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Membrane binding, endocytic trafficking and intracellular fate of high-affinity antibodies to gangliosides GD1a and GM1

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ABSTRACT

Gangliosides are glycolipids embedded in the outer leaflets of the plasma membrane. Antibodies against GM1 and GD1a gangliosides are associated with selective dysfunction of motor axons in peripheral neuropathies, and differential endocytic processing of antibodies to gangliosides represent a critical modulator of site-specific injury in Guillain-Barré syndrome. In addition, antibodies to glycolipids have emerged as an attractive tool for therapeutic interventions in cancer. In this work, we have investigated the binding, endocytosis and intracellular fate of high-affinity antibodies to gangliosides GD1a and GM1 both in epithelial and neuronal-like cells. Live cell imaging and fluorometric analysis showed that, after specific plasma membrane binding, a fraction of antibody to GD1a was slightly but rapidly internalized by a dynamin 2-independent pathway and then accumulated in the endocytic recycling compartment. We also show that internalization of antibody to GD1a is regulated by ADP-ribosylation factor 6. Surprisingly, experiment of cellular antibody uptake performed at 16 °C, widely used to accumulate the endocytic cargo in sorting endosomes, showed that the antibody to GD1a remained mostly localized at the plasma membrane, supporting the presence of selective mechanisms for cell internalization of antibody-ganglioside complex. In contrast, antibody to GM1 was endocyted in epithelial cells but remained at the plasma membrane of neuronal-like cells. Together, these results provide additional evidences about the molecular mechanisms that operate in the uptake and intracellular trafficking dynamics of antibodies to glycolipids and have significant translational implications for the understanding of clinical characteristics of anti-ganglioside antibody-mediated neuropathies and for the development of novel therapeutics targeting.

1. Introduction

Gangliosides, a large family of sialic acid bearing glycosphingolipids, are amphipathic molecules distributed on the external leaflet of the plasma membrane of nearly all vertebrate cells with particular abundance in brain and the nervous system where they contribute from 10 to 12% of the total lipid content [1]. Gangliosides have been implicated in many physiological processes, including growth, differentiation, migration and apoptosis through modulating both cell signalling processes and cell-to-cell and cell-to-matrix interactions [2–4]. Moreover, gangliosides have been associated with a wide range of pathological processes, being receptors for viruses (e.g. simian virus 40), toxins (e.g. cholera, tetanus and botulinum toxins) and antibodies [5–7].

Antibodies to gangliosides, in particular to GD1a, GM1 and GQ1b, have been associated with a wide range of clinically identifiable acute and chronic neuropathy syndromes [8,9]. In particular, antibodies against GM1 and GD1a gangliosides are associated with selective dysfunction and injury of motor axons in the acute motor axonal neuropathy subtype of Guillain-Barré syndrome. Gangliosides GD1a, GM1 and GD1b are highly enriched at or near the nodes of Ranvier, a critical region for generation and propagation of action potentials [10,11]. It was recently demonstrated that nodal disruption via complement pathway represents a common injury mechanism in immune-mediated neuropathies associated with autoantibodies to gangliosides GM1, GD1a or GD1b, which was associated with a minimal anti-ganglioside antibody uptake. In contrast, it was observed that the fast anti-ganglioside antibody internalization at the motor nerve terminal greatly attenuates complement-mediated injury [11,12]. Thus, these results point out that differential endocytic processing of antibodies to gangliosides represent a critical modulator of site-specific injury in Guillain-Barré syndrome and related neuropathies.

The aim of this study was to investigate cellular binding and endocytic destination of neuropathy-associated antibodies to GD1a and GM1 gangliosides. The high-affinity antibodies to GD1a (IgG class) used in this work were previously obtained using mice genetically engineered to lack complex ganglioside [13,14] and one of them was

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found to induce axonal neuropathy in mice [8] and rats [11], while the high-affinity and high-specificity antibodies to GM1 used, were found to reversely block muscle action potentials in muscle/spinal cord cocultures and to stain both the nodes of Ranvier and paranodal Schwann cells in human spinal roots [15]. By biochemical techniques, immunofluorescence and time-lapse confocal microscopic analysis, we performed both in neural and genetically modified epithelial cell lines a detailed and comparative study to investigate plasma membrane binding and molecular requirements for cell internalization/plasma membrane residence of antibodies to GD1a and GM1. Understanding these mechanisms is crucial for elucidating the pathological implications of anti-ganglioside antibodies in neuropathies and for the development of novel therapeutics targeting.

2. Materials and methods

2.1. Antibodies and plasmids

Two mouse monoclonal antibodies to ganglioside GD1a and a mouse monoclonal antibody to ganglioside GM1 were generated in knockout mice lacking the gene coding for UDP-GalNAc:LacCer/GM3/GD3*N*-acetylgalactosaminyltransferase (β4GalNAcT-I): i) IgG1 isotype anti-GD1a/GT1aα (Ab1-GD1a) [13], ii) IgG2b isotype anti-GD1a/GT1b/GT1aα (Ab2-GD1a) [13] and iii) IgG2b anti-GM1 (Ab2-GM1) [15]. Alexa Fluor 568 Antibody Labeling Kit from Invitrogen was used to label Ab1-GD1a following manufacturer's protocol to generate Alexa^{568}-Ab1-GD1a (^{568}Ab1-GD1a).

Haemagglutinin (HA)-Arf6 Q67L and (HA)-Arf6 T27N were received from J. Donaldson (NHLBI, National Institutes of Health, Bethesda, MD, USA) and pGFP-Rab11a from M. Colombo (Universidad Nacional de Cuyo, Mendoza, Argentina). Plasmids coding for GFP-dynamin 2-wild type (WT) and GFP-dynamin 2-K44A were supplied by J. Bonifacino (NICHD, National Institutes of Health, Bethesda, MD, USA). The construct containing the cDNA coding for the *N*-terminal domain (cytosolic tail, transmembrane domain, and a few amino acids from the stem region) of β4GalNAcT-I fused to the *N*-terminus of the CFP (β4GalNAcT-I-CFP) was obtained by subcloning the corresponding cDNA fragments into the plasmid pECFP-N1 (Clontech, Mountain View, CA, USA) [16]. The plasmid encoding PH-PLCδ1-GFP (a chimeric protein containing the phosphatidylinositol 4,5-biphosphate (PtdIns(4,5)P_2) specific pleckstrin homology domain of phospholipase Cδ1 fused to GFP) was received from M. Lemmon (University of Pennsylvania School of Medicine, Philadelphia, USA).

2.2. Cell culture and transfection

CHO-K1 cells (ATCC, Manassas, VA, USA) genetically modified to express gangliosides GM1 and GD1a (CHO-K1$^{GD1a/GM1+}$) [17] and neuroblastoma derived cell line Neuro-2a (ATCC, Manassas, VA, USA) that endogenously express gangliosides GD1a and GM1, were maintained at 37 °C, 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and antibiotics (100 μg/mL penicillin and 100 μg/mL streptomycin). Cells were transfected with 0.5–1.0 μg/35 mm dish of the indicated plasmid using linear polyethylenimine (Sigma-Aldrich, MO, USA).

2.3. Cell labeling and internalization assays

Cells transiently transfected with plasmids indicated above or mock transfected cells were incubated at 4 °C for 15 min to inhibit intracellular transport. Then, cells were incubated at 4 °C for 45 min with appropriate dilutions of ^{568}Ab1-GD1a or Ab2-GD1a in order to label GD1a ganglioside or Ab2-GM1 to label GM1 ganglioside expressed on the cell surface. Afterwards, cells were washed three times with cold DMEM to remove unbound antibody, fixed in 1% paraformaldehyde in PBS for 10 min at 4 °C or transferred to 37 °C or 16 °C fresh pre-warmed DMEM to allow antibody internalization for different times and finally fixed. For transferrin internalization, cells were first incubated for 90 min at 37 °C in DMEM without FBS. Then washed and incubated at 4 °C for 15 min followed by 45 min in cold DMEM containing 10 μg/mL Alexa^{647}-transferrin (^{647}transferrin) (Molecular Probes, Eugene, OR, USA) and ^{568}Ab1-GD1a. Finally, cells were transferred to 37 °C or 16 °C pre-warmed DMEM without FBS supplemented with 5 μg/mL ^{647}transferrin and fixed at different times. Alexa^{488}-conjugated goat anti-mouse IgG was used for co-localization assays in experiments performed at 16 °C. Alexa^{546}-conjugated goat anti-mouse IgG was used to detect Ab2-GD1a and Ab2-GM1.

2.4. Confocal immunofluorescence microscopy

Cells grown on coverslips were washed twice with DMEM, fixed in 1% paraformaldehyde in PBS for 10 min at 4 °C. Where indicated, cells were permeabilized with 0.1% Triton X-100/200 mM glycine in PBS for 2 min at room temperature and the secondary antibodies Alexa^{488}-conjugated goat anti-mouse IgG or Alexa^{546}-conjugated goat anti-mouse IgG, both diluted at 1:1000, were used for detection of primary antibodies. After final washes with PBS, cells were mounted in FluorSave reagent (Calbiochem, EMD Biosciences, La Jolla, CA, USA). Expression of β4GalNAcT-I was detected by the intrinsic fluorescence of CFP. Dynamin 2-K44A and dynamin 2-wild type were detected by the intrinsic fluorescence of GFP. Confocal images were collected using an Olympus FluoView™FV1000 laser-scanning confocal microscope (Olympus, Tokyo, Japan) equipped with an argon/helium/neon laser and a 63 × 1.4 numerical aperture, oil immersion objective. Single confocal sections of 0.8 μm were taken parallel to the coverslip (*xy* sections). Images were acquired and processed with the FV10 lsm image software and ImageJ software. Final images were compiled with Adobe Photoshop CS6. The fluorescence micrographs shown in this manuscript are representative of at least three independent experiments.

2.5. Live cell imaging

Time-lapse experiments were performed in CHO-K1$^{GD1a/GM1+}$ cells grown for 24 h in LabTek II coverglass. Cells were first incubated at 4 °C in cold phenol red-free DMEM (prf-DMEM) for 15 min and then an appropriate dilution of ^{568}Ab1-GD1a in cold medium was applied to cells and incubated for 45 min at 4 °C. Then cells were washed with cold medium to remove unbound antibody and fresh pre-warmed prf-DMEM at 16 °C was added. The temperature was maintained for 13 min. After that period of time, the medium was removed and replaced with fresh pre-warmed prf-DMEM at 37 °C and the temperature was maintained until the end of the experiment. Confocal stacks were acquired on FluoView™FV1000 confocal microscope with 63 × 1.4 numerical aperture, oil immersion objective.

2.6. Fluorometric assay

CHO-K1$^{GD1a/GM1+}$ cells were grown in 96-well plates for 24 h. Cells were first incubated at 4 °C in cold prf-DMEM for 15 min and then an appropriate dilution of ^{568}Ab1-GD1a in cold medium was applied to cells and incubated for 45 min at 4 °C (input). After this period of time the fraction of unbound antibody was recovered, cells were extensively washed with cold medium and fresh 16 °C pre-warmed prf-DMEM was added and incubated for 30 min. Medium containing antibody released during this 30 min incubation at 16 °C was recovered and fresh 37 °C pre-warmed prf-DMEM was added for another 30 min. Finally, medium containing antibody released during this 30 min incubation at 37 °C was recovered and the fluorescence in each recovered fraction was measured using a FluoroMax-P spectrofluorometer (HORIBA Jobin Yvon SAS,

Longjumeau Cedex, France). Initial binding was calculated subtracting the fluorescence signal obtained for the fraction of unbound antibody from the input signal.

2.7. Other experimental procedures

Metabolic labeling, lipid extraction and chromatography were performed mostly as described [18]. To reduce ganglioside and the neutral glycosphingolipid content, cells were treated with 2.4 μM of d,l-threo-1-phenyl-2-hexadecanoylamino-3-pyrrolidino-1-propanol-HCl (P4) (Matreya Inc., PA, USA) as previously described [17].

3. Results

3.1. Ab1-GD1a specifically binds to GD1a expressed at the cell surface of CHO-K1 cells and a minor fraction is internalized

First, we tested surface binding of the monoclonal antibody Ab1-GD1a conjugated with Alexa Fluor^{568} (^{568}Ab1-GD1a) in a GD1a-expressing CHO-K1 cell clone (CHO-K1$^{GD1a/GM1+}$) already established in our laboratory by stable expression of β4GalNAcT-I and β3GalT-IV [17,19]. This antibody also recognizes GT1aα ganglioside [13]. However, it should be mentioned that CHO-K1$^{GD1a/GM1+}$ cells express GM3, GM2, GM1 and GD1a gangliosides (Fig. 1A, lane 2). Briefly, CHO-K1$^{GD1a/GM1+}$ cells were incubated for 15 min at 4 °C to inhibit intracellular transport and then with ^{568}Ab1-GD1a for 45 min at 4 °C in order to label GD1a ganglioside expressed on the cell surface. Then, cells where extensively washed with cold DMEM to remove unbound antibody and fixed or transferred to 37 °C pre-warmed DMEM for different times to restore transport and thereby allow endocytosis. Confocal microscopic analysis revealed that ^{568}Ab1-GD1a bound to cells at 4 °C had a punctate plasma membrane distribution. No binding was detected in CHO-K1 wild type cells (CHO-K1$^{GD1a/GM1-}$) (Fig. 1B), which predominantly express ganglioside GM3 [20] and Fig. 1A, lane 1. Surprisingly, 5 min after shifting the temperature to induce endocytosis, total fluorescence associated to the antibody was drastically reduced to nearly 25% (relativized to 4 °C condition) and began to show a cytoplasmic and perinuclear distribution. After 15 min at 37 °C, and at longer periods of time (30 min and 45 min), the intracellular pool of ^{568}Ab1-GD1a was mainly detected in the perinuclear region (Fig. 1C). Controls to further demonstrate specificity of binding and internalization of antibodies to gangliosides are shown in Supplementary Figs. 1 and 2. These results indicate that ^{568}Ab1-GD1a is partially internalized after binding GD1a ganglioside at the plasma membrane.

3.2. Internalized Ab1-GD1a co-localizes with recycling endosome markers but not with a Golgi complex resident protein

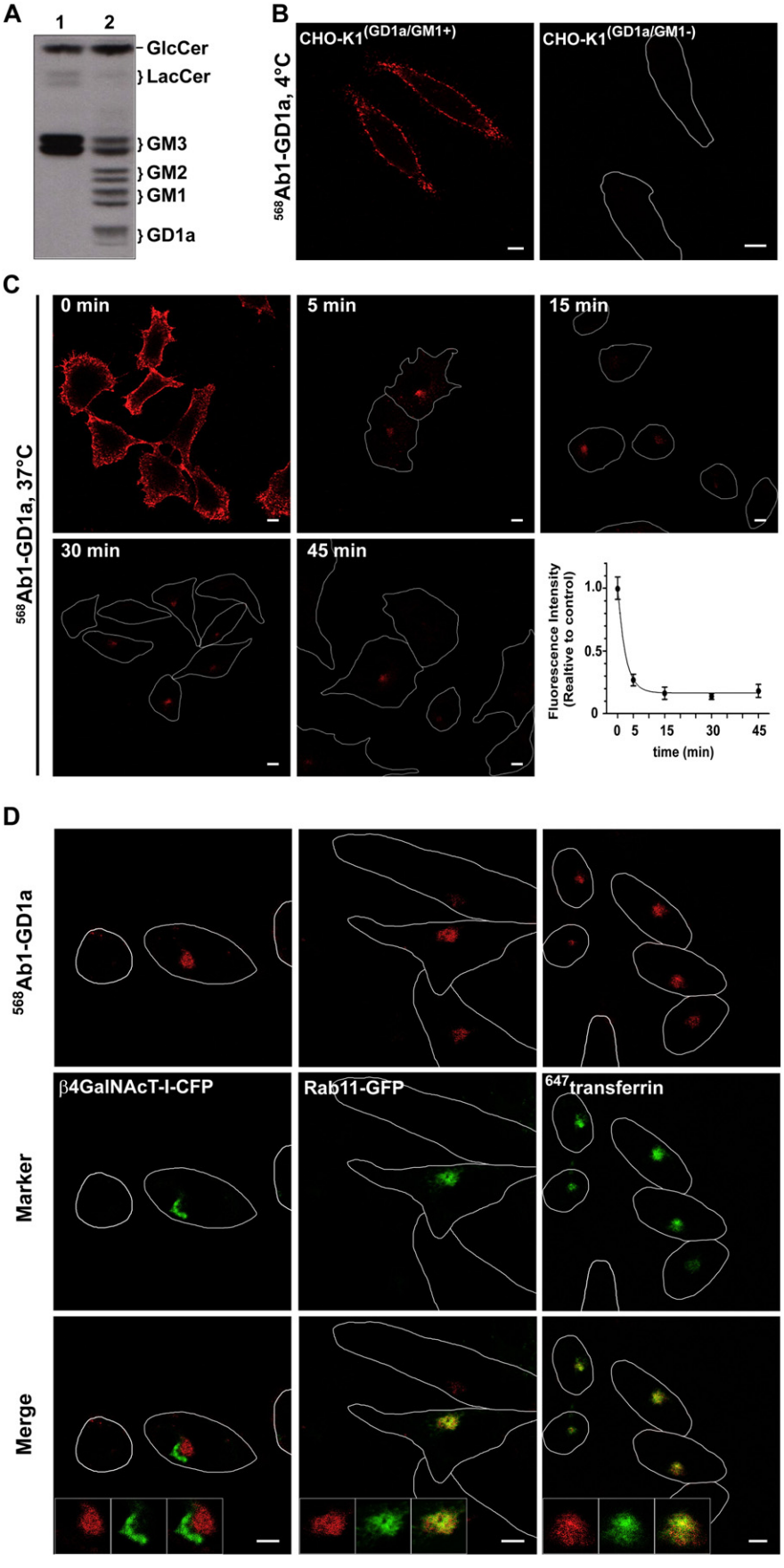
As described above, the ^{568}Ab1-GD1a internalized fraction was mostly located in a perinuclear region. In CHO-K1 cells, this intracellular localization resembles the typical pericentriolar distribution of Golgi complex or recycling endosomes [21,22]. To identify this juxtanuclear compartment, we performed colocalization analysis with markers of both recycling endosome and Golgi complex (Fig. 1D). After 30 min of internalization, no colocalization was observed between ^{568}Ab1-GD1a and β4GalNAcT-I-CFP, a Golgi complex marker. On the other hand, we observed extensive colocalization between ^{568}Ab1-GD1a and the GTPase Rab11, a well-established recycling endosome marker [21]. Additionally, we also found significant overlapping of ^{568}Ab1-GD1a with co-endocyted ^{647}transferrin in a perinuclear compartment, demonstrating that the major ^{568}Ab1-GD1a endocyted fraction was present in the recycling endosomes of CHO-K1 cells.

3.3. Ab1-GD1a uptake is a dynamin 2-independent process and depends on Arf6-GTPase activity

Many endocytic pathways are involved in the access of different cargo molecules into the cells. These entry routes could be classified based on their needs of the large GTPase dynamin 2 activity for vesicle scission (Fig. 2A). To explore the endocytic pathway taken by ^{568}Ab1-GD1a to reach recycling endosomes, we first tested the dependence on dynamin 2 activity for the internalization process. For this purpose, CHO-K1$^{GD1a/GM1+}$ cells were transiently transfected to express dynamin 2 fused to GFP in its wild type (WT) form or a GTP binding and hydrolysis deficient mutant, dynamin 2-K44A (K44A), which is unable to drive receptor-mediated endocytosis via clathrin-coated pits [23]. After 30 min of endocytosis at 37 °C, a fraction of ^{568}Ab1-GD1a was delivered to recycling endosomes of WT and K44A dynamin 2 transfected cells (Fig. 2B and C). As control, we investigated ^{647}transferrin internalization and found that it was not perturbed when the WT form of the GTPase was overexpressed, contrasting with a potent inhibition when transfected with the mutant variant, as previously reported [23] (Fig. 2B and C). Given that internalization of ^{568}Ab1-GD1a do not require dynamin 2, it is highly probable that endocytosis of this anti-ganglioside antibody in CHO-K1 cells occurs by a clathrin- and caveolin 1-independent mechanism.

Taken these results into account, we decided to investigate if the small GTPase Arf6 had a role in this uptake since Arf6-associated endocytosis appears to be a dynamin independent process and these endosomes can communicate and partially overlap with transferrin-positive compartments [24]. The GTPase Arf6 in its GTP bound state indirectly regulates PtdIns(4,5)P_2 levels at the plasma membrane through activation of phosphatidylinositol 4-phosphate 5-kinase (PI(4)P5K) [25]. Under this condition some plasma membrane proteins lacking cytoplasmic clathrin/AP2-targeting sequences are internalized in Arf6-GTP/PtdIns(4,5)P_2 endosomes [24,26]. The following inactivation of Arf6 and loss or modification of PtdIns(4,5)P_2 allow these endocytic vesicles to deliver their cargo in part, back to the plasma membrane whereas a fraction could fuse with the classical early endosomal compartment and traffic to late endosomes/lysosomes for degradation [24]. In

Fig. 1. Cell surface binding, endocytosis and subcellular localization of a monoclonal antibody to GD1a/GT1aα gangliosides (^{568}Ab1-GD1a) in CHO-K1 cells. A. TLC showing ganglioside expression patterns of wild type CHO-K1 cells (CHO-K1$^{GD1a/GM1-}$, lane 1) and CHO-K1 cells genetically modified to express a-Series complex gangliosides (CHO-K1$^{GD1a/GM1+}$, lane 2). Gangliosides were metabolically labeled with [^{3}H]galactose, purified, chromatographed on high performance thin layer chromatography plate and visualized by fluorography. The position of co-chromatographed ganglioside standards is indicated with brackets. B. CHO-K1 cells genetically modified to express a-Series complex gangliosides (CHO-K1$^{GD1a/GM1+}$, left panel) and wild type CHO-K1 cells (CHO-K1$^{GD1a/GM1-}$, right panel) were incubated for 15 min at 4 °C followed by 45 min incubation with ^{568}Ab1-GD1a at 4 °C. Then, cells were washed and fixed. Cell boundaries (white lines) are indicated. Scale bars: 5 μm. C. CHO-K1$^{GD1a/GM1+}$ cells were labeled as indicated in B and then fixed (0 min) or transferred to 37 °C pre-warmed DMEM for different times. Quantification of total immunofluorescence signal (means +/− SEM) that remained associated with the cellular fraction at each time is shown. The values were relativized to the fluorescence intensity obtained for the initial binding condition (0 min). Representative confocal sections of 0.8 μm from the different times taken parallel to the coverslip are shown. Cell boundaries (white lines) are indicated. Scale bars: 5 μm. D. CHO-K1$^{GD1a/GM1+}$ cells transiently transfected to express β4GalNAcT-I-CFP (pseudo-colored green, first column) or Rab11-GFP (second column) or not transfected (third column) were incubated for 15 min at 4 °C and then with ^{568}Ab1-GD1a (and 10 μg/mL ^{647}transferrin, third column only) for 45 min at 4 °C. Then, cells were washed and transferred to 37 °C pre-warmed DMEM (or 37 °C pre-warmed DMEM supplemented with 5 μg/mL ^{647}transferrin, third column only) for 30 min. Finally, cells were fixed. Insets in merged panels show details of colocalization with higher magnification. Representative confocal sections of 0.8 μm taken parallel to the coverslip are shown. Cell boundaries (white lines) are indicated. Scale bars: 5 μm.


A
1
2
GlcCer
LacCer
GM3
GM2
GM1
GD1a
B
CHO-K1(GD1a/GM1+)
CHO-K1(GD1a/GM1-)
568Ab1-GD1a, 4°C
C
0 min
5 min
15 min
30 min
45 min
568Ab1-GD1a, 37°C
Fluorescence Intensity (Realtive to control)
1.0
0.5
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time (min)
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568Ab1-GD1a
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β4GalNAcT-I-CFP
Rab11-GFP
647transferrin
Merge

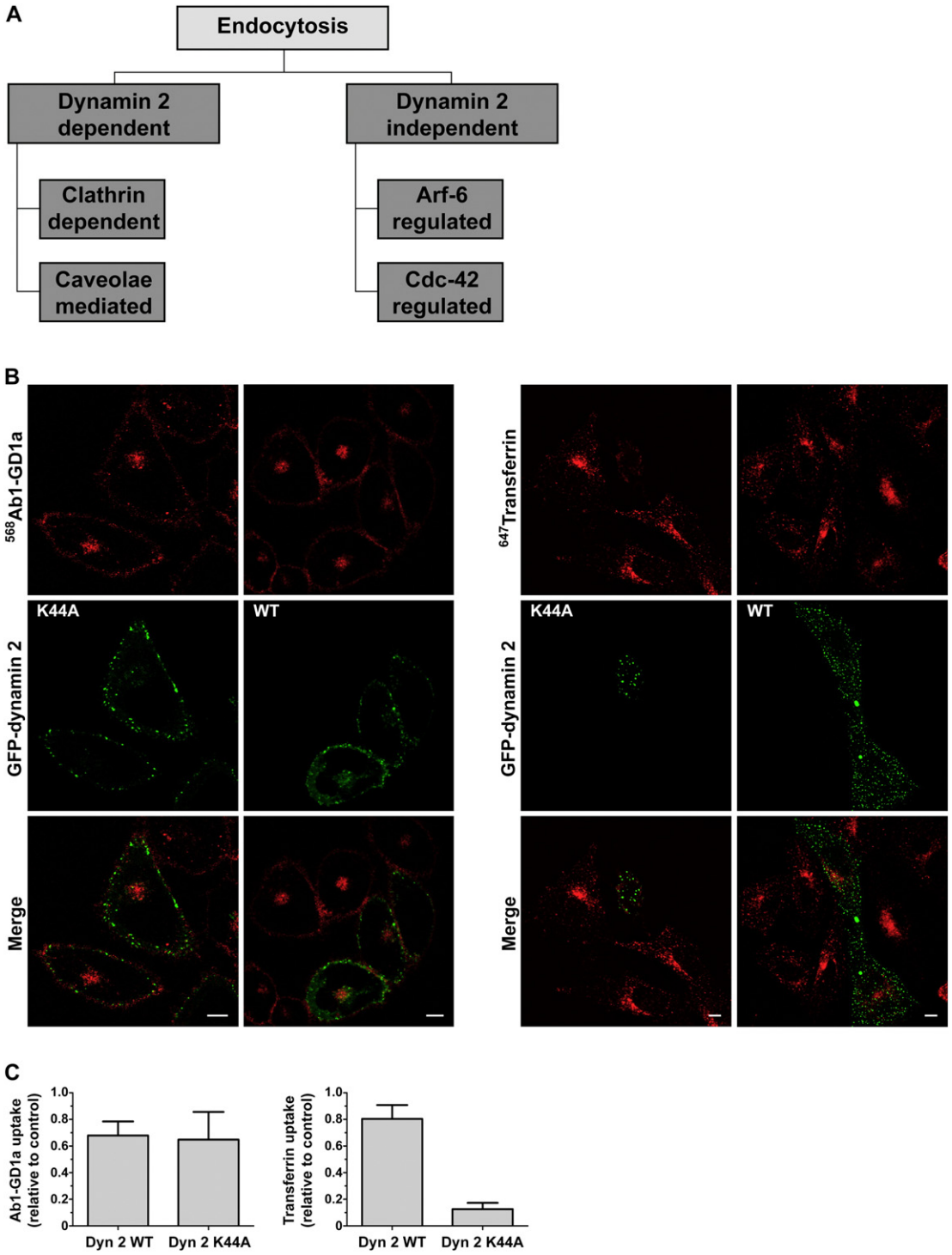


Fig. 2. Ab1-GD1a uptake is dynamin 2-independent. A. Schematic representation of the main cellular endocytic routes and their dependence on dynamin 2 GTPase. B. CHO-K1$^{GD1a/GM1+}$ cells grown on coverslips and transiently transfected to express dominant negative GFP-dynamin 2-K44A (K44A, first column) or GFP-dynamin 2-wild type (WT, second column) were incubated 15 min at 4 °C and then labeled for 45 min with ^{568}Ab1-GD1a in cold DMEM. After that, the medium was replaced by fresh 37 °C pre-warmed DMEM and incubated at that temperature for 30 min. In another experiment, CHO-K1$^{GD1a/GM1+}$ cells expressing GFP-dynamin 2-K44A (third column) or GFP-dynamin 2-wild type (fourth column) were first incubated for 90 min in DMEM without FBS and then incubated at 4 °C for 45 min in DMEM containing both 10 μg/mL ^{647}transferrin and ^{568}Ab1-GD1a. After that, cells were transferred to 37 °C pre-warmed DMEM without FBS supplemented with 5 μg/mL ^{647}transferrin for 30 min. Representative confocal sections of 0.8 μm taken parallel to the coverslip are shown. Scale bars: 5 μm. C. Quantification of fluorescence intensity signal (means +/− SEM) corresponding to ^{568}Ab1-GD1a endocyted fraction at recycling endosomes. The values were relativized to the fluorescence intensity obtained for non-transfected cells (arbitrarily taken as 1).

contrast, when GTP-GDP cycle of Arf6 is blocked by means of overexpression of Arf6 Q67L (a GTP hydrolysis deficient mutant), cells accumulate numerous PtdIns(4,5)P_2-enriched, actin-coated vacuolar structures that sequester cargo molecules which normally traffic through the Arf6 endosomal recycling pathway and then suffer extensive homotypic fusion resulting in larger vesicles [27] (Fig. 3A). To

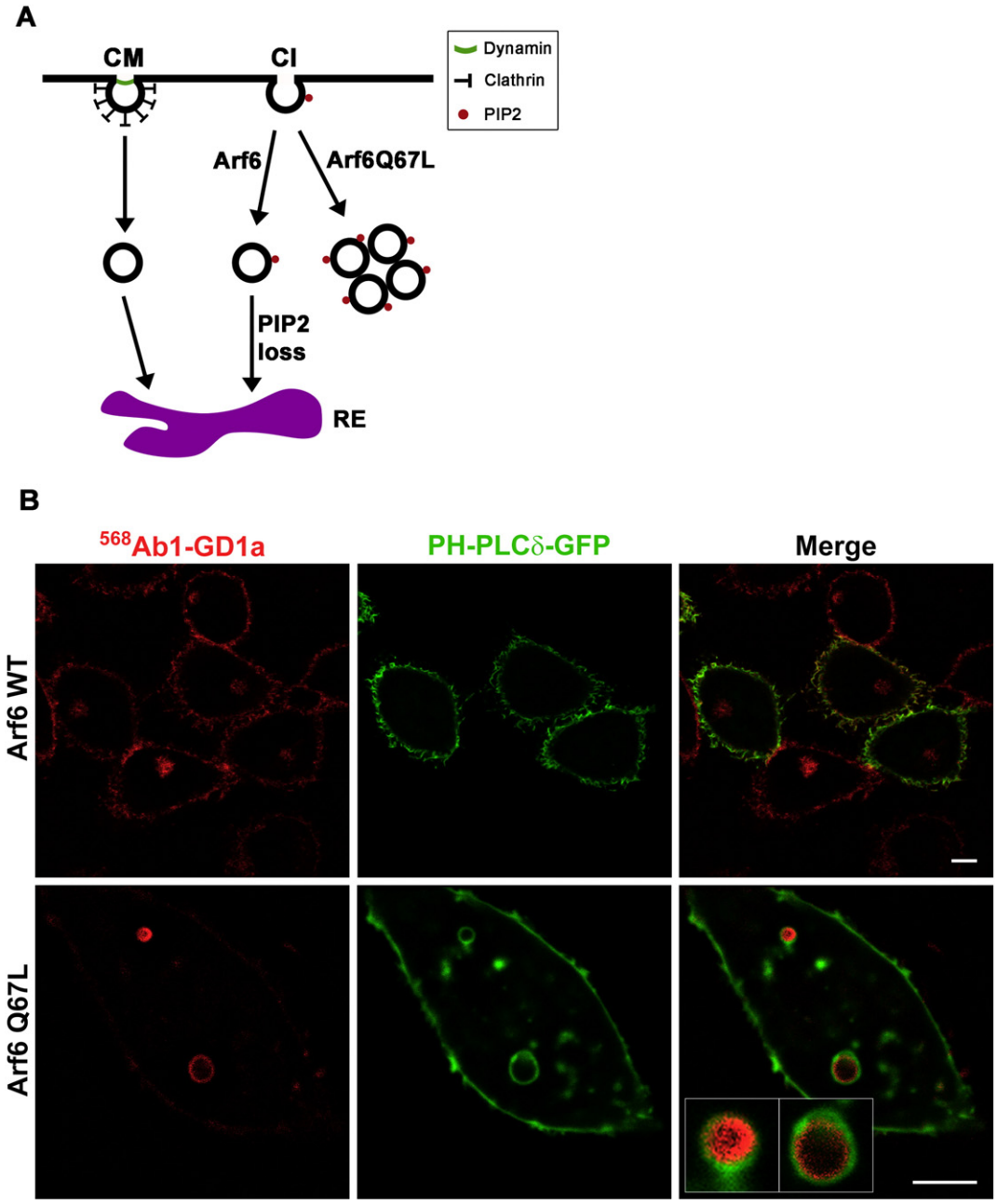


Fig. 3. Ab1-GD1a endocytosis is Arf6-dependent. A. After endocytosis through the clathrin independent (CI) endocytic pathway that depends on the activity of the Arf6 GTPase, the loss of PIP2 on Arf6 derived endosomes allows, in part, the fusion of these endocytic vesicles with the Rab5/EEA1 positive early endosomal compartment, thus merging the CI and clathrin mediated (CM) endocytic cargo at recycling endosomes (RE). When the GTPase hydrolysis deficient mutant of Arf6 (Arf6Q67L) is overexpressed, the cargo molecules that traffic through this pathway are sequestered and accumulated in vacuolar structures that then suffer extensive homotypic fusion. B. CHO-K1$^{GD1a/GM1+}$ cells co-transfected to express the plekstrin homology domain of phospholipase Cδ1 fused to GFP (PH-PLCδ1-GFP), and either the wild-type version of Arf6 (Arf6 WT, top row) or its GTP hydrolysis deficient mutant (Arf6 Q67L, bottom row), were incubated 15 min at 4 °C and then labeled for 30 min with ^{568}Ab1-GD1a in cold phenol red-free DMEM. After that, the temperature was changed to 37 °C to allow endocytosis. Representative confocal sections of 0.8 μm taken parallel to the coverslip are shown. Insets in merged panels show details at higher magnification. Scale bars: 5 μm.

investigate whether this pathway is involved in the internalization of ^{568}Ab1-GD1a, cells were co-transfected to express a PtdIns(4,5)P_2 sensor as the plekstrin homology domain of phospholipase Cδ1 [28–30] fused to GFP (PH-PLCδ-GFP) and either the wild-type version of Arf6 or its mutant counterpart Arf6 Q67L. Results shown in Fig. 3B clearly demonstrate that ^{568}Ab1-GD1a is internalized and accumulated in PtdIns(4,5)P_2 decorated vesicles in Arf6 Q67L expressing cells, suggesting a role for Arf6 in the endocytic uptake of this anti-ganglioside antibody.

3.4. Ab1-GD1a remains associated to the plasma membrane at 16 °C

To further explore the initial events involved in ^{568}Ab1-GD1a uptake, we performed endocytic experiments at 16 °C, a widely used technique to accumulate the endocytic cargo at early endosomal compartments [6,31,32]. Briefly, CHO-K1$^{GD1a/GM1+}$ cells were incubated at 4 °C with ^{568}Ab1-GD1a and then fixed (0 min) or transferred to 16 °C pre-warmed DMEM to allow endocytosis for 10 and 20 min. Finally, cells were fixed and incubated with Alexa^{488}-conjugated

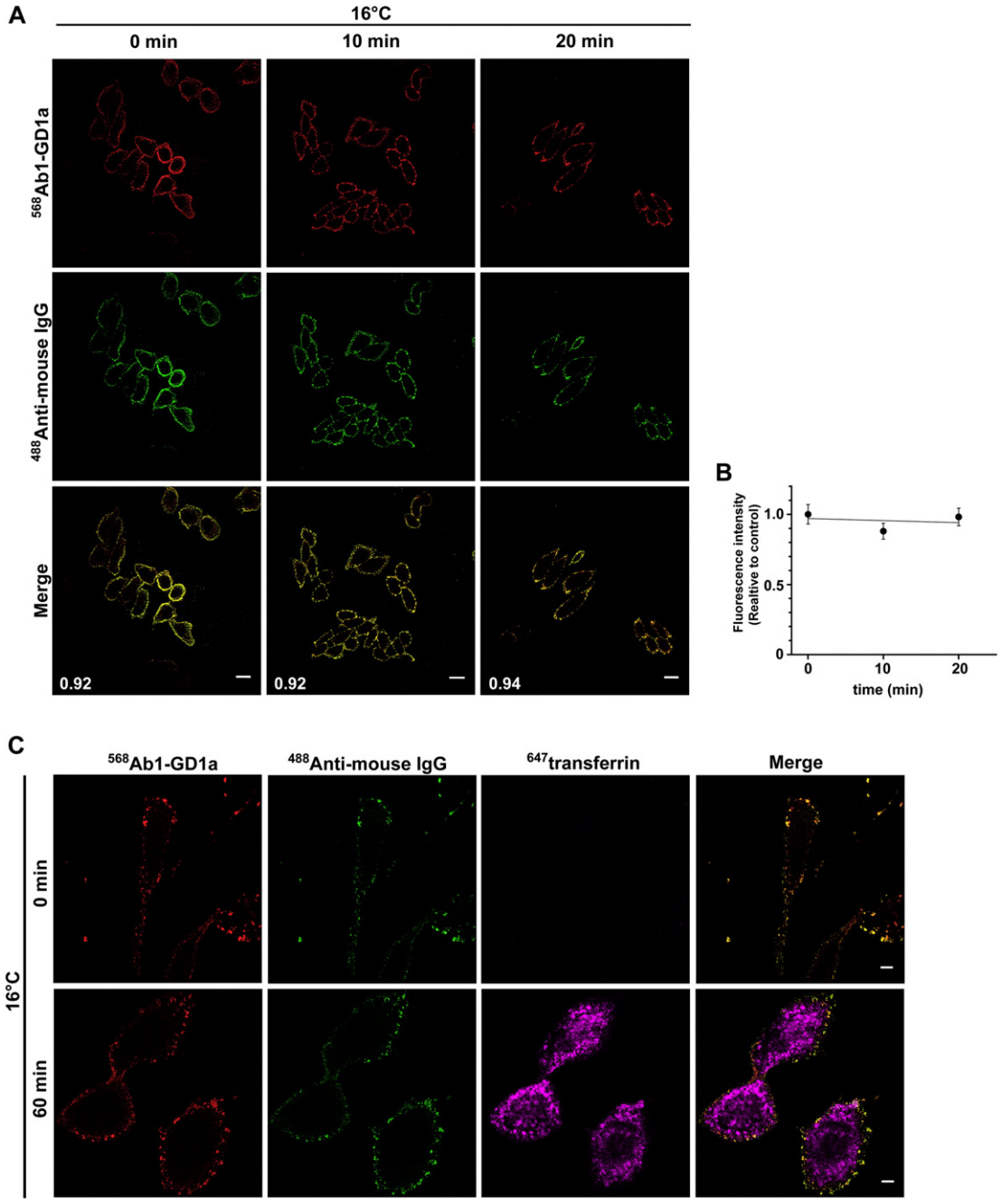


Fig. 4. Plasma membrane binding and differential cellular fate of Ab1-GD1a and transferrin in CHO-K1 cells at 16 °C. A. CHO-K1$^{GD1a/GM1+}$ cells were incubated for 15 min at 4 °C followed by 45 min incubation with ^{568}Ab1-GD1a at 4 °C. Then, cells where washed 3 times with cold DMEM and fixed or transferred to 16 °C pre-warmed DMEM for 10 and 20 min. After each period of time cells were fixed and incubated with Alexa^{488}-conjugated goat anti-mouse IgG. Representative confocal sections of 0.8 μm from the different times taken parallel to the coverslip are shown. Manders' colocalization coefficients are shown in merged panels. Scale bars: 15 μm. B. Quantification of total immunofluorescence signal (means +/− SEM) that remained associated with the cellular fraction at each time is shown. The values were relativized to the fluorescence intensity obtained for the initial binding condition (0 min). C. CHO-K1$^{GD1a/GM1+}$ cells were first incubated for 90 min in DMEM without FBS. Then incubated at 4 °C for 45 min in DMEM containing 10 μg/mL ^{647}transferrin and an appropriate dilution of ^{568}Ab1-GD1a. After that, cells were washed with cold DMEM, fixed (0 min) or transferred to 16 °C pre-warmed DMEM without FBS and supplemented with 5 μg/mL ^{647}transferrin for 60 min. Finally, cells were incubated with Alexa^{488}-conjugated goat anti-mouse IgG. Scale bars: 5 μm.

goat anti-mouse IgG to assess colocalization with any antibody remaining at the cell surface. We observed that a major fraction of ^{568}Ab1-GD1a remained associated to the plasma membrane at 16 °C (Fig. 4A) and no endocytic structures were evident, contrasting with previous findings which showed that a monoclonal antibody to ganglioside GD3 (R24) [22] and a polyclonal antibody to ganglioside GM1 (Ab1-GM1) [6] were internalized at this temperature and accumulated in sorting endosomes. Total immunofluorescence signal associated to the cellular fraction at each endocytic time remained unchanged (Fig. 4B). In control experiments, cells previously treated with ^{568}Ab1-GD1a to label the total pool of GD1a followed by incubation with Alexa^{488}-conjugated goat anti-mouse IgG showed access of the secondary antibody to intracellular compartments only under permeabilization conditions (Supplementary

Fig. 3), ruling out the possibility that cellular permeabilization occurred by the fixation procedure. Also, to prove that endocytic mechanisms are active at 16 °C, ^{647}transferrin was added to the culture medium (Fig. 4C) because it is known that transferrin is endocyted and accumulated in the early endosomal compartment at this temperature. As expected, transferrin was internalized and accumulated in vesicular structures scattered all over the cytoplasm, whereas ^{568}Ab1-GD1a endocytosis was completely blocked, maintaining a high co-localization with Alexa^{488}-conjugated goat anti-mouse IgG at the cell surface even after 60 min of incubation at 16 °C (Fig. 4C). The selective blockade of ^{568}Ab1-GD1a endocytosis at 16 °C and its rapid signal reduction at 37 °C were confirmed by time-lapse confocal fluorescence microscopy and fluorometric assays (Fig. 5A and B, respectively). Together, these results further support the presence of selective mechanisms for cell internalization of antibody-ganglioside complex, which are evidenced by a differential internalization at 16 °C.

3.5. Characterization of binding, endocytosis and intracellular fate of two different antibodies to GD1a in CHO-K1 cells

In order to determine whether the behavior of ^{568}Ab1-GD1a regarding membrane binding, internalization and intracellular fate is shared with another monoclonal antibody to GD1a, we included in the analysis the antibody Ab2-GD1a, an IgG2b immunoglobulin isotype which recognize GD1a/GT1b/GT1aα gangliosides. Briefly, CHO-K1$^{GD1a/GM1+}$ cells were incubated for 15 min at 4 °C to inhibit intracellular transport and then with Ab2-GD1a for 45 min at 4 °C. Later, cells were washed with cold culture medium to remove unbound antibody and fixed or transferred to pre-warmed medium at 37 °C for different times to restore transport and thereby allow endocytosis. Finally, cells were fixed, permeabilized and immunostained. Confocal microscopic analysis revealed that Ab2-GD1a, like Ab1-GD1a, bound in a punctuate pattern to cells at 4 °C and after restoring endocytosis by shifting the temperature to 37 °C, it began to show a perinuclear distribution and the total level of antibody signal was drastically reduced (Fig. 6A). As demonstrated for Ab1-GD1a, Ab2-GD1a was also accumulated in PtdIns(4,5)P_2 decorated vesicles in Arf6 Q67L expressing cells, and its endocytosis was significantly reduced in cells overexpressing a dominant negative form of Arf6 (Arf6 T27N) (Fig. 6B and C). Together, these results suggest that both antibodies to GD1a (Ab1-GD1a and Ab2-GD1a) share similar processes of binding, endocytosis and cellular fate.

3.6. Characterization of membrane binding and cellular fate of Ab2-GD1a in neuroblastoma cells

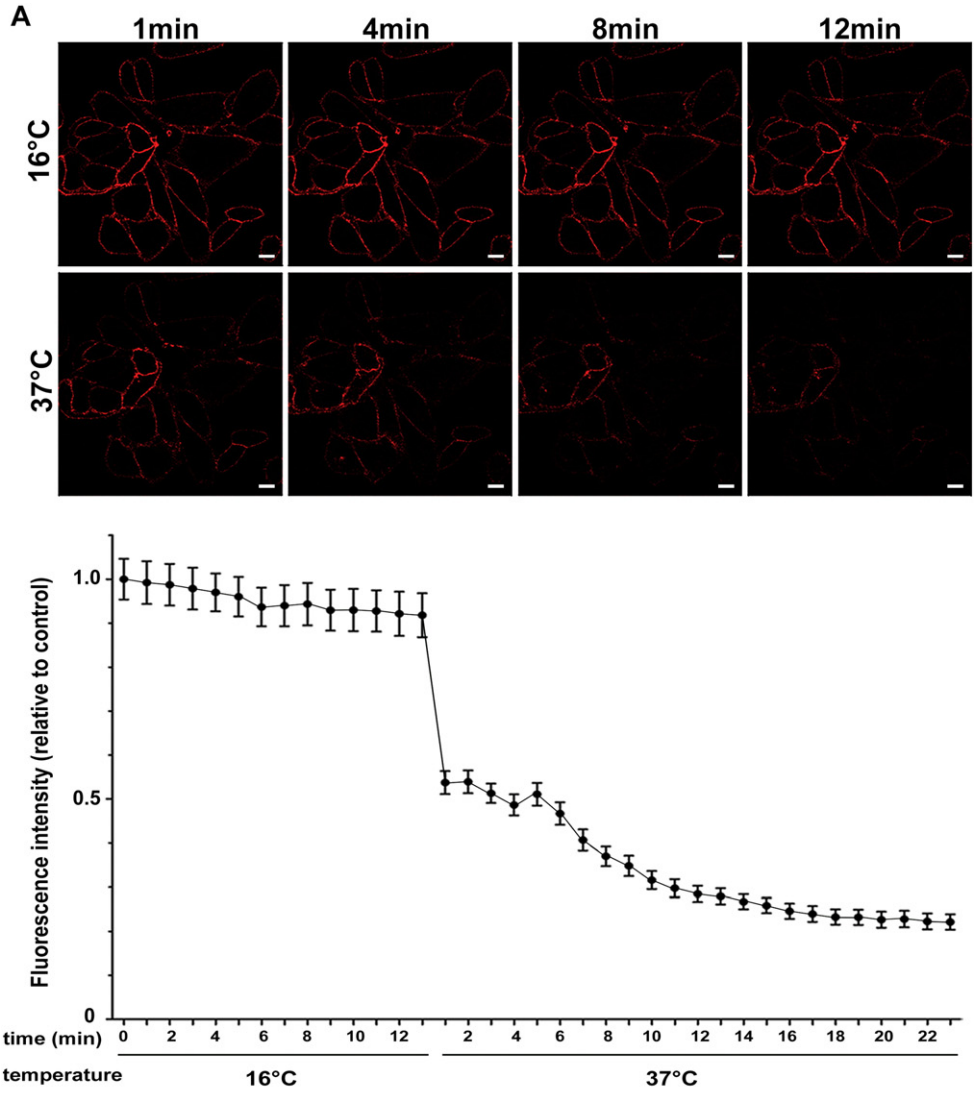
It was already demonstrated that an antibody to ganglioside GM1 shows a differential internalization and intracellular sorting in a cell-type depending fashion [6]. Briefly, it was found that antibody to GM1 was efficiently endocyted in epithelial CHO-K1 cells but only slightly internalized in the neuroblastoma cell line Neuro-2a, which mainly express gangliosides GD1a and GM1 [33,34]. With these antecedents, we decided to explore if this behavior is extended to other antibodies to gangliosides, particularly to Ab2-GD1a antibody. Ab2-GD1a bound efficiently at the cell surface of Neuro-2a cells at 4 °C (Fig. 6D, 0 min). When the temperature was changed to 37 °C the Ab2-GD1a initial fluorescence signal was moderately reduced (Fig. 6D and E) compared with the drastic reduction reported in CHO-K1$^{GD1a/GM1+}$ cells (Fig. 6A). Thus, these results point out that the antibodies to gangliosides may have differential endocytic behaviors or cellular processing, which could represent a critical aspect to be considered in the analysis of their pathogenic role and therapeutic uses.

3.7. A monoclonal antibody to GM1 shows a dissimilar behavior with antibodies to GD1a in both epithelial and neuronal cells

Finally, we explored if kinetic parameters of membrane binding and endocytosis of antibodies to GD1a is specific for this ganglioside/antibody complex or is rather a general phenomenon also observed for other antibodies to a-Series gangliosides. Thus, we analyzed in CHO-K1 and Neuro-2a cells the membrane binding kinetics of Ab2-GM1, a subclass IgG2bκ monoclonal antibody that strongly reacts with ganglioside GM1 [35]. Ab2-GM1 antibody was found to prolong the muscle action potential intervals at the neuromuscular junctions and to stain both the nodes of Ranvier and paranodal Schwann cells in human spinal roots [15]. CHO-K1$^{GM1/GD1a+}$ cells were incubated for 15 min at 4 °C to inhibit intracellular transport and then with Ab2-GM1 antibody for 45 min at 4 °C. After washing to remove unbound antibody, cells were fixed (0 min) or transferred to 37 °C pre-warmed DMEM (Fig. 7A) or 16 °C pre-warmed DMEM (Fig. 7E) for different times to restore endocytosis. Confocal microscopic analysis revealed that Ab2-GM1 antibody bound to CHO-K1$^{GM1/GD1a+}$ cells with a punctate plasma membrane distribution at 4 °C. Immediately after shifting the temperature to 37 °C to induce endocytosis (5 min), the presence of the antibody in a perinuclear region became evident with a moderate reduction in total immunofluorescence signal (remaining 70% of the initial signal, 0 min) keeping close to that value at longer periods of time (15 min and 30 min) (Fig. 7A and C), contrasting with fluorescence signal associated with antibodies to GD1a (Ab1-GD1a, Fig.1C and Ab2-GD1a, Fig. 6A) that is drastically reduced within the first minutes. On the other hand, when shifting the temperature to 16 °C, the total immunofluorescence signal remained almost unchanged and associated to plasma membrane (Fig. 7E and F), as also seen for antibody to GD1a (Ab1-GD1a, Figs. 4 and 5). Controls of binding and internalization specificity of antibodies to gangliosides are shown in Supplementary Figs. 1 and 2. We also observed in neuroblastoma cells that after binding and shifting the temperature to 37 °C, Ab2-GM1 antibody remained associated to the cell surface (Fig. 7 B) and the total immunofluorescence signal was unchanged (Fig. 7D), contrasting with antibody to GD1a (Ab2-GD1a, Fig. 6D and E). Thus, we observed that under identical experimental conditions, both the time of residence at the cell surface and the endocyted fraction of antibodies to GD1a and GM1 were notoriously dissimilar, suggesting differential processing that could depend not only on the anti-ganglioside antibody but also on the cell type. These findings could help to better understand not only the role and differential pathogenic effects of anti-ganglioside antibodies in neuropathies but also to improve their use as therapeutic agents.

4. Discussion

Antibodies to gangliosides, in particular to GM1, GD1a and GQ1b, have been associated with a wide range of neuropathy diseases, including the Guillain-Barré and Miller Fisher syndromes [9]. In addition, and given the aberrant glycosylation pattern of glycolipids expressed on the surface of most cancer cells, a substantial number of passive and active immunotherapies against these lipids have been developed, which have shown promising results in clinical trials [36,37]. In this sense, dinutuximab (a chimeric monoclonal antibody to GD2 ganglioside) was approved in 2015 by the US Food and Drug Administration (FDA) and is currently used in a combination immunotherapeutic regimen for the treatment of children with high-risk neuroblastoma [38]. More recently, antibodies to glycolipids have also emerged as an attractive tool for the targeted delivery of cytotoxic agents, thereby providing a rationale for future therapeutic interventions in cancer [36]. In this scenario, it is essential to understand mechanisms by which antibodies to glycolipids, mainly those against gangliosides, recognize their epitopes, the


A
1min
4min
8min
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16°C
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Fluorescence intensity (relative to control)
1.0
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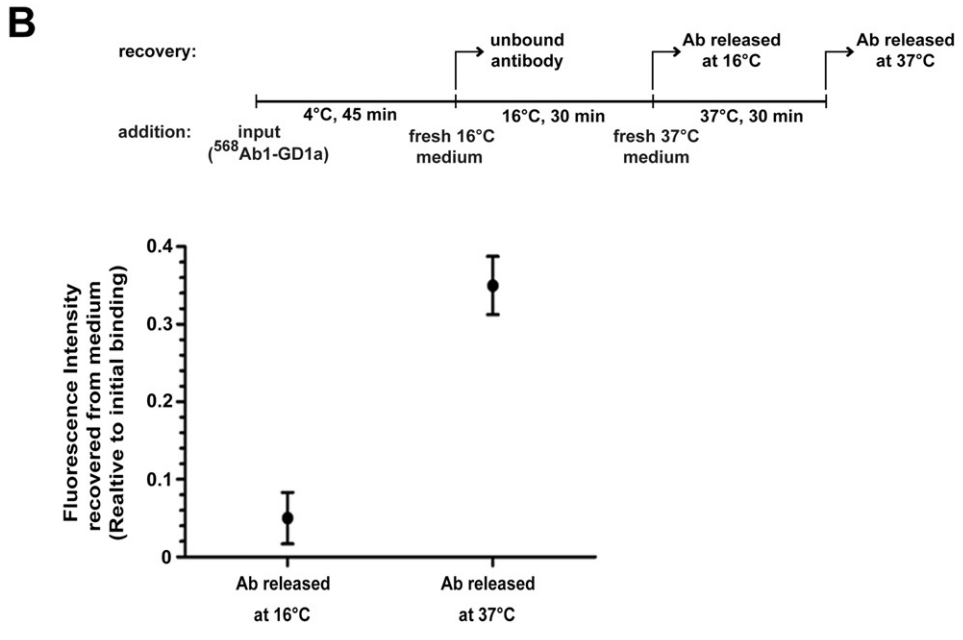
B
recovery:
unbound antibody
Ab released at 16°C
Ab released at 37°C
addition:
input (^{568}Ab1-GD1a)
4°C, 45 min
fresh 16°C medium
16°C, 30 min
fresh 37°C medium
37°C, 30 min
Fluorescence Intensity recovered from medium (Realtive to initial binding)
0.4
0.3
0.2
0.1
0
Ab released at 16°C
Ab released at 37°C

cellular fate and the physiopathological consequences. This information has important translational implications for the understanding of clinical characteristics of anti-glycolipid antibody-mediated neuropathies and for the development of novel therapeutics targeting.

According to information obtained from our and other laboratories, antibodies to gangliosides do not share common features like membrane binding, cellular endocytosis and immune response (Fig. 8). Rather, they behave in an epitope- and cell type-dependent manner. In this way, we demonstrated in CHO-K1 and SK-Mel 28 melanoma cells that a mouse monoclonal antibody to GD3 was specifically endocyted bound to the ganglioside, sorted to early and recycling endosomes and transported back to the plasma membrane by clathrin-coated vesicles [22]. In contrast, an antibody to GM1 (Ab1-GM1) was rapidly and specifically endocyted in CHO-K1 cells whereas it showed a reduced internalization in COS-7 cells and neural cell lines SH-SY5Y and Neuro-2a [6] (Fig. 8). This information was considered of biological and immunological significance since it provided for the first time evidences about internalization, intracellular fate and endocytic pathways of antibody to GM1 obtained from rabbits with experimental Guillain-Barré syndrome. Later on, it was described that rapid anti-ganglioside antibodies uptake at the motor nerve terminal membrane attenuates complement-mediated injury, in contrast to the extensive damage seen at nodes of Ranvier, where antibody uptake was observed to be minimal [11,12]. Moreover, it was recently demonstrated in mice that a high proportion of plasma circulating antibodies to gangliosides are mainly cleared by binding to neuronal gangliosides at motor nerve terminals of neuromuscular junctions [39]. This neuronal endocytosis represents a major pathway by which pathogenic anti-ganglioside antibodies, and potentially other ganglioside binding proteins, are cleared from the systemic circulation and also covertly delivered to the central nervous system.

In this work, we extended our analysis of antibodies against gangliosides, investigating binding, uptake and cellular fate of antibodies to the disialo-ganglioside GD1a. We found that, in CHO-K1 cells, the antibody to GD1a bound to living cells at 4 °C had a punctate plasma membrane distribution. After induction of endocytosis by changing the temperature to 37 °C, a fraction of antibody to GD1a was internalized being initially found in vesicles all over the cytoplasm and then accumulated in a pericentriolar compartment characterized as recycling endosome. Unexpectedly, a major fraction of the antibody was released from cells at 37 °C and found at the extracellular medium. In this regard, it has been widely documented that a considerable fraction of the gangliosides produced by a cell is released to the extracellular milieu, and potentially, can be transferred to the plasma membrane of other cells [40–42]. Thus, it is highly probable that antibody to GD1a is being cleared from plasma membrane bound to the shed ganglioside. Moreover, it was demonstrated that gangliosides are able to spontaneously transfer between membranes at elevated temperatures [43] and the rate of transfer is dependent on both temperature and the physical state of donor and acceptor membranes [44], which is in agreement with experiments performed at 16 °C where the antibody to GD1a remained mostly localized at the plasma membrane (Figs. 4 and 5) and in contraposition to the significant release observed at 37 °C.

Experiments shown in Fig. 2 indicate that the endocytosis of the antibody to GD1a in CHO-K1 cells do not require dynamin 2, suggesting a clathrin and caveolin 1-independent mechanism. Taking these results into account and considering that internalization of many GPI-anchored proteins and acylated proteins (both types of molecules attached to the cell membrane by lipid anchors) occurs by a clathrin-independent pathway regulated by the small GTP-binding protein Arf6 [24,26], we investigated whether this small GTPase participates in the internalization of antibody to GD1a. Thus, we examined the effect of expression of the constitutively active GTPase-deficient Arf6 mutant on antibody to GD1a endocytosis, and found that the antibody was mainly associated with vacuolar structures containing PtdIns(4,5)P_2. On the other hand, overexpression of a dominant negative form of the GTPase caused a reduction in the internalization of antibody to GD1a. Thus, these results strongly suggest that the antibody-ganglioside complex is being internalized through an Arf6-associated pathway. Rapid Arf6-mediated endocytosis has been demonstrated to occur in growth cones from neurons during development [45] and the participation of Arf6 in anterograde and retrograde trafficking was found in axons and growth cones from adult neurons [46]. Arf6 also has a role in the recruitment of clathrin coat proteins to membranes where synaptic vesicle recycling occurs at least in part by increasing PtdIns(4,5)P_2 levels [47]. Taken into consideration these and other antecedents, Arf6 could be involved in the rapid anti-ganglioside antibodies uptake at the motor nerve terminal membrane, attenuating complement-mediated injury [11,12]. By contrast, there is little information regarding exocytic and endocytic transport at nodes of Ranvier and, particularly, there is no literature about participation of Arf6 at this specialized neuronal region. Thus, examination of Arf6-mediated membrane endocytosis/recycling pathway at these neuronal sites is of interest since the minimal uptake of antibodies to gangliosides observed at nodes of Ranvier could eventually be associated with the absence of Arf6-mediated membrane endocytosis, which remains to be investigated.

As mentioned, neosynthesized gangliosides observed in oncogenic processes show antigen specificity and, therefore, they are attractive candidates for the design of cancer immunotherapies. In this sense, the information reported in this and other studies [6,22,48] has also important conceptual and translational implications for the development of innovative therapeutics targeting [49]. Thus, whereas many antibodies to gangliosides have specific binding, different modes of action are possible depending on the desired function of the antibody (i.e. cellular internalization for delivering a drug versus sustained presence on the cell surface for immune-mediated tumor cell killing).

Supplementary data to this article can be found online at doi:10.1016/j.bbamem.2016.10.020.

Fig. 5. Endocytosis and fate of Ab1-GD1a at different temperatures assessed by time-lapse confocal fluorescence microscopy and fluorometric assays. A. CHO-K1$^{GD1a/GM1+}$ cells grown in LabTek II coverglass were labeled for 45 min at 4 °C with ^{568}Ab1-GD1a, then washed and the temperature was shifted to 16 °C for 13 min. Immediately after, the temperature was changed to 37 °C and maintained until the end of the experiment. Quantification of total immunofluorescence signal (means +/− SEM) that remained associated to the cellular fraction at each time is shown. The values were relativized to the fluorescence intensity obtained for the initial binding condition (16 °C, 0 min). Scale bars: 10 μm. B. CHO-K1$^{GD1a/GM1+}$ cells were grown in 96-well plates for 24 h. Pre-warmed medium was added (addition) or collected (recovery) at different times during the experiment. First, cells were labeled at 4 °C for 30 min with ^{568}Ab1-GD1a in phenol red-free DMEM (input). After that, culture medium was recovered (unbound antibody); cells were extensively washed and fresh 16 °C pre-warmed phenol red-free DMEM was added for 30 min. Medium containing antibody released during this 30 min incubation at 16 °C was recovered (Ab released at 16 °C), and fresh 37 °C pre-warmed phenol red-free DMEM was added for other 30 min. Finally, medium containing antibody released during this 30 min of incubation at 37 °C was collected (Ab released at 37 °C). The fluorescence in each recovered fraction was measured using a FluoroMax-P spectrofluorometer. Values are means +/− SEM relativized to initial antibody binding (arbitrarily taken as 1 and calculated subtracting the fluorescence signal obtained for the fraction of unbound antibody from the input signal) from two independent experiments; n = 3 for each time condition.

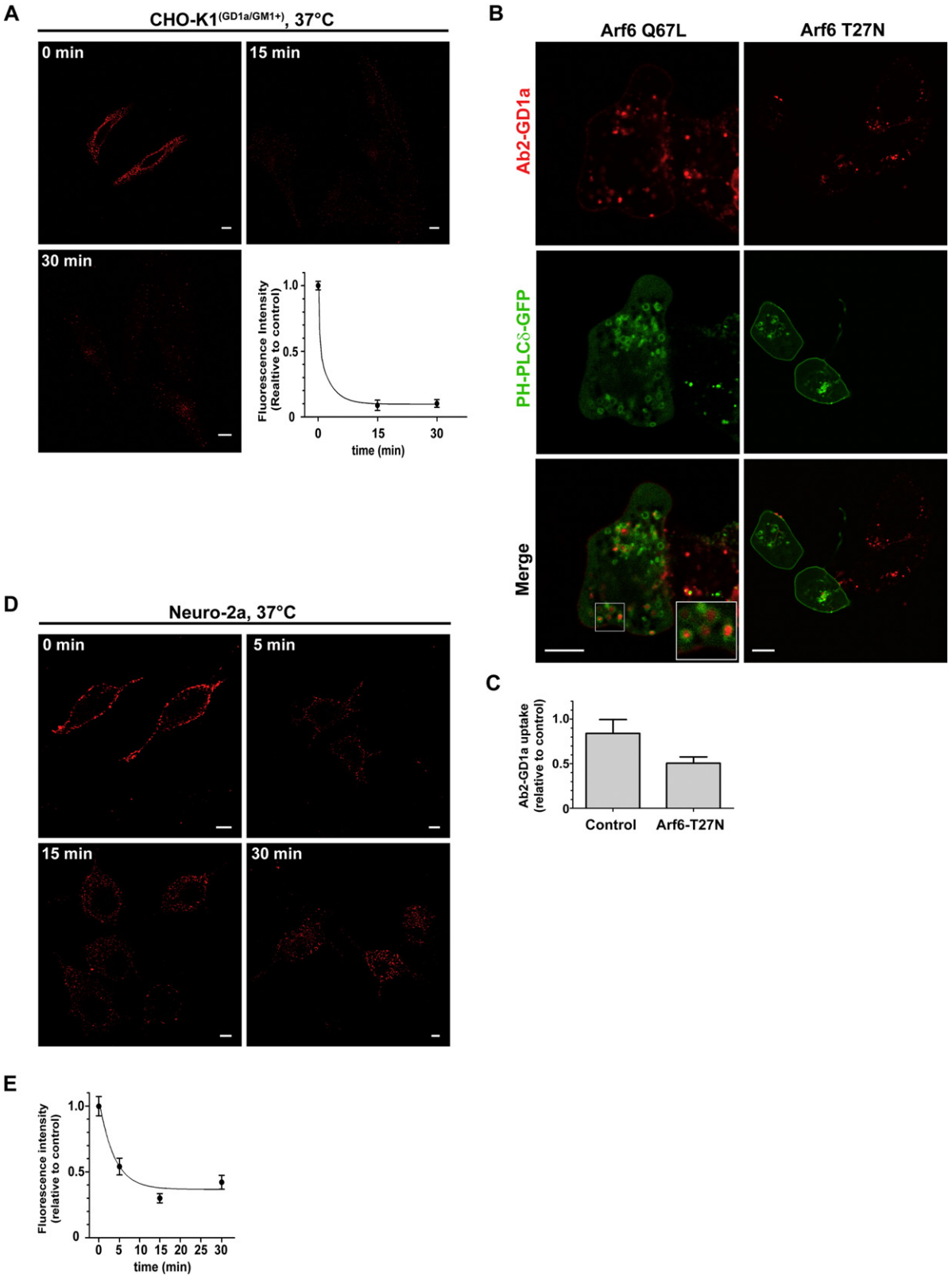

Fig. 6. Cell surface binding and fate of a monoclonal antibody to gangliosides GD1a/GT1b/GT1aα (Ab2-GD1a) in CHO-K1 $^{GD1a/GM1+}$ and Neuro-2a cells. CHO-K1$^{GD1a/GM1+}$ cells (A) or Neuro-2a cells (D) were incubated for 15 min at 4 °C and then with Ab2-GD1a for 45 min at 4 °C. Then, cells were washed with cold DMEM and fixed at 4 °C (0 min) or transferred to 37 °C pre-warmed medium for different times. Finally, cells were fixed, permeabilized and incubated with Alexa^{546}-conjugated goat anti-mouse IgG. Representative confocal sections of 0.8 μm from different endocytic times taken parallel to the coverslip are shown. Scale bars: 5 μm. Quantification of total immunofluorescence signal (means +/− SEM) that remained associated to CHO-K1$^{GD1a/GM1+}$ (A) or Neuro-2a (E) cellular fraction at each time is shown. The values were relativized to the fluorescence intensity obtained for the initial binding condition (0 min), arbitrarily taken as 1. CHO-K1$^{GD1a/GM1+}$ cells were co-transfected to express PH-PLCδ1-GFP and either a dominant positive version of Arf6 (B, Arf6 Q67L, left column) or a dominant negative mutant of the GTPase (B, Arf6 T27N, right column). After 24 h of transfection, cells were labeled with Ab2-GD1a at 4 °C, washed and transferred to 37 °C to allow endocytosis for 30 min. Alexa^{546}-conjugated goat anti-mouse IgG was used to detect Ab2-GD1a. Scale bars: 5 μm. Inset on merged panel shows details with higher magnification. Quantification of Ab2-GD1a endocyted immunofluorescence signal in Arf6 T27N transfected cells (means +/− SEM) relativized to the fluorescence intensity obtained for the non-transfected cells is shown (C).

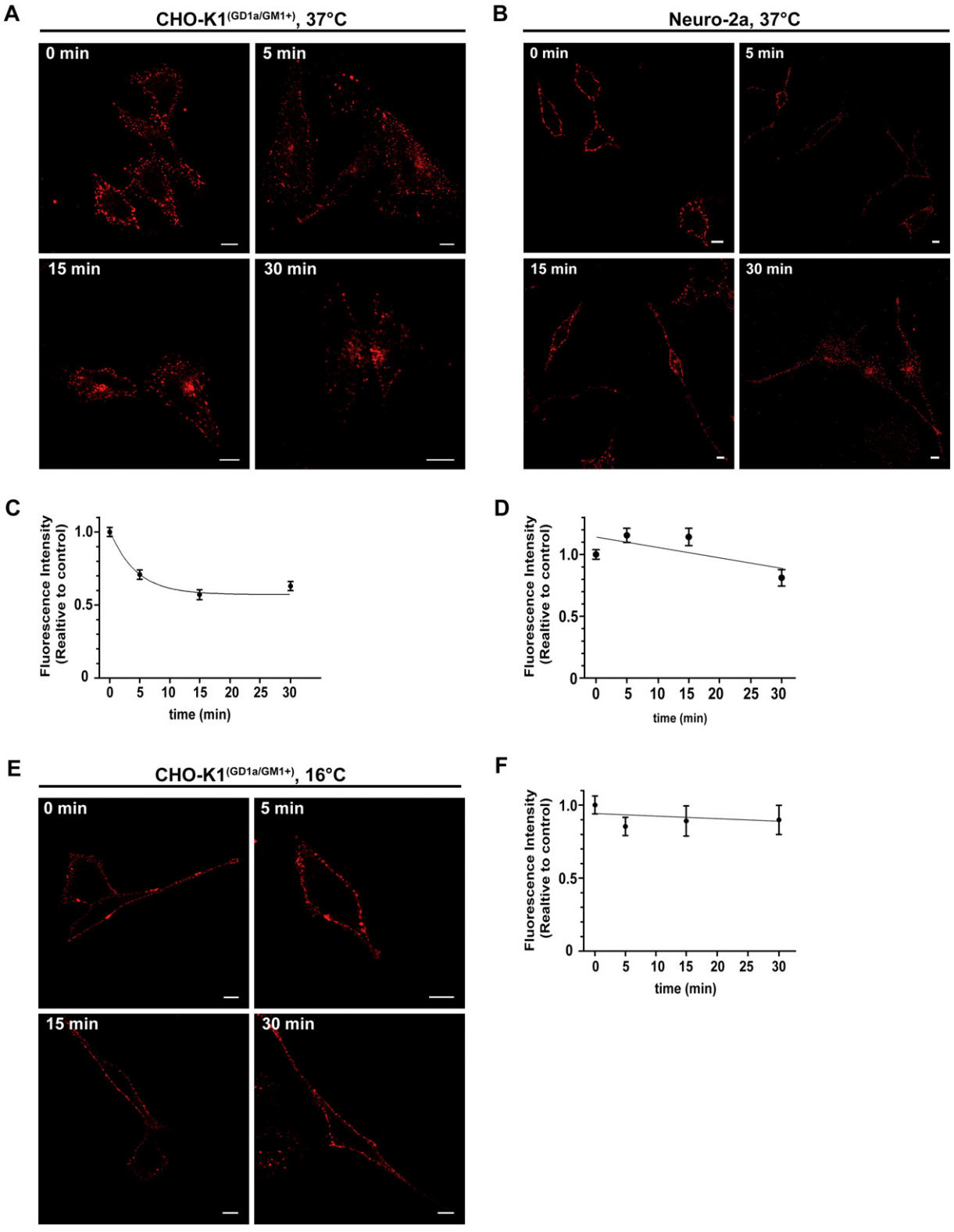

Fig. 7. Cell surface binding and fate of a monoclonal antibody to ganglioside GM1 (Ab2-GM1) in CHO-K1 $^{GD1a/GM1+}$ and Neuro-2a cells. CHO-K1$^{GD1a/GM1+}$ cells (A) and Neuro-2a cells (B) were incubated at 4 °C for 15 min and then with an appropriate dilution of an anti-GM1 antibody (Ab2-GM1) for 45 min at 4 °C. After that, cells were fixed at 4 °C (0 min) or transferred to 37 °C pre-warmed DMEM to allow endocytosis. After that, cells were permeabilized and incubated with Alexa^{546}-conjugated goat anti-mouse IgG. Scale bars: 5 μm. Total Ab2-GM1 immunofluorescence signal associated to CHO-K1$^{GD1a/GM1+}$ cells (C) and Neuro-2a cells (D) was quantified. Endocytosis at 16 °C was performed in CHO-K1$^{GD1a/GM1+}$ cells (E) and the total Ab2-GM1 immunofluorescence signal associated with the cellular fraction at each endocytic time was quantified (F). In all cases fluorescence intensity values were referred to the signal obtained for the initial binding condition (0 min, arbitrarily taken as 1).

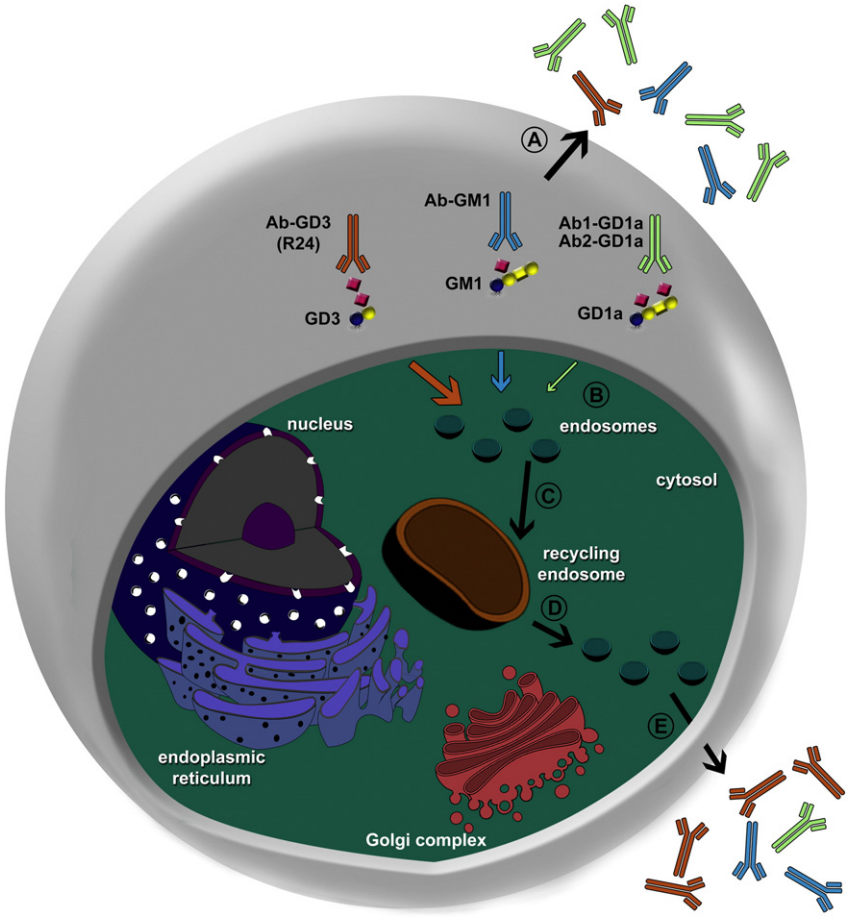

Fig. 8. Schematic representation of the cellular fate and processing of different anti-ganglioside antibodies in CHO-K1 cells. The fate of three different anti-ganglioside antibodies in CHO-K1 cells is represented. Arrow A represents the dissociation from plasma membrane to extracellular medium, the main fate of antibodies to GD1a ganglioside. The thickness of arrows from plasma membrane to endosomes (B) represents the amount of endocytosis of the ganglioside-antibody complex. Arrows C, D and E show the main intracellular pathway followed by these antibodies after internalization.

Transparency document

The Transparency document associated with this article can be found, in online version.

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