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The complex organization and apical positioning of the Golgi complex is dispensable for polarized apical trafficking and polarity in hepatoma HepG2 cells at steady state

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ABSTRACT

The Golgi complex in mammalian cells forms a pericentrosomal ribbon of laterally linked cisternal stacks that, in polarized epithelial cells, is typically orientated towards the apical plasma membrane domain. However, whether the ribbon structure or apical positioning of the Golgi complex is required for its function in the polarized sorting and trafficking of apical plasma membrane proteins and lipids is not known. Here, we show that extracellular acidosis causes a reversible temperature-dependent fragmentation and dispersion of Golgi membranes in polarized human hepatoma HepG2 cells, the extent of which correlates with decreasing pH values and exposure times. Acidosis does not prevent the de novo biogenesis of Golgi membranes, albeit fragmented, as evidenced by brefeldin A washout experiments. Low temperature-mediated slowdown of trafficking reveals that newly synthesized apical plasma membrane proteins pass through the fragmented Golgi membranes. No missorting or intracellular accumulation of apical plasma membrane proteins is observed at steady state. Furthermore, the metabolism of a ceramide analog to form (glyco)sphingolipids and the polarized trafficking of these metabolites is not inhibited. Our data suggest that the typical ribbon structure and apical positioning of the Golgi complex can be uncoupled from its main functions relating to apical plasma membrane polarity.
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INTRODUCTION

The Golgi apparatus, discovered in the 19th century by Camillo Golgi, plays a pivotal role in diverse cellular processes. These include posttranslational protein and lipid modification, (glyco)sphingolipid metabolism, protein sorting and vesicular trafficking and, more recently, cell division. In mammalian cells, the Golgi apparatus consists of compositionally and functionally distinct cisternal stacks (cis, medial and trans) and a trans-Golgi network. The cisternal stacks are laterally linked to form a lace-like ribbon structure. In addition to this structural complexity, the Golgi complex is typically positioned near the centrosome in a manner that depends on the activity of the minus end-directed microtubule-based motor protein dynein (Hehnly et al., 2010). Furthermore, in transiently polarized cells (e.g., migrating fibroblasts, T-lymphocytes) and in permanently polarized cells (e.g., neurons, epithelial cells) both the Golgi ribbon and centrosome display a polarized position facing the leading edge, immunological synapse, or “apical” plasma membrane domain. The functional relevance of the high degree of structural complexity and the typical (polarized) pericentrosomal localization of the mammalian Golgi complex remains not well understood.

Previous studies indicate that the trafficking of proteins in yeast or mammalian cells, as such, does not appear to require Golgi ribbon and/or pericentrosomal localization (Preuss et al., 1992; Cole et al., 1996; Diao et al., 2003; Yadav et al., 2009, Koegler et al., 2010). However, directional post-Golgi trafficking towards the leading edge and cell migration were shown to require Golgi complex-associated CLASP proteins, which regulate Golgi ribbon formation (Miller et al., 2009). Furthermore, the depletion of GMAP210 or Golgin-160, two structural proteins of the golgin family, efficiently disrupted the ribbon-like structure of the Golgi complex and resulted in the appearance of isolated ministacks (Yadav et al., 2009). While this did not prevent general protein transport to the cell surface, it impaired the ability of migrating fibroblast cells to secrete vesicular stomatitis virus G protein in a
directional manner toward the leading edge and to migrate in a wound-healing assay (Yadav et al., 2009). Posttranslational protein and/or lipid glycosylation has been proposed to be important for the polarized sorting and trafficking from the Golgi complex to the apical plasma membrane (reviewed in Weisz and Rodriguez-Boulan, 2009). The disruption of the Golgi ribbon in HeLa cells was reported to associate with a nonuniform distribution of Golgi resident enzymes and Golgi-specific defects in protein glycosylation (Puthenveedu et al., 2006), although others did not find a correlation between the absence of a Golgi ribbon and a general defective posttranslational processing (including glycosylation) of secretory cargos at the Golgi complex (Marra et al., 2007). Collectively, these data suggest that the Golgi ribbon and/or polarized distribution of the Golgi complex ensure the targeted delivery of membranes to selected sites at the cell surface (reviewed in Sütterlin and Colanzi, 2010).

It is unclear whether the subapical position of the centrosome and the Golgi ribbon architecture is functionally relevant for apical trafficking and the relatively stable plasma membrane polarity displayed by epithelial cells. For instance, the Madin-Darby canine kidney (MDCK) II/J clone maintains a perinuclear organization of the centrosome and Golgi ribbon, as opposed to a polarized apical surface orientated organization, but exhibited normal steady-state polarity and trafficking of apical and basolateral proteins (Grindstaff et al., 1998). Similarly, human hepatoma HepG2 cells that stably express a PKA type II-displacing peptide maintained a perinuclear centrosome position but exhibited normal bile canalicular polarity and a normal polarized trafficking of apical bile canalicular proteins (Wojtal et al., 2007). However, in the latter cells the trafficking of the apical resident ABCB1 protein to the apical plasma membrane was somewhat delayed, and stimulation of apical plasma membrane development by oncostatin M was inhibited (Wojtal et al., 2007). Whether a complex Golgi architecture and a polarized subcellular distribution of the Golgi complex are required for proper apical trafficking and polarity in hepatocytes (and epithelial cells in general) is not known.
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Here, we report a dramatic yet reversible loss of pericentrosomal Golgi membrane organization in polarized human hepatoma HepG2 cells when exposed to extracellular acidosis, commonly observed during ischemia and hypoxia. The loss of Golgi complex organization did not affect apical protein sorting, steady-state apical polarity, and (glyco)sphingolipid metabolism, sorting and trafficking. The molecular mechanisms by which acidosis may induce Golgi membrane fragmentation, and the relevance of the typical ribbon structure and apical positioning of the Golgi complex for its main functions relating to apical plasma membrane polarity in hepatocytes and other cell types is discussed.

MATERIALS AND METHODS

Materials

6-(N-[7-nitrobenz-2-oxa-1,3-diazol-4-yl]amino) hexanoic acid (C6-NBD) was obtained from Molecular Probes (Eugene, OR). DMEM and HBSS were from GIBCO-BRL (Paisley, Scotland). FCS was bought from BioWhittaker (Verviers, Belgium). Brefeldin A (Prod. No. B7651) was from Sigma (Zwijndrecht, Netherlands). Inhibitors were from Calbiochem (Darmstadt, Germany). All other chemicals were of analytical grade.

Cell culture

HepG2 cells were cultured at 37°C in a 5% CO2-containing, humidified atmosphere in high glucose (4500mg/l) DMEM, supplemented with 10% (v/v) heat-inactivated (at 56°C) FCS, 2mM L-glutamine, 100IU/ml penicillin, and 100µg/ml streptomycin. Media were changed every other day. For experiments, cells were plated onto ethanol-sterilized glass coverslips and cultured for 2–3 days.
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Extracellular acidosis
Cells were incubated in DMEM at pH 5.5, 6.0 and 7.4 in a 5% CO2-containing humidified atmosphere at the temperature and for the time intervals as indicated. In some experiments, cells were subsequently washed and incubated in DMEM pH 7.4 at the temperature and for the time intervals as indicated.

Immunofluorescence
HepG2 cells were fixed with 4% paraformaldehyde (PFA) at room temperature for 20 min., washed, and permeabilized with 0.1% Triton X-100 at 37°C for 10 min. Antibodies were diluted in HBSS. Cells were next incubated with the primary antibodies, diluted in HBSS, at 37°C for 60 min., washed and incubated with appropriate secondary antibodies (Alexa Fluor, Molecular Probes, Eugene, OR) at 37°C for 30 min. Primary antibodies used: rabbit polyclonal anti-giantin (Golgi marker; Eurogentec), anti-acetylated α-Tubulin (Zymed, Invitrogen), anti-MRP2 (ABCC2, Alexis) and monoclonal anti-MDR1 (C219, Abcam). Actin was detected by TRITC-phalloidin (Sigma); nucleus was visualized by the DNA-binding dye Hoechst 33528. All coverslips were mounted and analyzed by fluorescence microscopy (Provis AX70; Olympus, New Hyde Park, NY).

Golgi biogenesis assay
Cells were incubated with 5µg/ml Brefeldin A (BFA) at 37°C for for 60 min. Cells were then exposed to an acidic environment as described above and BFA was washed out with HBSS for the indicated time intervals. Cells were then fixed and immunolabeled with antibodies against the Golgi marker giantin.

Cell Labelling and Lipid Transport Assays

Synthesis of C6-NBD sphingolipids
C6-NBD-ceramide (C6-NBD-Cer) was synthesized from C6-NBD and D-sphingosine as described elsewhere (Babia et al., 1994).
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Insertion and back-exchange of fluorescent lipids.

Cells were washed three times with cold PBS. C6-NBD-Cer was added to cold Hanks' buffered salt solution (HBSS) by means of ethanol injection. Lipids from a stock solution in chloroform/methanol (2:1 vol/vol) were dried under nitrogen and solubilised in absolute ethanol. The ethanol solution was subsequently injected into HBSS under vigorous vortexing. The final concentration of ethanol did not exceed 0.5% (vol/vol). When required, C6-NBD-lipids present in the outer leaflet of the plasma membrane were removed by a back-exchange procedure. To this end the cells were incubated for 30 min. at 4°C with 5% BSA in HBSS, followed by washing with cold HBSS. This procedure was repeated once.

Apical delivery of de novo-synthesized lipids.

Cells were plated on coverslips and labelled with C6-NBD-Cer for 60 min. at 4°C. To allow for synthesis of C6-NBD-GlcCer or C6-NBD-SM and their subsequent transport, incubation was carried out at 37°C for 60 min. in HBSS containing 5% BSA. The apical labelling of the bile canalicular structures was determined semi quantitatively by assessing the percentage of bile canaliculi that was NBD positive (Zegers and Hoekstra, 1997). The bile canaliculi, which are easily visualized under phase contrast by their microvillar appearance, were classified as NBD positive or negative under epifluorescence illumination. Cells were examined using an epifluorescence microscope (Provis AX70; Olympus, New Hyde Park, NY).

Metabolism of C6-NBD-Cer and basolateral delivery of de novo-synthesized sphingolipids.

Cells were plated in culture dishes and labelled with C6-NBD-Cer for 60 min. at 4°C. The cells were then incubated for 60 min. at 37°C in HBSS, containing 5% BSA. Subsequently they were washed and scraped from the culture dish. Lipids from the incubation medium and cells were extracted and quantified as described below.
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Lipid extraction and quantification.

Lipids from cells and back-exchange media were extracted according to the method of Bligh and Dyer (1959). NBD lipids were separated by TLC and quantified fluorometrically as described previously (Kok et al., 1991).

RESULTS

Extracellular acidosis induces a pH-, time- and temperature-dependent reversible fragmentation and dispersion of the Golgi apparatus

In order to examine the effect of extracellular acidosis on the organization of the Golgi complex, human hepatoma cells were incubated in DMEM with a pH of 7.0, 6.0 or 5.5 at 37°C for different time intervals. At each time point, cells were then fixed and (immuno) labelled with antibodies against the Golgi-resident protein giantin and the nuclear dye Hoechst 33528. As shown in Figure 1, a 60 min. exposure to extracellular acidosis (pH 5.5) induced severe fragmentation of the Golgi complex and dispersion of Golgi membranes throughout the entire cell. No fragmentation of the Golgi complex was observed at alkaline pH (i.e., above 7.4) (data not shown). When examined as a function of time, stretching of the Golgi apparatus (30 min.) appeared to precede fragmentation and dispersion (60 min.; Fig. 2). Extracellular acidosis-induced Golgi fragmentation and dispersion occurred in a temperature-dependent manner as little or no fragmentation was observed at 16°C (Fig. 3). Exposure to extracellular acidosis under the conditions of these experiments did not inhibit cell viability as evidenced by a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (data not shown).

In order to determine whether the extracellular acidosis-induced fragmentation and dispersion of the Golgi apparatus was a reversible process, cells were exposed to an acidic environment at 37°C for 60 min., followed by incubation at pH 7.4 for 0, 30 or 60 min. As shown in Figure 4, clustering of Golgi fragments was readily observed after a 30 min. recovery and no Golgi fragments were observed after a 60 min.
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Figure 1. Structure of the Golgi complex in polarized HepG2 cells cultured in different pH. Cells were incubated in DMEM pH 7.4, 6.0 and 5.5 for 60 minutes at 37°C, fixed and labelled with the Golgi marker Giantin; DNA was labelled with Hoechst. pH 5.5 induced severe fragmentation of the Golgi complex and dispersion of Golgi membranes throughout the entire cell.

Figure 2. Structure of the Golgi complex in polarized HepG2 cells during extracellular acidification as a function of time. Cells were incubated in DMEM pH 5.5 for 0, 30 and 60 minutes at 37°C, fixed and stained with the Golgi marker Giantin; DNA was labelled with Hoechst. Stretching of the Golgi apparatus (30 min.) appeared to precede full fragmentation and dispersion (60 min.).
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recovery. The recovery of the Golgi organization was much less efficient at 16°C, indicating temperature-dependency (Fig. 5). These data demonstrate that exposure of hepatoma cells to severe extracellular acidosis in a controllable fashion perturbs the structural organization of the Golgi apparatus.

**Extracellular acidosis does not inhibit Golgi biogenesis**

In order to investigate whether extracellular acidosis inhibited the process of Golgi biogenesis, cells were treated with the fungal metabolite brefeldin-A. Treatment with brefeldin A inhibits ADP-ribosylating factors that are associated with Golgi membranes and results in the merging of Golgi membranes with the endoplasmic reticulum (Fujiwara et al., 1988; Lippincott-Schwartz et al., 1989). Fully consistent with this, brefeldin A caused a complete disassembly of the Golgi apparatus in HepG2 cells (Fig. 6A). Brefeldin A was subsequently washed out and cells were allowed to recover in normal medium at 37°C for 60 min. at pH 7.4 (Fig. 6C) or pH 5.5 (Fig. 6B). The Golgi structure reappeared at pH 7.4 (Fig. 6C), while at pH 5.5 Golgi remained fragmented and dispersed, however a discrete giantin-labeled Golgi vesicles were seen (Fig. 6B). These data indicate that extracellular acidosis does not inhibit Golgi biogenesis but, rather, prevents the assembly of individual Golgi elements into a single larger complex.

**Extracellular acidosis does not perturb the overall organization of the microtubule network**

Because the organization of the Golgi requires an intact microtubule cytoskeleton, we next investigated the effects of extracellular acidosis on the appearance of the microtubule network. For this HepG2 cells were exposed to extracellular acidosis (Fig. 7B) or normal medium (Fig. 7A), fixed and double labeled with antibodies against tubulin and the Golgi resident protein giantin. Exposure to extracellular acidosis did not visibly alter the overall organization of the microtubule network, and
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Figure 3. Acid-induced Golgi dispersion is temperature dependent. The polarized HepG2 cells were incubated in DMEM pH 5.5 for 60 minutes at 4°C, 16°C and 37°C. The Golgi showed little or no fragmentation at temperatures below 16°C.

Figure 4. The recovery of the acid-dispersed Golgi structure in a function of time. Polarized HepG2 cells were incubated in pH 5.5 for 60 minutes at 37°C to induce the Golgi dispersion. The cells were next washed and incubated in pH 7.4 culture medium for 0, 30 and 60 min. at 37°C, fixed and labeled with Giantin and Hoechst. The recovery of the Golgi is readily observed after 30 minutes, while after 60 min. the normal Golgi structure was restored.
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the dispersed giantin-labeled Golgi fragments followed the overall pattern of the microtubule network (Fig. 7B).

**Protein transport through fragmented Golgi elements is maintained during extracellular acidosis**

We next used the extracellular acidosis-induced Golgi fragmentation as a tool to address the Golgi structure-function relationship with regard to apical trafficking and polarity. The Golgi plays a major role in the polarized trafficking of proteins and lipids. First, we determined whether newly synthesized proteins pass through the fragmented Golgi membranes during extracellular acidosis. Protein transit through the Golgi is rapid and can not be detected under steady state conditions at 37°C but can be followed upon lowering the temperature, which slows down trafficking in the Golgi. Therefore, cells were exposed to extracellular acidosis for 60 min. to induce Golgi fragmentation (see Fig. 1) and subsequently incubated at 16°C for 4 hours. Cells were then fixed and immunolabeled with antibodies against MRP2 or MDR1, two bile canalicular plasma membrane resident proteins. As shown in Figure 8, both MRP2 (A, B) and MDR1 (C, D) colocalized with Giantin-positive Golgi membranes of untreated (A, C) and extracellular acidosis-treated (B, D) cells. No colocalization of MRP2 or MDR1 with giantin was observed when cells were pre-treated with the protein synthesis inhibitor cycloheximide, and both MRP2 and MDR1 disappeared from giantin-positive Golgi membranes after release of the low temperature block (data not shown). These data indicate that fragmentation and dispersion of the Golgi complex during extracellular acidosis does not prevent passage of newly synthesized MRP2 and MDR1.

**Direct apical trafficking of MRP2 and MDR1 is maintained during extracellular acidosis**

In cells exposed to extracellular acidosis, MRP2 and MDR1 were readily observed at the apical bile canalicular plasma membrane domains of the cells, whereas no labeling
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Figure 5. The recovery of the acid-dispersed Golgi structure in a function of temperature. Polarized HepG2 cells were incubated in pH 5.5 for 60 min. at 37°C to induce the Golgi dispersion. The cells were next washed and incubated in pH 7.4 culture medium at 4°C, 16°C and 37°C for 60 min., fixed and labeled with Giantin and Hoechst. The recovery of the Golgi structure is less efficient at temperatures lower than 16°C.

Figure 6. Golgi complex biogenesis during acidosis in HepG2 cells. Polarized HepG2 cells were preincubated with 5µg/ml Brefeldin A at 37°C (A) for 60 min. to disassemble the Golgi structure. Cells were next washed and incubated in DMEM pH 7.4 (C) and 5.5 (B) at 37°C for 60 min. to allow the biogenesis of the Golgi. The Golgi structure was restored at pH 7.4 (C). At pH 5.5 the giantin-labelled vesicles reappeared, however remained dispersed.
of the basolateral surface was detected (Fig. 8). This suggests that extracellular acidosis-induced fragmentation and dispersion of the Golgi does not interfere with the polarized distribution of MRP2 and MDR1 to the apical domain. Newly synthesized MDR1 has been demonstrated to travel from the Golgi to the apical surface via a direct pathway, i.e., not involving prior delivery to the basolateral surface. To investigate whether this direct trafficking is maintained during extracellular acidosis, cells were exposed to an acidic environment, fixed and immunolabeled with an anti-MDR1 antibody (4E3) that recognizes an extracellular epitope on MDR1. While after permeabilization of the fixed cells with Tx100 MDR1 was clearly observed at the apical surface of the cells (Fig. 9A), omitting the permeabilization step resulted in a lack of antibody reactivity both in neutral and acidic conditions (Fig. 9B and C, respectively), which is indicative for the absence of a basolateral pool of MDR1, and suggests that MDR1 follows a direct pathway to the apical surface also during extracellular acidosis.

Figure 7. Structure of microtubules during acidosis in HepG2 cells. Polarized HepG2 cells were incubated in DMEM pH 7.4 (A) and 5.5 (B) at 37°C for 60 minutes to allow the Golgi dispersion. Cells were fixed and stained with antitubulin and giantin antibodies. The acid treatment caused Golgi stacks dispersion but did not alter the microtubule network.
Figure 8. Newly synthesized apical proteins reach apical membrane despite acid-dispersed Golgi structure. Polarized HepG2 cells were incubated at pH 7.4 and pH 5.5 at 37°C for 60 minutes to allow the Golgi dispersion and subsequently incubated in the same media at temperature 16°C for 4 hours to slow down the protein transit through Golgi. Cells were fixed and stained with anti-MRP2 (A, B) and anti-MDR1 (C, D) antibodies. Both MRP2 and MDR1 colocalized with Giantin-positive Golgi membranes of untreated (A, C) and extracellular acidosis-treated (B, D) cells.
Figure 9. Trafficking of apical proteins to apical membrane in acid-treated cells. Polarized HepG2 cells were incubated in DMEM pH 7.4 and 5.5 at 37°C C for 60 minutes, fixed and processed for immunofluorescence with (A) and without the permeabilization step (B, C) with an anti-MDR1 antibody (4E3) that recognizes the extracellular epitope of MDR1. The acid treatment did not affect the direct apical trafficking route of MDR1 protein.

(Glyco)sphingolipid metabolism and trafficking is not affected during extracellular acidosis

The sorting and polarized trafficking of several newly synthesized resident apical plasma membrane proteins involves their partitioning in (glyco)sphingolipid-enriched membrane microdomains or lipid rafts in Golgi membranes (Äit Slimane et al., 2003), and (some) sphingolipids, including raft-associated sphingomyelin, are synthesized in the Golgi complex.
To investigate whether extracellular acidosis interfered with the metabolism of ceramide to sphingomyelin and glucosylceramide (the precursor for all higher glycosphingolipids), cells were exposed to extracellular acidosis or to neutral medium and incubated with 4µM C₆-NBD-Cer for 30 min. at 4°C, washed, and again incubated at 37°C for another 60 min. in HBSS. This procedure results in the accumulation of the lipid analogue in the Golgi apparatus, followed by its conversion to C₆-NBD-SM and –GlcCer (Zegers et al., 1998). The de novo synthesis of C₆-NBD-SM and -GlcCer from C₆-NBD-Cer was not affected by acidification (Table 1). Thus, in both control and treated cells, ~55% of the C₆-NBD-Cer was converted to C₆-NBD-SM and -GlcCer, in a ratio of 3:1, respectively, similarly as reported previously (Zegers and Hoekstra, 1998).

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<tr>
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<th>pH 7.4</th>
<th>pH 5.5</th>
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<tr>
<td>Total SPL product</td>
<td>61 ± 1.7</td>
<td>52 ± 5.5</td>
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<tr>
<td>Total SM</td>
<td>74 ± 3.5</td>
<td>77 ± 2.9</td>
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<tr>
<td>Total GC</td>
<td>26 ± 3.5</td>
<td>24 ± 2.9</td>
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Table 1. C₆-NBD-Cer metabolism in HepG2 cells under neutral and acidic conditions. The percentage of C₆-NBD-Cer that was metabolized (total SPL product), and the fraction of that which is represented by C₆-NBD-SM or –GC (total SM and total GC) is depicted.

In order to determine the polarized trafficking of fluorescent sphingolipid metabolites to the apical and basolateral surface domains, 5% BSA (w/v) was added to the basolateral medium during the 37°C incubation step. BSA captures C₆-NBD-lipid that arrives at the basolateral surface, thereby preventing re-entry of the lipid analogues and subsequent apical delivery via transcytosis (van IJzendoorn and Hoekstra, 1997). As BSA does not have access to the apical lumens (van IJzendoorn and Hoekstra, 1997) it, therefore, allows for the study of direct Golgi-to-apical surface and Golgi-to basolateral surface trafficking of C₆-NBD-lipids. To determine Golgi-to-apical trafficking, cells were washed three times with ice-cold HBSS following the
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37°C incubation step and the percentage of NBD-labelled apical plasma membrane domains was determined by fluorescence microscopy as described in Materials and Methods. As shown in Figure 10A, in control cells and in acidosis-treated cells, ~52% of the BCs were positively labelled with NBD-lipids. In the absence of BSA in the basolateral medium, thereby allowing newly synthesized lipids to reach the apical plasma membrane via the basolateral surface and subsequent transcytosis, ~76% of the BCs were NBD-positive in both untreated and treated cells. Quantification of the percentage of newly synthesized C6-NBD-SM and -GlcCer that was delivered to the basolateral surface showed that in control cells ~25 and 30% of the newly synthesized C6-NBD-SM and -GlcCer, respectively, were transported to the basolateral surface. In acidosis-treated cells this percentage was ~18 and 27%, respectively (Fig. 10B). We conclude that sphingolipid metabolism and polarized sphingolipid trafficking is not significantly affected in cells that have been exposed to extracellular acidosis.

DISCUSSION

Microcirculatory disturbances such as ischemia or hypoxia can have deleterious effects with regard to the viability and function of hepatocytes and often result in oxidative stress and severe acidosis. Little is known with regard to the effect of acidosis on organelles that contribute to (apical) plasma membrane dynamics. Here, we demonstrated that the typical ribbon architecture and polarized pericentrosomal organization of the Golgi complex is lost in polarized human hepatoma HepG2 cells when exposed to severe extracellular acidosis. The extent of the phenotypic effect correlated with decreasing pH values, time and temperature, and was fully reversible (in a time- and temperature-dependent manner) upon shifting to neutral extracellular pH. Extracellular acidosis often rapidly causes intracellular acidification. Identical results were obtained when cytosol pH was reduced while maintaining normal extracellular pH, by using an NH₄Cl prepulse followed by washes and incubation in pH 7.4 buffer in which sodium was replaced by choline (thereby effectively inhibiting
Figure 10. Trafficking of C6-NBD-sphingolipids to the bile canaliculi (BC) in HepG2 cells. (A) Trafficking of newly synthesized and transcytosing C6-NBD-sphingolipids to BC. Control HepG2 and acid-treated HepG2 cells were labelled with 4µM C6-NBD-Cer for 30 min. at 4°C, washed, and incubated at 37°C for another 60 min. in the presence or absence of BSA, which captures any C6-NBD-lipid arriving at the basolateral surface. All cells were subsequently subjected to a BSA back exchange to remove basolaterally localized lipid analogue. The apical labelling of the bile canalicular structures was then determined semiquantitatively by assessing the percentage of bile canaliculi that was NBD positive. The bile canaliculi, which are easily visualized under phase-contrast by their microvillar appearance, were classified as NBD positive or negative under epifluorescence illumination. (B) Trafficking of newly synthesized C6-NBD-sphingolipids to BC. Control HepG2 and acid-treated HepG2 cells were labelled with 4µM C6-NBD-Cer for 30 min. at 4°C, washed, and incubated at 37°C for another 60 min. in the presence of 5% BSA. Lipids were extracted from the BSA fractions and the cells, and quantified as described in Materials and Methods. Hatched and coloured bars represent C6-NBD-sphingomyelin (S) and -GlcCer (G), respectively. The sphingolipid metabolism and polarized sphingolipid trafficking is not significantly altered in cells exposed to extracellular acidosis in comparison to control conditions.
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the activity of Na⁺/H⁺-exchanger proteins) (not shown) (Sandvig et al., 1987). Cell viability was not affected and no stimulation of apoptosis was noted (not shown). These data indicate that acidosis vividly perturbs the spatial organization and ribbon architecture of the Golgi complex in a regulated manner. Extracellular acidosis did not prevent the biogenesis of Golgi membranes from the endoplasmic reticulum in a brefeldin A-washout experiment, indicating that extracellular acidosis perturbed the organization of Golgi membranes after their formation. Many organelles, including the Golgi complex, require microtubules and the microtubule-based and minus end-directed motor protein dynein to cluster near the centrosome (Teuling et al., 2008). Moreover, the merging of isolated Golgi stacks to form an interconnected ribbon structure requires microtubule-associated CLASP proteins. Microtubules and microtubule motor proteins therefore control both the pericentrosomal localization and the ribbon architecture of the Golgi complex. No effects on the overall organization of the microtubule network were observed in acidosis-treated cells and Golgi markers, although dispersed, followed the overall pattern of the microtubule network. Of interest, the Golgi phenotype in acidosis-treated cells is very similar to that observed in cells that overexpress dynamitin (p50), a subunit of dynactin which, in turn, is a multisubunit complex that plays an accessory role in dynein function (Burkhardt et al., 1997; Quintyne et al., 1999; our unpublished data). Moreover, under low pH conditions (recombinant) dynein light chain LC8 (DYNLL1) is protonated at His55 in vitro, thereby converting LC8 from a dimer into a folded monomer and affecting its function (Nyarko et al., 2005; Mohan et al., 2006). LC8 associates with the Golgi complex (Varma et al., 2010). The dimer-monomer ratio of LC8 is also regulated by phosphorylation, and a phosphomimetic mutation at Ser88 promotes dissociation of LC8 to a monomer and subsequent dissociation from the dynein complex in vitro (Hall et al., 2008). The phosphomimetic Ser88 mutation was demonstrated to open an entrance into the dimer interface for water molecules, which disturb the hydrogen bond network around His55 and is expected to raise the protonation ratio of His55 (Xiao et al., 2010). It can therefore be hypothesized that
extracellular acidosis, through protonation and monomerization of LC8 inhibits the motor function of dynein and, consequently, the spatial distribution and ribbon architecture of the Golgi complex. Experiments to determine whether acidosis-treated cells display an increased ratio of LC8 monomers, and to determine the effects of dimerization-incompetent LC8-Ser88 mutants on the spatial distribution and ribbon architecture of the Golgi complex are ongoing in our laboratory. These experiments are expected to help elucidate the molecular mechanism by which acidosis interferes with the spatial organization and ribbon-structure of the Golgi complex.

The pronounced effects of acidosis on the spatial distribution and ribbon architecture of the Golgi complex allowed us to correlate these parameters with Golgi functions related to apical plasma membrane trafficking and polarity. Indeed, in polarized cells the Golgi ribbon and centrosome are typically positioned near the apical plasma membrane domain. Furthermore, the Golgi ribbon and/or polarized pericentrosomal distribution of the Golgi complex is believed to ensure the targeted delivery of membranes to selected sites at the cell surface (reviewed in Sütterlin and Colanzi, 2010). Under the conditions and within the time frame of the experiments described in this study, we found no evidence for loss of apical plasma membrane polarity, missorting of apical plasma membrane proteins to the basolateral domain, or the metabolism of sphingolipids that are believed to play a role in the sorting and trafficking of newly synthesized apical plasma membrane proteins. These data suggest that the assembly of a Golgi ribbon and the apical positioning of the Golgi complex is dispensable for polarized apical trafficking and polarity at steady state, at least in hepatoma HepG2 cells. It should be noted, however, that a prolonged exposure of HepG2 cells to severe extracellular acidosis did result in apical plasma membrane defects (Golachowska et al., chapter 4 in this thesis) but, given the disparity in onset, it is unclear whether this is a direct consequence of the observed Golgi alterations. Possibly, a structure-function relationship becomes more obvious in cells displaying a higher degree of plasticity with regard to plasma membrane polarity, such as during directionally migration, during the epithelial polarization process, or in neuronal cells.
which must continuously remodel their synapse membranes (Sütterlin and Colanzi, 2010), and we expect that in such cells acidosis is more likely to provoke deleterious effects.

Golgi fragmentation is a common and early feature in multiple aging-related neurodegenerative diseases (Fan et al., 2008) such as amyotrophic lateral sclerosis (ALS), corticobasal degeneration, Alzheimer's disease, spinocerebelar ataxia type 2 (SCA2) and Parkinson's disease (Gonatas et al., 2006; Lee et al., 2006; Fujita et al., 2006). The precise mechanism(s) that cause(s) Golgi complex fragmentation in these neurodegenerative diseases remain(s) unknown (Nakagomi et al., 2008). Loss of dynein function has been correlated with the induction of neurodegenerative diseases such as ALS (Teuling et al., 2008), and the interaction between dynein-dynactin complexes was reported to be attenuated in aged monkey brains, suggesting that dynein dysfunction exists in aged brains (Kimura et al., 2007; Kimura et al., 2009). Intriguingly, neurodegenerative disease, as well as general ageing, has been associated with an accumulation of metabolic (mitochondrial) damage and increased production of lactic acid, a natural by-product of glycolysis, and acidosis has been suggested to play a role in the development of (some) neurodegenerative diseases (Pirchl et al., 2006). Particularly interesting is that mitochondrial fragmentation, which leads to intracellular acidification (Johnson and Nehrke, 2010), is an early and causal event in neurodegeneration (Knott et al., 2008). It can be speculated that acidosis, as a result of mitochondrial dysfunction and accumulation of lactic acid, (possibly via effects on dynein (see above)) perturbs neuronal Golgi organization and position and thereon dependent (polarized) functions of axons and presynaptic terminals (Fan et al., 2008) or other functions of the Golgi complex. Future studies are needed to address these possibilities.