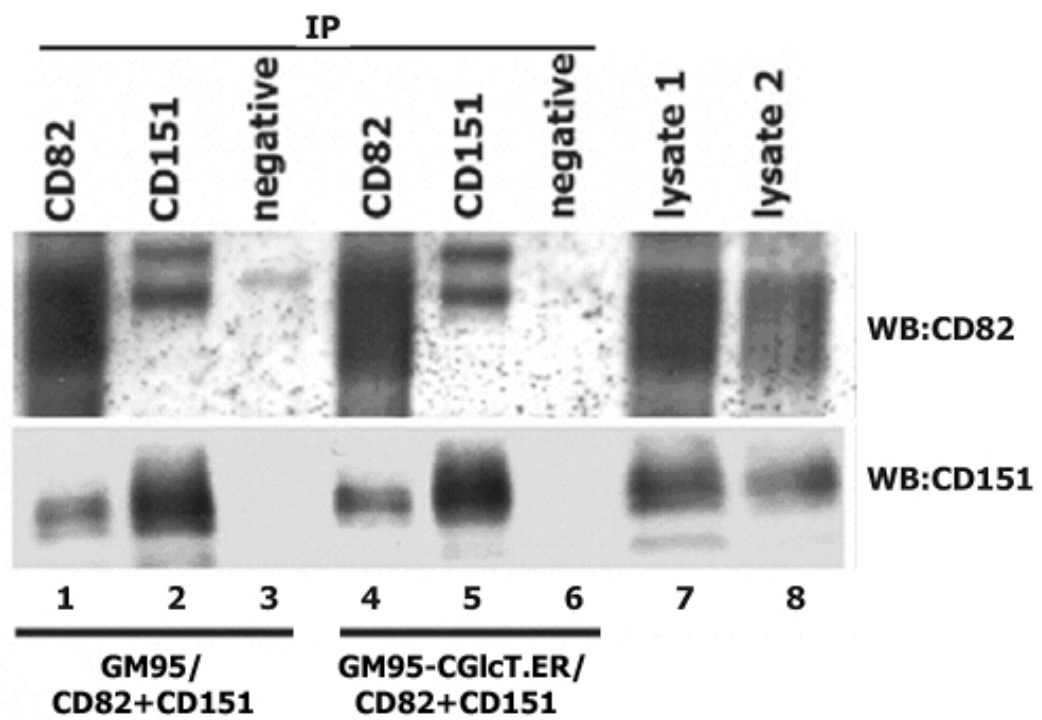


Figure 6.

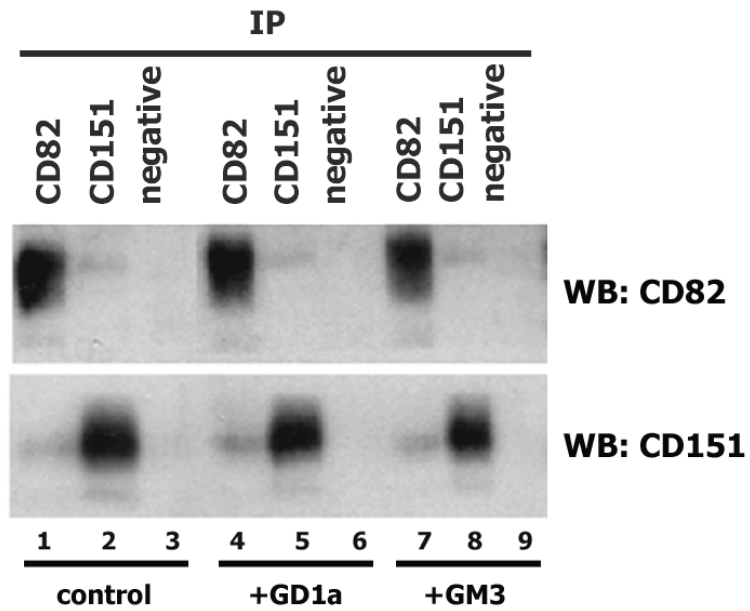
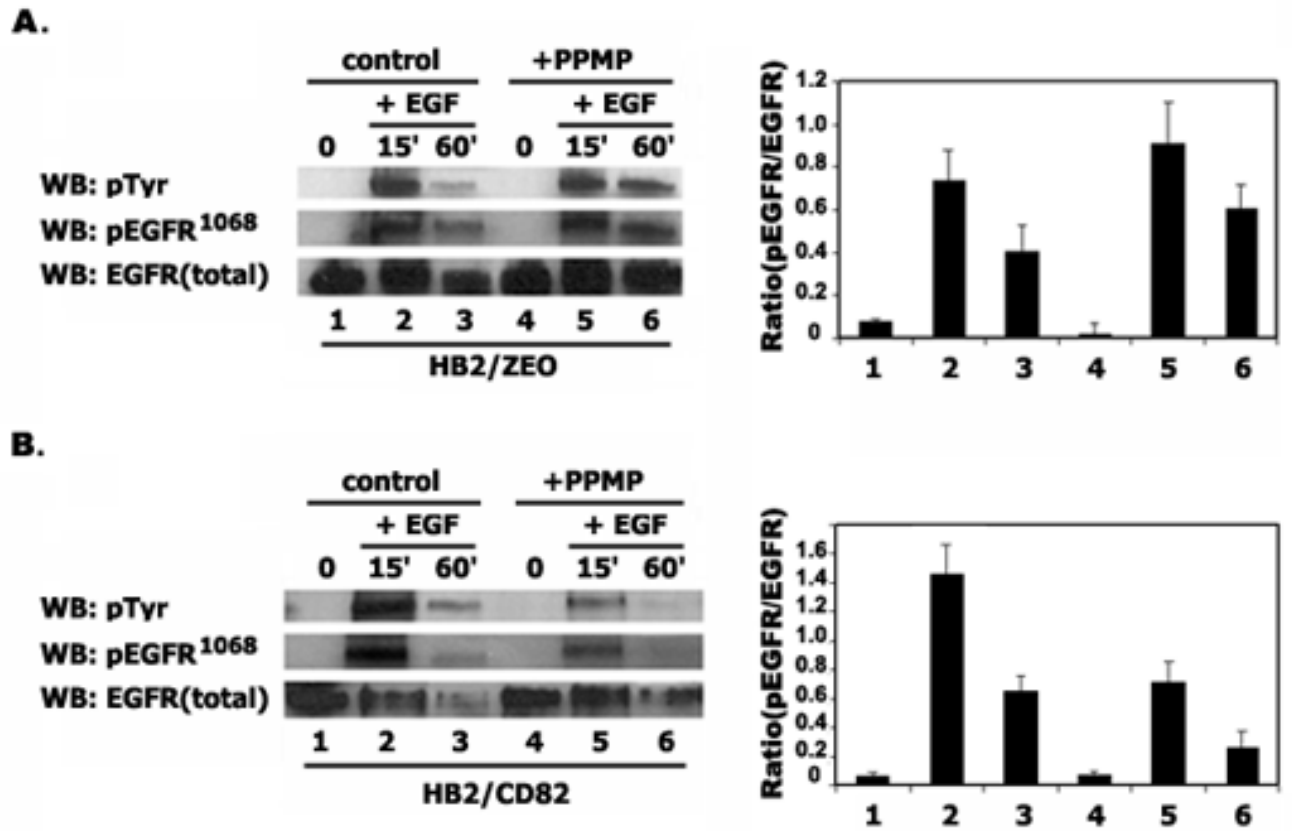


Figure 7.



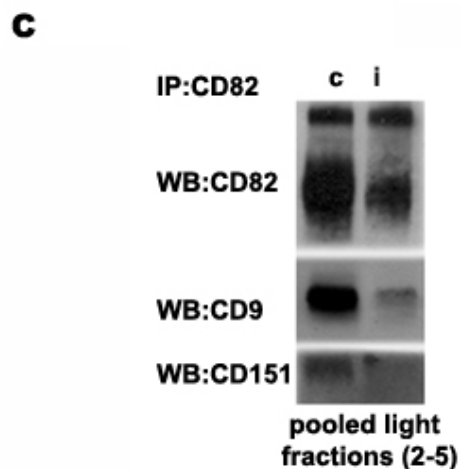
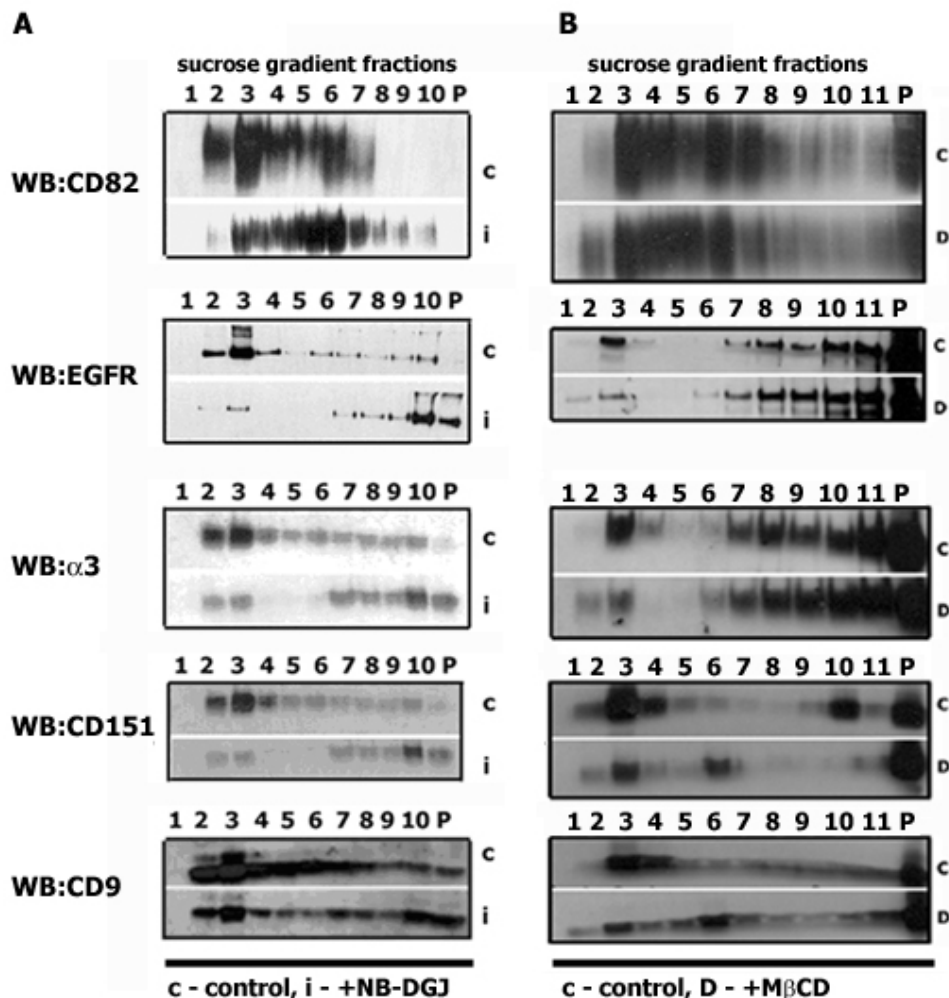


Figure 9.

