FORUM REBOUND ORIGINAL RESEARCH COMMUNICATION

Abnormal Golgi pH Homeostasis in Cancer Cells Impairs Apical Targeting of Carcinoembryonic Antigen by Inhibiting Its Glycosyl-Phosphatidylinositol Anchor-Mediated Association with Lipid Rafts

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Abstract

Aims: Carcinoembryonic antigen (CEACAM5, CEA) is a known tumor marker for colorectal cancer that localizes in a polarized manner to the apical surface in normal colon epithelial cells whereas in cancer cells it is present at both the apical and basolateral surfaces of the cells. Since the Golgi apparatus sorts and transports most proteins to these cell surface domains, we set out here to investigate whether any of the factors commonly associated with tumorigenesis, including hypoxia, generation of reactive oxygen species (ROS), altered redox homeostasis, or an altered Golgi pH, are responsible for mistargeting of CEA to the basolateral surface in cancer cells.

Results: Using polarized nontumorigenic Madin-Darby canine kidney (MDCK) cells and CaCo-2 colorectal cancer cells as targets, we show that apical delivery of CEA is not affected by hypoxia, ROS, nor changes in the Golgi redox state. Instead, we find that an elevated Golgi pH induces basolateral targeting of CEA and increases its TX-100 solubility, indicating impaired association of CEA with lipid rafts. Moreover, disruption of lipid rafts by methyl- β -cyclodextrin induced accumulation of the CEA protein at the basolateral surface in MDCK cells. Experiments with the glycosylphosphatidylinositol (GPI)-anchorless CEA mutant and CEA-specific GPI-anchored enhanced green fluorescent protein (EGFP-GPI) fusion protein revealed that the GPI-anchor was critical for the pH-dependent apical delivery of the CEA in MDCK cells.

Innovation and Conclusion: The findings indicate that an abnormal Golgi pH homeostasis in cancer cells is an important factor that causes mistargeting of CEA to the basolateral surface of cancer cells *via* inhibiting its GPI-anchor-mediated association with lipid rafts. *Antioxid. Redox Signal.* 30, 5–21.

Keywords: cancer, protein trafficking, lipid rafts, apical targeting, Golgi pH, hypoxia, carcinoembryonic antigen

Introduction

THE PLASMA MEMBRANE OF ALL polarized epithelial cells is divided into distinct apical and basolateral surfaces that are characterized by having a distinct set of proteins and lipids, and thus, also different functions. To maintain such functional polarity, cells have evolved various ways to de-

liver newly synthesized secretory and membrane proteins into these two distinct membrane domains with high accuracy (17). The sorting machinery itself resides mainly in the trans-Golgi compartment and utilizes various recognition motifs, such as N- and/or O-linked glycans (6, 54, 58) or the glycosylphosphatidylinositol (GPI)-anchor added post-translationally to various proteins on cleavage of their

Sponsoring Peers: Marc Fransen, Mary E. Choi, Kristian Prydz, and Michael Caplan (see Review Comments in shaded box).

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Innovation

Loss of cell polarity is a common phenomenon during carcinogenesis. Usually, it is maintained by polarized transport of proteins from the Golgi to the cell surface. Our findings indicate that neither hypoxia, the redox state, nor reactive oxygen species, but rather, a sufficiently acidic pH of the Golgi lumen is needed for faithful targeting of carcinoembryonic antigen (CEA) to the apical surface of epithelial cells. These findings together with the observation that the Golgi luminal pH is abnormal in many cancer cell types help to understand why CEA, and perhaps other apically targeted glycosylphosphatidylinositol-anchored membrane proteins are mislocalized in cancer cells.

carboxyl-terminal transmembrane domains (36, 37). GPI-anchored proteins typically associate with sphingolipid- and cholesterol-rich lipid rafts (8, 35, 74). Such lipid raft association is believed to be crucial for the apical targeting of some, but not all, GPI-anchored proteins.

Carcinoembryonic antigen (CEACAM5, here referred to as CEA) is a widely used follow-up marker for colorectal cancer (21). CEA appears to have multiple functions, including a role in immunological defense, cell signaling, and cell adhesion (24). Moreover, when overexpressed, it was shown to inhibit apoptosis, anoikis, cell polarity, and differentiation (16, 29, 50, 75, 81), and to promote tumorigenesis in nude mice (69). Importantly, in normal epithelial cells, CEA is localized almost exclusively at the apical plasma membrane; whereas in cancer cells and tissues, it shows, apart from an increased expression (20, 23, 24, 30), a nonpolarized distribution, that is, it is present at both the apical and basolateral surfaces (1, 4, 39, 47).

Currently, it is unclear why CEA shows this nonpolarized distribution and is mistargeted to the basolateral surface in cancer cells. Previously, it was shown that factors such as hypoxia, generation of reactive oxygen species (ROS), and an altered redox potential are all hallmarks of tumorigenesis (70–72). Thus, they may impair Golgi homeostasis, thereby affecting apical delivery of CEA. In support of this, it was shown that hypoxia impairs Golgi-mediated glycosylation events (5, 18, 32, 33, 61, 73, 82, 84). An altered Golgi pH homeostasis is another factor that may affect apical delivery of CEA, as it has been previously shown to impair correct glycosylation, sorting, and transport of secretory and membrane proteins (9, 27, 55, 62, 63). Moreover, Golgi luminal pH, which is usually slightly acidic (pH 6.5–6.0 along the cis-trans axis), is in many cancer cell types abnormally high (2, 63, 76), indicating that organelle acidification is defective in cancer cells. In this article, we investigated whether and how hypoxia, ROS, the Golgi redox state, or an altered Golgi pH homeostasis contribute to the observed mistargeting of CEA in cancer cells.

Results

Normal and cancer cells differentially target CEA to the apical and basolateral surface

First, we verified the apical and/or basolateral expression of CEA in normal epithelia *in vivo* by staining normal and colorectal cancer tissue sections with the anti-CEA antibody

Rebound Track

This work was rejected during standard peer review and rescued by Rebound Peer Review (*Antioxid Redox Signal* 16: 293–296, 2012) with the following serving as open reviewers: Marc Fransen, Mary E. Choi, Kristian Prydz, and Michael Caplan.

Marc Fransen (marc.fransen@kuleuven.be): I am a qualified reviewer (per *Antioxid Redox Signal* 16: 293–296, 2012) and move to rescue this article that was rejected during the regular peer review process after reviewing all versions of the article and detailed reviewer comments. The manuscript authored by Kokkonen and coworkers is an interesting study aiming at understanding the molecular mechanisms underlying the mistargeting of carcinoembryonic antigen (CEA), a glycosylphosphatidylinositol (GPI)-anchored protein, to the basolateral surface in cancer cells. Given that hypoxia, altered redox state, and altered Golgi pH homeostasis are all hallmarks of tumorigenesis, the authors focused on these parameters. First, they established and validated a new experimental setup. Next, by employing various microscopic, cell biological, and biochemical approaches, they identified disturbances in Golgi luminal pH, but not hypoxia or Golgi redox state, as the causative factor for altered CEA localization. In addition, the authors also demonstrated that elevated Golgi pH impairs the association of CEA with membrane rafts, and that mistargeting of CEA to the basolateral membrane is not due to immature N-glycosylation, a pH-sensitive event. Together, these novel findings provide a molecular explanation for CEA mislocalization in cancer cells. Whether or not disturbances in Golgi pH also affect the targeting of other endogenous GPI-anchored membrane proteins remains to be established. However, given that CEA is an extensively studied molecule with proven functions in multiple cancer types, the manuscript is of broad interest to researchers in the field. As, in my opinion, (i) the authors have properly addressed all genuine comments and criticisms raised by the previous reviewers, and (ii) the key findings reported are novel, relevant, and sound, I fully support acceptance with the provision that the authors make the suggested “editorial” changes. Therefore, in the interest of science, I take full responsibility to rescue this work from rejection.

Mary E. Choi (mec2025@med.cornell.edu): I am a qualified reviewer (per *Antioxid Redox Signal* 16: 293–296, 2012) and move to rescue this article that was rejected during the regular peer review process after reviewing all versions of the article and detailed reviewer comments. The manuscript by Kokkonen *et al.* is an interesting study that examines the mechanism of the loss of polarity in colorectal cancer cells involving impaired apical targeting of CEA, known as a tumor marker for colorectal cancer with well-established functions in multiple cancer cell types. Loss of epithelial cell polarity is a key feature implicated in tumorigenesis, cancer cell migration, and metastasis and as such, the studies herein are of high significance to the advancement of our understanding of cancer biology. CEA is a GPI-anchored glyco-protein, usually expressed on the apical surface of epithelial cell membranes, that is mistargeted to the basolateral surface

(COL-1). As expected, the CEA protein localized exclusively at the apical surface in normal noncancerous acinar epithelial cells (Fig. 1A), that is, the plasma membrane facing the acinar lumen. By contrast, in cancer tissue specimens, CEA was detected at both the apical and basolateral cell surfaces (Fig. 1A). The insert in Figure 1A (right) shows staining of both the basolateral and apical membrane domains of the columnar epithelial cells.

We next assessed whether this discrepant localization of CEA is mimicked in cultured polarized noncancerous Madin-Darby canine kidney (MDCK) cells expressing CEA, and in intact human CaCo-2 colorectal cancer cells. By using confocal microscopy and Z-stack imaging, we found that CEA localizes almost exclusively at the apical surface in noncancerous polarized MDCK cells, that is, above the tight junction marker protein ZO-1 (Fig. 1B). In CaCo-2 colorectal cancer cells, however, CEA is localized at both the apical and basolateral surfaces of the cells (Fig. 1C, arrows). Quantification by immunoblotting (Fig. 1D, E) revealed that 91% of CEA released from the cell surface by using phosphatidylinositol specific phospholipase C (PI-PLC) was present at the apical surface in polarized MDCK cells, whereas roughly equal amounts (49% vs. 51%) of CEA in CaCo-2 cells were recovered from the apical and basolateral surfaces, respectively.

To verify that our experimental setup can also detect basolateral CEA in MDCK cells if/when present, we treated MDCK cells with 10 mM brefeldin A (BFA) for 24 h. This drug selectively blocks apical, but only slightly, if at all, affects basolateral transport (40, 42). We found that most (89%) of the CEA protein in BFA-treated MDCK cells was recovered from the basolateral surface (Fig. 1D, E), that is, somewhat higher than observed for most other proteins (40). The exact reason for this is unclear but may be specific for GPI-linked proteins and involve, in addition to inhibition of total secretion, a delay in ER exit, as shown for the proteoglycan serglycin (78), or the fact that GPI-linked protein can reach the apical membrane also *via* transcytosis (3, 57). Note also that there is a shift from higher to lower molecular weight CEA species between nontreated and BFA-treated MDCK cells (210 kDa vs. 180 kDa). Given that CEA is a heavily N-glycosylated protein (49, 80), it is likely that this size decrease is either due to the impaired processing of core (high mannose) N-glycans to mature complex type N-glycans in the Golgi (see further details below) or that CEA by-passes the Golgi *via* alternate trafficking pathways in BFA-treated cells (59).

Nevertheless, these data indicate that CEA localizes identically both *in vivo* and in cultured cells, thereby validating the suitability of our experimental setup for dissecting why CEA is mistargeted to the basolateral surface in cancer cells.

Apical targeting of CEA is Golgi pH dependent

An impaired pH homeostasis due to enhanced glycolysis (the sc. Warburg effect) is frequently associated with malignant tumors, in both hypoxic and normoxic cancer cells (76). We and others have also previously shown that in many cancer cells the luminal pH of acidic organelles is abnormally high compared with noncancerous cells (2, 63, 76). To verify whether this pH difference also exists in the Golgi lumen between noncancerous MDCK cells and CaCo-2 colorectal cancer cells, we determined the pH of the Golgi lumen (pH-

of cancer cells. The Golgi apparatus is responsible for the apical and basolateral sorting of proteins. Using biochemical and biological approaches, the authors provide solid evidence that the sorting mechanism of CEA depends on alterations in Golgi pH, but not changes in the Golgi redox state or hypoxia. The studies elucidate several key findings that are innovative: (i) elevated Golgi luminal pH induces basolateral targeting of CEA, (ii) this occurs *via* inhibition of its GPI anchor-mediated association with lipid rafts, (iii) CEA associates with lipid rafts in a Golgi pH-dependent manner but oligomerization of CEA is not affected by the Golgi luminal pH, and (iv) immature N-glycans are not responsible for the increased basolateral delivery of CEA. Given that the Golgi luminal pH is abnormal in various cancer cell types, the findings in this article delineate a mechanism for CEA mislocalization in cancer cells that may be potentially applicable more broadly to how other apically targeted GPI-anchored membrane proteins are mislocalized in cancer cells and the loss of epithelial cell polarity contributing to tumorigenesis. Therefore, I fully support acceptance, and in the interest of science, I take full responsibility to rescue this work from rejection.

Kristian Prydz (kristian.prydz@ibv.uio.no): I am a qualified reviewer (per *Antioxid Redox Signal* 16: 293–296, 2012) and move to rescue this article that was rejected during the regular peer review process after reviewing all versions of the article and detailed reviewer comments. The work by Kokkonen *et al.* is experimentally complete, addressing adequately how different mechanisms previously reported to influence protein sorting in the secretory pathway of normal epithelial cells contribute to sorting of the GPI-linked protein CEA. They also address how this sorting could be disturbed in cancer cells by addressing changes reported to occur in the Golgi apparatus of cancer cells. The studies point to the increase in pH observed in the lumen of the Golgi apparatus in cancer cells as the main factor. Also, the engineered protein, where EGFP was fused to the ceaGPI anchor, was affected. The glycans attached to the protein did not seem to contribute to the apical sorting, because inhibition of their synthesis does not disturb apical transport. The pH effect did not influence the oligomerization state of the protein, thus the multimeric state of the protein was not a part of the pH effect. The effect of the pH increase in the Golgi lumen seemed to work by reducing the association of the GPI-linked protein, as shown by reduced association with detergent insoluble membrane extracts. The findings were also supported by co-localization with cholera toxin protein chains. In addition, neither hypoxia nor changes of redox potential could affect CEA sorting. A few minor comments have been submitted to the authors. Based on what has been mentioned earlier, I fully support acceptance with the provision that the authors make the suggested “editorial” changes. Therefore, in the interest of science, I take full responsibility to rescue this work from rejection.

Michael Caplan (michael.caplan@yale.edu): I am a qualified reviewer (per *Antioxid Redox Signal* 16: 293–296, 2012) and move to rescue this article that was rejected during the regular peer review process after reviewing all

FIG. 1. Localization of CEA in normal and cancer tissues as well as in cultured cells. (A) Colon tissue specimens cut longitudinally into 5- μ m sections were processed for immunostaining with the monoclonal anti-CEA antibody (COL-1) followed by peroxidase conjugated anti-mouse secondary antibody and DAB staining. Both normal (left) and colorectal carcinoma sections (right) were obtained from the same patient. Note the almost exclusive apical localization (brown color) of CEA in normal colon and apical/basolateral localization (arrows) of CEA in the cancer tissue section. (B, C) Z-axis projections from a stack of images taken from fully polarized MDCK cells and CaCo-2 cells, which were stained with anti-CEA and anti-ZO-1 (tight junction marker) antibodies. They confirm the almost exclusive apical localization of CEA in MDCK cells, whereas CaCo-2 cells grown on normal cell culture plates express CEA on both the apical and basolateral surface. (D, E) Western blotting and quantification of the CEA protein released from both apical and basolateral surfaces of MDCK/CEA cells using PI-PLC (see also original immunoblot files in supplementary material). Ten millimolars of BFA were added to the cells 24 h before the addition of PI-PLC. The samples were processed for Western blotting, as described in the "Materials and Methods" section. The band intensities were quantified by using the ImageJ software (mean $\pm$ SD, $n=3$). AP, apical; BFA, brefeldin A; BL basolateral; CEA, carcinoembryonic antigen; COL-1, anti-CEA antibody; DAB, 3,3'-diaminobenzidine; MDCK, Madin-Darby canine kidney; PI-PLC, phosphatidylinositol specific phospholipase C. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

versions of the article and detailed reviewer comments. The manuscript by Kokkonen *et al.* elucidates mechanisms responsible for the sorting of the GPI-linked protein CEA in normal *versus* tumor epithelial cells. The authors demonstrate that, as expected, CEA is apical in normal colonic epithelia, whereas it is apical and basolateral in colon cancer cells. Since the Golgi constitutes the major site of sorting, the authors propose that differences in the Golgi luminal environment might account for these differences. They examine the behavior of CEA in Madin-Darby canine kidney (MDCK) and CaCo-2 cells. Although CEA is apical in MDCK cells, it is apical and basolateral in CaCo-2 cells. Through a series of exhaustive and high-quality experiments, they demonstrate that this is not attributable to differences in the Golgi redox environment, nor is it due to differences in CEA glycosylation. They provide quantitative evidence that the Golgi lumen pH is more acidic in MDCK than in CaCo-2 cells. Agents that alkalinize Golgi pH cause a partial randomization of CEA distribution in MDCK cells. They show that lipid rafts are critical factors in apical sorting of the GPI-linked CEA protein, and that Golgi alkalinization perturbs CEA raft association. These results constitute an important advance in our understanding of the cell biological properties of CEA. Further, they provide novel, well-documented, and extremely interesting insights into the mechanisms through which the Golgi environment influences its sorting functions. Consequently, this manuscript is of broad scientific interest and significance. Therefore, in the interest of science, I take full responsibility to rescue this work from rejection and I recommend that it be accepted in its current form.

G) by using a ratiometric and Golgi-localized pH-sensitive probe pHluorin (44). We found (Fig. 2A) that the Golgi pH (pH-G) was on average 0.5 pH units lower in MDCK cells than that in CaCo-2 cells (pH 6.2 ± 0.16 vs. 6.71 ± 0.28 ; mean $\pm$ SD).

To test whether this Golgi pH difference contributes to the observed basolateral localization of CEA in CaCo-2 cells, we next utilized known pH gradient-dissipating reagents such as concanamycin A (Con A, an inhibitor of the vacuolar V-ATPase) and ammonium chloride (NH_4Cl , a weak base). Treatment of MDCK cells for 24 h with these substances increased the pH-G by 0.8 (Con A) and 0.2 (NH_4Cl) pH units (pH-G 7.02 ± 0.06 and 6.41 ± 0.10) compared with untreated cells, respectively (Fig. 2A). A similar increase in the pH-G was detected with both substances after a 4-h treatment (data not shown). Subsequent analyses by immunofluorescence microscopy of cells treated with the two reagents revealed that they both also increased the amount of the CEA protein at the basolateral membrane of MDCK cells, that is, at the site of cell-cell "contacts" (Fig. 2B, arrows). No such basolateral accumulation of CEA was detected in untreated control cells. Z-axis projections extracted from the whole set of image stacks further confirmed the presence of CEA at both the apical and basolateral surfaces in Con A-treated MDCK cells, but not in control cells, in which CEA was localized only on top of the ZO-1 tight junction marker (Fig. 2C, Con A). In addition, Z-stack images taken from CEA-expressing individual cells treated with Con A also showed the presence of CEA at the basolateral

FIG. 2. Effect of Golgi pH on the apical targeting of CEA. (A) Golgi pH measurements in MDCK and CaCo-2 cells with the ratiometric pHluorin probe. Cells were grown on optical cell culture plates (Ibidi™, diameter 30 mm) and stably transfected with the ratiometric Golgi-localized pHluorin plasmid (see the Material and Methods section). The graph shows the pH values (calculated from the intensity ratios) for each cell measured (3000 cells/cell line or treatment). Note that in CaCo-2 cells, the Golgi pH is on average 0.5 U higher than that of MDCK cells. Treatment of MDCK cells with pH gradient-dissipating drugs, NH₄Cl and Con A increased Golgi pH by 0.2 and 0.8 pH units, respectively. (B) MDCK cells were untreated and treated, with NH₄Cl (10 mM) or Con A (50 ng/mL) for 20 h. Then, cells were fixed before staining with the anti-CEA and ZO-1-specific antibodies before imaging with a confocal microscope. Note that in the treated cells, CEA is concentrated in the areas of cell-cell contacts (top, white arrows), indicating the accumulation of CEA to the basolateral surface. No such accumulation is detected in untreated cells. CEA (red color), ZO-1 (green color). Scale bar 20 μm. (C) Z-axis side projection showing the CEA (red) either on top of the tight junction marker ZO-1 in untreated MDCK cells (control) or on both sides of the tight junction marker in Con A-treated cells (Con A). (D) Serial Z-stack images of a single cell treated with Con A. The figure shows the localization of CEA protein (red) in areas devoid of the ZO-1 protein (green) and also in areas where the ZO-1 is present (apical surface). Note also the apparent lack of intracellular CEA protein (*) in the sections. (E) Quantification of apical and basolateral CEA in MDCK cells grown and treated or not with NH₄Cl and Con A for 16 h. CEA protein was released from both the apical and basolateral membranes with the help of PI-PLC. After precipitation, the fractions were subjected to SDS-PAGE and Western blotting with the anti-CEA antibody. Quantification of the bands was performed as described earlier. The results are presented as percentages of the total amount (apical plus basolateral) of the CEA in the cells. Con A, concanamycin A; NH₄Cl, ammonium chloride; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

surface (Fig. 2D), that is, in areas devoid of the tight junction marker protein (ZO-1). We also did not observe marked intracellular accumulation of CEA in Con A-treated MDCK cells examined ($n = 5$). Finally, quantification of the CEA protein at both surfaces by immunoblotting revealed that NH₄Cl and Con A increased basolateral CEA by two- and three-fold (NH₄Cl

from 9% to 18%; Con A from 9% to 26%), respectively (Fig. 2E). Thus, in Con A-treated cells, roughly one third of the apically localized CEA protein was redirected to the basolateral surface. These calculations assume that the one-to-one ratio in the distribution of the protein between the two membrane surfaces represents its nonpolarized delivery to the cell surface.

Hypoxia, generation of ROS, and an altered redox potential are other factors commonly associated with the cancer cell phenotype. Given that the Golgi lumen has an unexpectedly high oxidative potential (65), and that all these parameters are closely connected and may thus affect Golgi pH, we next cross-tested their effects on each of these parameters in MDCK cells. To accomplish this, we grew cells under moderate hypoxia (20 h at 5% O₂), or we treated cells separately with a known antioxidant (100 μ M ascorbic acid), a reductant (5 mM dithiothreitol [DTT]), or an oxidant (100 μ M H₂O₂) as well as with the pH gradient-dissipating compound Con A (50 ng/mL). First, Golgi redox state measurements using a ratiometric, pH-insensitive Golgi-specific roGFP2 as a probe together with automated high-content imaging analyses showed that the redox potential (depicted as roGFP2

intensity ratio) was decreased by ascorbic acid, DTT, and moderate hypoxia (16 h at 5% O₂); whereas it was increased by H₂O₂, as expected (Fig. 3A). Intact CaCo-2 cancer cells also had a slightly higher Golgi redox potential than untreated MDCK cells, despite their similar Golgi redox buffering capacity (tested with increasing amounts of DTT (5–50 mM), data not shown). Second, we measured cellular ROS levels in untreated and treated MDCK cells by using the CellROX red assay kit. As expected, we found that hypoxia, DTT, and ascorbic acid moderately decreased cellular ROS levels (Fig. 3B), whereas Con A and H₂O₂ increased them either slightly or markedly, respectively. The ROS levels were also higher in intact CaCo-2 cells than in untreated MDCK cells. Third, Golgi pH measurements in untreated and treated MDCK cells (Fig. 3C) revealed that neither hypoxia nor

FIG. 3. Effect of hypoxia, ROS, and the Golgi redox state on the apical targeting of CEA. Redox state (A), ROS level (B), and Golgi pH (C) measurements in MDCK cells treated with the depicted substances/conditions, and in intact CaCo-2 cells. Cells were grown and transiently transfected with the required plasmid encoding either Golgi-localized roGFP2 or the pHluorin fluorescent variants. Cellular ROS levels were determined by using the CellROX Deep Red kit. All measurements were carried out in triplicate (mean $\pm$ SD) on 96-well plates by using a high-content imaging system (5000–15,000 cells/measurement). The statistical significance (marked as *) between the control and test group denotes T-test *p*-values as follows: ****p* < 0.001, ***p* < 0.005, **p* < 0.05, ^{NS}*p* > 0.05. (D) Localization of CEA in polarized MDCK cells under different experimental conditions. The figures show piled confocal z-stack images and their side projections in MDCK cells treated with the depicted substances. Note that CEA is localized almost exclusively at the apical surface of the cells except when the cells were treated with either Con A or H₂O₂. These two substances were also the only ones that increased the pH of the Golgi lumen (Fig. 3C). ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

ascorbic acid perturbed the Golgi luminal pH, whereas Con A (as expected, see Fig. 2A) and, quite unexpectedly, also H_2O_2 increased the pH-G by about 0.8 and 1.2 pH units, respectively. The Golgi luminal pH in intact CaCo-2 cells (Fig. 3C) was also ~ 0.5 pH units higher than in untreated MDCK cells (combined data from Fig. 2A).

Next, we treated cells with hypoxia, ascorbic acid, N-acetylcysteine (another antioxidant), DTT, and H_2O_2 , and we followed the appearance of CEA in the basolateral surface of polarized MDCK cells. Confocal microscopy and Z-stack imaging revealed that neither hypoxia, ascorbic acid, N-acetylcysteine, nor DTT (a reductant) affected the apical localization of CEA (Fig. 3D). Based on the z-axis projections, in all these cases, CEA localized almost exclusively at the apical surface of MDCK cells. However, H_2O_2 redirected a proportion of CEA to the basolateral surface of MDCK cells.

Taken together, these data show that only two of these treatments, Con A and hydrogen peroxide, were able to reroute CEA to the basolateral surface of MDCK cells. The fact that they both also increased the Golgi luminal pH suggests that the Golgi luminal pH is the common denominator for the increased targeting of CEA to the basolateral surface in MDCK cells. In further support of this, Con A did not alter the ROS levels or the Golgi redox state in the treated cells while H_2O_2 did.

An elevated Golgi pH does not impair apical targeting of endogenous transmembrane proteins in MDCK cells

CEA possesses a C-terminal GPI anchor, which is considered important for its localization. However, other proteins also showing a polarized distribution do not contain a

GPI anchor. To understand whether the elevated Golgi pH also affects the distribution of proteins without a GPI anchor, we examined whether Con A impairs apical targeting of the two known GPI-anchorless apical membrane proteins gp114 (19) or gp135 (43) in MDCK cells. Both before and after Con A treatment, the two proteins were found almost exclusively at the apical surface of MDCK cells (Fig. 4A). Western blotting data supported this and revealed that more than 95% of both proteins were present on the apical surface in cells treated or not with Con A (Fig. 4B). These data indicate that apical targeting of these two proteins is pH independent, and that the elevated Golgi pH specifically impairs targeting of GPI-anchored membrane proteins such as CEA.

Immature N-glycans are not responsible for the increased basolateral delivery of CEA

N-glycans are known to play an important role in apical targeting of some membrane proteins (6, 11, 28, 54). Since CEA has 28 potential N-glycosylation sites (49, 80), which are structurally altered in cancers or in cells with elevated Golgi luminal pH (62, 63), we investigated next whether altered N-glycans are responsible for the accumulation of CEA in the basolateral surface of MDCK cells. To accomplish this, we treated cells for 16 h with a set of N-glycosylation inhibitors, and we followed whether they increase basolateral targeting of CEA in polarized MDCK cells. Quite surprisingly (Fig. 5A), none of the inhibitors used (swainsonine [SW], a Golgi α -mannosidase II inhibitor; benzyl-N-acetyl- α -galactosaminide [BGN], an inhibitor of galactosyltransferase and sialyltransferase; deoxymannojirimycin [DMJ], an ER/Golgi α -mannosidase I inhibitor; castanospermine [CSP],

FIG. 4. Localization of known endogenous apical membrane proteins (gp114 and gp135) in ConA-treated MDCK cells. (A) Indirect immunofluorescence staining of the proteins in Con A-treated MDCK cells with the anti-gp114 or anti-gp135 antibodies. The cells were co-stained with the anti ZO-1 antibody (green) followed by anti-mouse Alexa Fluor-488 or anti-rabbit Alexa Fluor-594 conjugated secondary antibodies. The figure shows the absence of these apical membrane proteins at the site of cell-cell contacts. Scale bar 20 μ m. (B) Quantification of the apical and basolateral gp135 and gp114 in MDCK cells treated or not with Con A. The cells were biotinylated, after which the proteins were solubilized and collected by using NeutrAvidin beads before analysis by SDS-PAGE and immunoblotting by using protein-specific antibodies. Each column represents an average ($\pm$ SD) of three independent experiments. Note that Con A does not increase basolateral targeting of the protein in either case. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 5. Elevated Golgi pH impairs association of CEA with lipid rafts.

(A) The role of mature N-glycans in apical targeting of CEA. Cells were grown as described earlier but in the presence of the various glycosidase inhibitors, after which CEA was released and collected from both the apical and basolateral surfaces by using PI-PLC. The amount of the CEA protein in both apical and basolateral surfaces was determined by immunoblotting. (B) Determination of TX-100 solubility of CEA in MDCK cells treated (24h) or not with NH₄Cl or Con A. TX-100-soluble (s) and insoluble (p) fractions were quantified after immunoblotting. (C) Sensitivity of the apical and basolateral CEA to TX-100 extraction. MDCK cells stably expressing CEA treated with Con A were extracted with cold TX-100 before fixation and staining with the COL-1 antibody (for a control, see also Fig. 3B top left). The figure shows a marked decrease in the amount of basolateral CEA after TX-100 extraction, whereas the apical CEA is still present. (D) TX-100 solubility of endogenous CEA in intact CaCo-2 cells. Cells were extracted by using cold TX-100 and processed thereafter for confocal microscopy as described earlier. Note the marked loss of basolateral CEA (green) after TX-100 extraction. (E) Quantification of the amount of TX-100 soluble CEA in both Con A-treated MDCK cells and untreated CaCo-2 cells. The relative intensity (unit/area) of apical and basolateral CEA was quantified from five different sets of Z-stack images (50 images per stack) by using the image quantification module of the Zen 2009 software. The statistical significance (marked as stars) denotes the T-test p -value: *** $p < 0.001$. CSP, castanospermine; DMJ, deoxymannojirimycin; SW, swainsonine; TX-100, Triton X-100. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

an ER glucosidase inhibitor) inhibited apical delivery of CEA in polarized MDCK. However, they increased the mobility of CEA during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), consistent with their ability to inhibit the processing of N-glycans in the Golgi (Fig. 5A). These data indicate that mature N-glycans are not required for the apical delivery of CEA in MDCK polarized cells.

CEA associates with lipid rafts in a Golgi pH-dependent manner

Apical targeting of proteins in epithelial cells commonly involves their association with cholesterol-rich lipid rafts in the Golgi (8, 46, 74). To investigate whether the elevated Golgi pH in CaCo-2 cancer cells increases basolateral delivery of CEA *via* inhibiting its association with lipid rafts, we first utilized the so called "cold Triton X-100 (TX-100) insolubility" assay in the presence of NH_4Cl and Con A. This assay is usually used to separate lipid raft-associated proteins from the nonassociated ones (34, 67). It relies on the notion that the detergent is able to solubilize only proteins that reside in cholesterol-poor membrane domains, leaving the cholesterol-rich membrane domains intact. Quantification of the TX-100 soluble and -insoluble fractions from both untreated and Con A-treated MDCK cells revealed that in untreated MDCK cells, 17% of the total CEA protein was TX-100 soluble, and thus not associated with lipid rafts (Fig. 5B). Caveolin 1, a known lipid raft marker (46, 67), was always recovered from the TX-100 insoluble fraction (data not shown), confirming that the extraction protocol reliably can separate lipid raft-associated protein from nonassociated ones. Quantification of NH_4Cl - and Con A-treated cells showed that TX-100 solubility of CEA was increased by ~ 1.5 and ~ 2 -fold (NH_4Cl , from 17% to 25%; Con A from 17% to 33%), respectively. In line with previous studies (9, 27, 55, 62, 63), both NH_4Cl and Con A reduced the size of CEA from 210 to 200 kDa and 180 kDa, respectively, in both TX-100 soluble and TX-100 insoluble fractions (Fig. 5B) due to the altered glycosylation at the elevated Golgi pH. Thus, in addition to increasing basolateral delivery of CEA, both NH_4Cl and especially Con A also increase the TX-100 solubility of CEA, suggesting that they impair the GPI anchor-mediated association of CEA with the lipid rafts.

In further support, confocal microscopy and Z-stack imaging showed that the basolateral CEA in both MDCK cells (Fig. 5C) and intact CaCo-2 cells (Fig. 5D) was mainly TX-100 soluble, whereas the apical CEA was more resistant to detergent extraction. Quantification of the Z-stack images collected from five different sets of pictures indicated that TX-100 extraction decreased the relative amount of basolateral CEA from 42% to 20% in MDCK cells and from 42% to 30% in CaCo-2 cells (Fig. 5E). Taken together, the data cited earlier suggest that the elevated luminal Golgi pH in cancer cells impairs the association of CEA with cholesterol-rich lipid rafts, thereby impairing its apical delivery to the cell surface.

We also utilized fluorescently labeled cholera toxin, a marker for lipid rafts, together with an anti-CEA antibody to verify the localization of CEA in the lipid rafts at the apical surface of untreated MDCK cells. Fluorescence microscopy revealed that CEA and cholera toxin show substantial colocalization (Fig. 6A), as assessed by the yellow color in the

merged figure. Treatment of MDCK cells with methyl- β -cyclodextrin (M β CD), a compound that depletes cellular cholesterol pools and thus disrupts lipid rafts, also increased CEA delivery to the basolateral surface of treated MDCK cells (Fig. 6B, for controls, see Figs. 1B, 2B, 2C, and 3D) similarly to previous results (15), thereby providing further proof for the association of CEA with lipid rafts.

Since many apical membrane proteins, including CEA, possess a GPI anchor (26, 37, 38, 77) that usually mediates their association with cholesterol-rich lipid rafts in the Golgi (8, 46, 74), we tested next whether the elevated Golgi pH increases basolateral accumulation of CEA *via* inhibiting its GPI anchor-mediated association with lipid rafts. Therefore, we removed the C-terminal GPI attachment site from CEA, and we expressed this truncated secretory CEA variant in both untreated and Con A-treated MDCK cells. Even though this maneuver slightly increased the basolateral delivery of this secretory variant (Fig. 6C), relative to the wild-type protein (Fig. 1), it also rendered apical delivery of this variant into a pH-independent process. In Con A-treated MDCK cells, more than 90% of the variant was still secreted through the apical surface (Fig. 6C), that is, at a comparable level to that seen with the wild-type protein (Fig. 1E). Moreover, when we tagged the enhanced green fluorescent protein (EGFP) fluorescent reporter protein N-terminally with a cleavable ER-targeting signal sequence, and C-terminally with the CEA-specific GPI-anchor attachment site, we found that the EGFP reporter protein was efficiently targeted to the apical surface in untreated MDCK, as assessed by confocal microscopy and Z-stack imaging (Fig. 6D). Intriguingly, Con A treatment also markedly increased accumulation of the reporter protein both intracellularly and at the basolateral surface (Fig. 6D) in the treated cells. Collectively, these observations provide strong support for the view that the elevated Golgi luminal pH impairs apical transport of CEA by inhibiting the GPI anchor-mediated association with lipid rafts.

Oligomerization of CEA is not affected by the Golgi luminal pH

Some apical membrane proteins, such as caveolin-1 and placental alkaline phosphatase, also oligomerize concomitant with their lipid raft association (52, 53, 67). To find out whether the Con A-induced basolateral delivery of CEA involves also changes in its oligomerization state, we utilized Blue Native polyacrylamide gel electrophoresis (BN-PAGE) as well as SDS-PAGE to identify CEA oligomers and monomers in the cells. For BN-PAGE, cell pellets were solubilized from both untreated and Con A-treated MDCK and CaCo-2 cells by using the TNE/TX-100 solubilization buffer, and cleared by centrifugation. Immunoblotting with the COL-1 antibody revealed that CEA migrated mainly as two major bands (Fig. 7A, B) with an estimated mw of 270 and 340 kDa, assuming the CEA protein is globular. The bands likely represent CEA dimers (the upper band) and CEA monomers (lower band) based on the use of bovine serum albumin (BSA) monomers and oligomers as a standard for globular proteins. The fact that the molecular sizes of CEA monomers and dimers on BN-PAGE do not exactly match those of the CEA monomer on SDS-PAGE (210 kDa, Fig. 7C) or its calculated dimer (420 kDa) likely reflects either the nonglobular nature of the CEA or the migration

behavior of the native CEA dimers on BN-PAGE. Nevertheless, in all the tested samples, both the suggested CEA monomers and dimers were present in the TX-100 insoluble CEA (associated with lipid rafts, apically targeted), whereas TX-100 insoluble CEA (nonassociated with lipid rafts, basolaterally targeted) existed only as dimer. Thus, we did not observe any marked difference in the oligomerization state of CEA between untreated and Con A-treated MDCK cells, nor between MDCK and CaCo-2 cells. These data indicate that the Con A-induced accumulation of CEA to the basolateral surface is not dependent on CEA's oligomerization state, but rather, on its GPI anchor-mediated association with lipid rafts.

Discussion

Given the importance of CEA for tumorigenesis and for its use as a follow-up marker for colorectal cancer, we set out to investigate the molecular cues that are responsible for its apical targeting in normal epithelia, and mistargeting to the basolateral surface in cancerous epithelia. We showed here that neither hypoxia nor a different redox potential typically detected in cancer cells is responsible for the basolateral targeting of CEA in MDCK cells and in CaCo-2 colorectal cancer cells. Instead, we show that an abnormal Golgi luminal pH, also a feature of cancer cells, was able to divert a substantial proportion of CEA to the basolateral surface of MDCK cells. The observation together with findings that intact CaCo-2 colorectal cancer cells expressing CEA in a nonpolarized manner also have an abnormally high Golgi luminal pH emphasize that the main cause for the non-polarized delivery of CEA is the abnormally high Golgi luminal pH in cancer cells.

Abnormal glycosylation is also inherently associated with tumorigenesis, and similarly dependent on Golgi acidity (64). Thus, maintenance of the two main Golgi functions—glycosylation and protein sorting—are both strictly dependent on proper Golgi pH homeostasis in the cells. Previously, glycosylation defective (Concanavalin A resistant) MDCK cells were shown to sort certain endogenous GPI-anchored proteins (gp35 and gp55) to the basolateral surface (37). The addition or removal of N-glycans of GPI-anchored proteins also changed their transport from basolateral surface to the apical surface and vice versa (6, 54). Based on our results, however, mature N-glycans do not act as apical targeting determinants in CEA, since the glycosylation inhibitors used

FIG. 6. GPI anchor-mediated association of CEA with lipid rafts is pH dependent. (A) Co-localization of CEA with the lipid raft marker cholera toxin (CT) subunit B. Intact MDCK cells were processed for immunofluorescence staining with the anti-CEA antibody and Alexa 488-conjugated cholera toxin. Note the partial co-localization of CEA with the lipid raft marker. (B) The effect of M β CD (Sigma) treatment on apical targeting of CEA in MDCK cells. Cells were treated with 10 mM M β CD for 2 h at +37°C before fixation and staining with the anti-CEA and anti-ZO-1 antibodies. Note the presence of basolateral CEA in the treated cells (for the control, see Fig. 1B). (C) Apical targeting of the GPI anchorless CEA. MDCK cells stably expressing the secretory CEA variant (CEAwoGPI) were grown and left untreated or treated with Con A, after which the variant CEA was collected from both the basolateral and apical media before analysis by SDS-PAGE and Western blotting. Note that Con A did not increase basolateral accumulation of this GPI anchorless CEA, in contrast to wild-type CEA. (D) Apical targeting of EGFP fusion protein tagged with the CEA-specific GPI anchor (EGFPceaGPI). MDCK cells stably expressing the fusion protein were treated with Con A before fixation and counterstaining (with the anti-ZO-1 antibody), and analysis by confocal microscopy and Z-stack imaging. Note that the fusion protein is misrouted from the apical surface (control) to the basolateral surface after Con A treatment (arrows). GPI, glycosylphosphatidylinositol; EGFP, enhanced green fluorescent protein; M β CD, methyl- β -cyclodextrin. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

FIG. 7. Oligomerization state of CEA in untreated and Con A-treated MDCK cells and in intact CaCo-2 cells. (A, B) Cells were grown and treated or not with Con A (50 ng/mL) before analysis of the CEA oligomers by Blue Native PAGE. Putative CEA oligomers were extracted from the cells by using TX-100 solubilization buffer (TX-100 soluble, **A**) and TX-100 insoluble (pellet) fractions (**B**) after solubilizing the latter with 0.2% SDS containing buffer. Both fractions were then subjected to BN-PAGE before analysis by Western blotting. Note that the Con A treatment did not affect the oligomerization state of the TX-soluble CEA protein (the upper band likely representing CEA dimers. The endogenous TX-100 soluble CEA protein in CaCo-2 cells also behaved as a dimer on BN-PAGE. The TX-100 insoluble fraction contained both CEA monomers (lower band) and dimers (upper bands). This is as expected due to the presence of SDS in the TNE buffer used for solubilization. BSA (monomer size 66 kDa) and its oligomers (132, 198, and 264 kDa, dimer, trimer, and tetramer, respectively) were used as standards for the globular proteins. As CEA is heavily glycosylated and thus likely not globular, it is retained in the gel relative to the globular BSA. This likely explains why the 180 or 210 kDa CEA monomer migrates as a 264 kDa globular protein on BN-PAGE. (**C**) Determination of the molecular weight of CEA monomers by SDS-PAGE. Analysis by SDS-PAGE of the CEA TX-100 insoluble fraction obtained from untreated and Con A-treated MDCK cells as well as from intact CaCo-2 cells. Based on the SDS-PAGE, the size of the CEA monomer in both untreated cell types is 210 kDa; whereas in the ConA-treated MDCK cells the CEA has an mw of 180 kDa, due to altered glycosylation. BN-PAGE, Blue Native polyacrylamide gel electrophoresis; BSA, bovine serum albumin.

did not impair apical targeting of CEA despite their ability to inhibit normal processing of N-glycans in the Golgi. Earlier studies have shown that about 8% of N-glycans carried by CEA in cancer tissue specimens are high-mannose type (31, 80). However, our current data showed that blocking the processing of high mannose-type N-glycans using DMJ (58) did not disrupt the apical delivery of CEA, nor did the BGN treatment [an inhibitor of α 2,3-sialylation and elongation of O-glycans (58)]. These data suggest that O-glycans, even if present in CEA, do not have a role in the apical targeting of CEA. This view is also consistent with previous findings with other GPI-anchored proteins (10). Finally, the data showing that cancer-associated CEA is mistargeted to the basolateral surface despite carrying complex type N-glycans (14, 80) further supports the view that mature N-glycans do not act as the primary apical targeting motifs for CEA.

Previously, it has been shown that CEA is associated with lipid rafts (13, 68). In further support, we showed that CEA is mostly TX-100 insoluble in untreated MDCK cells, and that M β CD, a disruptor of lipid rafts, increased its appearance at the basolateral surface in MDCK cells, perhaps by inhibiting general apical transport capacity of MDCK cells (60).

Moreover, we showed that the GPI-anchorless CEA is targeted to the apical surface in a pH-independent manner, and that the apical transport of CEA-specific GPI anchor containing EGFP reporter protein is, in turn, pH sensitive. These observations indicate that the C-terminal GPI-anchor plays a major role in the apical targeting of CEA in normal epithelial cells and is needed for the association of CEA with lipid rafts in a pH-dependent manner. An important question remaining then is how the elevated pH in the Golgi lumen impairs the association of CEA with lipid rafts. One obvious possibility is that it generally inhibits intra-Golgi transport. However, the two endogenous membrane proteins (gp114 and gp135) in MDCK cells (19, 83) were efficiently targeted to the apical surface even in Con A-treated cells. Therefore, this possibility seems unlikely. The fact that we did not observe any intracellular accumulation of CEA on Con A treatment also supports this view. Nevertheless, given that alternate transport routes to the apical surface exist (12, 59, 78), it may be that the transport route used by CEA, but not that used by gp114 or gp135, is specifically inhibited by Con A. In agreement with this view, we also observed accumulation of the GPI-anchored enhanced green fluorescent protein (EGFP-GPI) fusion protein

in some intracellular sites, in contrast to CEA. If the apical transport for CEA is, indeed, impaired, then compensatory transport to the basolateral surface is required. This might explain the absence of intracellular CEA in Con A-treated MDCK cells. The difference in the behavior of CEA and EGFP-GPI protein is unclear, but it may involve the need of having the highly glycosylated luminal domain for the maintenance of membrane transport from the Golgi to the cell surface. Also, in earlier studies, the addition or removal of the GPI-anchor from a different target protein variably did or did not interfere with the apical transport of the target protein (36, 79). Elevated Golgi pH also seemed to induce a stricter apical transport of the GPI-anchorless CEA variant. This is similar to what was observed with Bafilomycin A1 treatment on both general protein and proteoglycan secretion, indicating that elevated Golgi pH increases general apical transport capacity of secretory cargo (22).

Both apical and basolateral GPI-anchored proteins are known to associate with lipid rafts (6, 52, 66), suggesting that lipid rafts, in general, also do not determine alone their apical localization in the cells. It likely involves some other cues that may be protein specific. For example, many apically sorted GPI-anchored proteins (*e.g.*, placental alkaline phosphatase) have been shown to oligomerize on their association with lipid rafts (45, 51–53). One possibility, thus, is that the lipid raft association of CEA involves its homo-oligomerization (7), similar to some other lipid raft-associated proteins (52, 53). In this scenario, the elevated Golgi pH either in Con A-treated MDCK cells or in intact CaCo-2 cells could interfere with such oligomer formation, and thus also lipid raft association. However, in contrast to earlier studies mentioned (45, 51–53), we did not observe marked changes in the oligomerization state of CEA between untreated and Con A-treated MDCK cells, as in both cases Blue Native PAGE revealed the presence of CEA dimers and monomers. This suggests that oligomerization itself does not play a major role in the apical targeting of CEA in MDCK cells.

On the basis of these findings, we propose that under normal conditions, that is, when the Golgi pH is sufficiently acidic ($\sim$ pH 6.0–6.5), CEA oligomerizes with itself and associates with lipid rafts, resulting in its sorting into apical transport vesicles and eventually to the apical plasma membrane. The GPI anchor is necessary for this to occur, as it is responsible for the association with lipid rafts. Thus, it contributes to the efficiency of apical sorting, whereas mature N-glycans do not seem to play any role in apical targeting of CEA. In contrast, when the Golgi pH is elevated such as in CaCo-2 cancer cells, or in Con A-treated MDCK cells, CEA cannot associate efficiently with lipid rafts irrespective of its oligomerization state, whereby it fails to be sorted into apical transport vesicles, and instead, may enter into basolateral vesicular carriers. The suggested view, thus, provides a direct link between abnormal Golgi pH homeostasis, mistargeting of CEA, and abnormal glycosylation in cancers, that is, factors that are all inherently associated with tumorigenesis and have also further consequences, for example, in the maintenance of cell polarity and tumor growth, cancer cell migration, and metastasis (14, 56). Mistargeting of CEA, in turn, can also contribute to the loss of cell polarity, given the many functions assigned to it thus far (16, 24, 29, 50, 69, 75, 81).

The main question that remains is how an elevated Golgi pH can impair lipid raft association of CEA, and perhaps of

other GPI-anchored proteins as well. At least two options can be envisioned, which are not mutually exclusive. First, an elevated Golgi pH contributes to the protonation state of the CEA polypeptide that then may delay homo-oligomerization and concomitant association of CEA with lipid rafts. Second, it may inhibit intra-Golgi transport of CEA, whereby it cannot undergo pH-dependent oligomerization and/or association with lipid rafts. Although there is no direct evidence for the former, the latter view is supported by the fact that influenza virus M2 protein (a proton channel, which also dissipates Golgi pH gradient) has been shown to delay apical transport kinetics of glycosylated membrane and secretory proteins (27). Overall, the current findings add a new layer toward the understanding of the variable membrane localization of proteins often seen in epithelial cancer cells.

Materials and Methods

cDNA plasmid constructs

cDNA encoding CEA (CEACAM5, kindly provided by Dr Clifford P. Stanners, Montreal, Canada) was subcloned into pcDNA3-vector (Invitrogen, Grand Island, NJ). The GPI-anchorless CEA variant (CEAwoGPI) was prepared by mutating Ala₆₇₇ encoding codon to STOP codon (5'-TAA-3') with the QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and the following primers 5'-CAAGAGCAT CACAGTCTCTTAATCTGGAACCTCTCCTGGT-3' and 5'-ACCAGG-AGAAGTCCAGATTAAGAGACTGTGATGCTCTTG-3' (Sigma-Genosys, Haverhill, United Kingdom). The mutant lacks an alanine residue (Ala₆₇₇, in which the GPI-anchor is usually attached) and the most distal 26 amino acids, including the GPI-anchor attachment signal (48). EGFP-GPI was constructed by PCR cloning of the CEA's N-terminal signal sequence (cleavable ER-targeting signal) and the C-terminal GPI-anchor attachment site (48) by using the pcDNA3CEA-plasmid as a template. The primers (Sigma-Genosys) used for PCR amplifications had the following sequence: 5'-AAAAAAGCTAGCATGGAGTCTCCCTCGGCCC-3', 5'-AAAAAAACCGGTTCAAT-AGTGAGCTTGGCAGTGG-3', 5'-AAAAAAGATCTGCATCTGGAACCTTCTCCTGGTC-T-3', and 5'-GGCAACTAGAAGG-CACAGTCGAGG-3', respectively. Novel *NheI*, *AgeI*, and *BglII* restriction enzyme cleavage sites (underlined in primer sequences) were created during the PCR. After digestion with *NheI* and *AgeI* (N-terminus) and *BglII* and *Apal* (C-terminus), the gel-purified dsDNAs were ligated into the N- and C-terminus of EGFP, respectively, by using the pEGFP-C1 vector (Clontech, Mountain View, CA). Generated plasmids were sequence verified by using the ABI Prism sequencer.

Cell transfections and selection of stable cell lines

MDCK cells (ATCC, Manassas, VA) were grown in Dulbecco's modified Eagle's medium supplemented with Glutamax DMEM (Invitrogen), 10% fetal bovine serum (HyClone, Cramlington, United Kingdom), and antibiotics (Penicillin-Streptomycin; Sigma-Aldrich, St. Louis, MO) in humidified conditions at +37°C/5% CO₂. MDCK cells grown 16–20 h in 35 mm plastic dishes were stably transfected with pcDNA3-CEA, CEAwoGPI, or EGFP-GPI plasmids. For transfections, we used a 1 μ g plasmid-DNA/dish and 5 μ L of the FuGENE HD

Transfection Reagent (Roche Diagnostics, Mannheim, Germany), unless stated otherwise. Transfected cells were selected against geneticin resistance (1 mg/mL of G418; Sigma-Aldrich) for 10 days with the necessary media changes. Cells were propagated in the presence of 0.5 mg/mL geneticin in the growth media.

Cell culture and cell treatments

Stably transfected MDCK cells were grown for a minimum of 6 days in Transwell filters (polyester, 24 mm diameter, 0.4 μ m pores; Corning, New York, NY) to obtain polarized confluent monolayers. When appropriate, cell monolayers were treated with various drugs for 16–24 h under normal cell culture conditions or under moderate hypoxia (5% O₂). Glycosylation inhibitors SW (5 μ g/mL), DMJ (100 μ g/mL), and CSP (5 μ g/mL) were purchased from Calbiochem (Darmstadt, Germany). BGN (2 mM), BFA (10 μ g/mL), and the vacuolar H⁺-ATPase-inhibitor Con A (50 ng/mL), NH₄Cl (10 mM), Con A (100 nM) DTT, M β CD (10 mM), and N-acetylcysteine (1 mM) were all purchased from Sigma-Aldrich, unless stated otherwise. Ascorbic acid (100 μ M) was purchased from AppliChem GmbH (Darmstadt, Germany), and Alexa 488-conjugated Cholera toxin subunit B (5 μ g/mL) was from Thermo Fisher Scientific (Cheshire, United Kingdom). The drugs were added to the culture medium for 16 h before analyses, unless stated otherwise. M β CD was added to the cells only for 2 h at +37°C.

Immunohistochemistry

Normal and colorectal tissue specimens were collected from patients who underwent surgery for colorectal carcinoma. Normal counterparts were taken at least 5–10 cm distance apart from carcinoma. Written consent was obtained from each patient, and the use of patient samples for research purposes has been approved by a local ethical committee under a license no 252/08/91. For immunostainings, 5- μ m-thick longitudinal tissue sections were taken from formalin-fixed paraffin tissue blocks. De-paraffinized and rehydrated sections were stained by using conventional procedures with the CEA-specific monoclonal antibody (COL-1; Zymed Laboratories, Carlsbad, CA) and horseradish peroxidase conjugated rabbit anti-mouse secondary antibody (Abliance, Compiegne, France). 3,3'-Diaminobenzidine (DAB; Sigma-Aldrich) was used as a substrate to identify the bound antibodies. Stained specimens were embedded in Immu-Mount, and they were photographed by using the Olympus BX 51 epifluorescence microscope with a normal light source, 20 $\times$ objective and a CCD camera.

Indirect immunofluorescence and confocal microscopy

MDCK/CEA cells grown on Transwell filters were treated or not with pH gradient-dissipating drugs for 24 h, and they were fixed with 4% paraformaldehyde. After blocking and permeabilization (1% BSA, 0.1% saponin in phosphate buffered saline [PBS]), cells were double stained with monoclonal anti-CEA antibody (COL-1) and polyclonal anti-ZO-1 antibody (both from Zymed Laboratories). Alexa Fluor 488-conjugated goat anti-rabbit and Alexa Fluor 546-conjugated goat anti-mouse antibodies (Molecular Probes, Eugene, OR) were used as secondary antibodies. Lipid rafts

were visualized by using Alexa Fluor 488-conjugated cholera toxin subunit B (5 μ g/mL, 30 min, +4°C; Thermo Fisher Scientific). After fixation and co-staining with the anti-CEA antibody, cells were embedded with Immu-Mount (Thermo Fisher Scientific) and stained specimens were examined and photographed by using the Olympus FluoView 1000 laser scanning confocal microscope equipped with a 60 $\times$ Oil objective and appropriate filters. For Z-stack imaging, Zeiss LSM710 confocal microscope and the 100 $\times$ oil immersion objective was used. Thirty to 50 sections at 0.3 μ m intervals were taken from each region of interest. The relative intensity (unit/area) of apical and basolateral surfaces was quantified from the Z-stack images by using the image quantification module of the Zen 2009 software.

Determination of Golgi pH, redox potential, and cellular ROS levels

Ratiometric pHluorin (44), which also contains 80 N-terminal amino acids from B4GalT-1 (GT-pHluorin) to allow targeting and retention in the Golgi membranes, was used to determine the Golgi luminal pH in MDCK and CaCo-2 cells. Briefly, MDCK cells were transfected by using the GT-pHluorin plasmid and electroporation with the nucleofector kit (Lonza, Basel, Switzerland) according to the manufacturer's suggestions. Stable MDCK transfectants grown in complete DMEM were selected against geneticin (G418, 0.5 mg/mL) for 10 days before the Golgi pH measurements. CaCo-2 cells were transiently transfected with Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific), as suggested by the manufacturer.

To measure Golgi luminal pH, stably GT-pHluorin-expressing cells were seeded on 96-well microscopy plates in complete DMEM for 6 h, after which the medium was changed to FluoroBrite™ DMEM (Thermo Fisher Scientific) to minimize background fluorescence. When appropriate, the drugs (10 mM NH₄Cl, 50 ng/mL Con A, 5 mM DTT, 100 μ M ascorbic acid, 100 μ M H₂O₂) were included in the buffers used. After equilibration (30 min, 5% CO₂/95% air at RT to keep the media pH at pH 7.2–7.4), Golgi pH was determined by taking 50 serial images from each well by using the Operetta high-content imaging system (PerkinElmer, Inc.). Appropriate filter sets for two different excitation wavelengths (420 and 470 nm excitation, 500–550 emission) were used to allow ratiometric quantification of the signal intensities with the in-built Harmony software package. Fluorescence intensity ratios were then converted to pH values by using a calibration curve. *In situ* pH calibration was carried out by using specified pH calibration buffers prepared in 125 mM KCl/20 mM NaCl/1.0 mM CaCl₂/1.0 mM MgCl₂. The pH was adjusted to 7.5, 6.5, and 5.5 with 20 mM HEPES, MOPS, or MES, respectively. All calibration buffers also contained 5 mM nigericin and 5 mM monensin to dissipate monovalent cation gradients.

Redox potential measurements were performed similarly, but by using the ratiometric Golgi-RoGFP2 construct (25, 41, 65). MDCK and CaCo-2 cells were transfected with Golgi-RoGFP2 by using the Lipofectamine 3000 transfection reagent, as described earlier. Cells were seeded on 96-well microscopy plates 6 h before measurement. RoGFP2 ratios for each sample were measured in triplicate with the Operetta high-content imaging system by using the same filter sets as

for GT-pHluorin and the Harmony software package (PerkinElmer, Inc.). The level of cellular ROS in MDCK and CaCo-2 cells was measured with a CellROX® Deep Red reagent kit (Thermo Fisher Scientific). Cells were cultured on 96-well plates as described earlier and treated (or not) with 100 μ M ascorbic acid, 100 μ M H₂O₂, 50 ng/mL Con A, or 5 mM DTT for 16 h before staining with 5 μ M reagent diluted in serum-free DMEM for 2 h in +37°C. After washing with 1 $\times$ PBS (pH 7.4) and Hoechst DNA counterstaining (10 μ g/mL), cell imaging was done with PerkinElmer Operetta as described earlier by using appropriate filter sets (620–640 ex, 650–760 em). Quantification of intensities was performed with the in-built Harmony software (PerkinElmer, Inc.).

Determination of the apical and basolateral distribution of proteins in MDCK cells

Cells grown on Transwell filters treated or not with various drugs were washed with PBS. GPI-anchored proteins were released from the apical and basolateral membranes into serum-free DMEM by using 100 mU/mL PI-PLC (Sigma-Aldrich). After 1 h incubation at +37°C/5% CO₂, the media were collected and cells were lysed with 2 $\times$ SDS sample buffer (4% SDS, 20% glycerol, 125 mM Tris pH 6.8, 100 mM dithiothreitol, bromophenolblue). The released CEA-protein was collected by immunoprecipitation with a polyclonal anti-CEA antibody (DAKO A/S, Glostrup, Denmark) and protein-A-sepharose (GE Healthcare Biosciences, Uppsala, Sweden). After washing with RIPA buffer (50 mM Tris pH 7.4, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1% Triton X-100) and 10 mM Tris pH 7.4, CEA was eluted from the beads with 2 $\times$ SDS sample buffer, heated, and subjected to SDS-PAGE (7.5% gel) and immunoblotting with monoclonal anti-CEA antibody (COL-1; Zymed Laboratories). Goat-anti-mouse IgG conjugated with horseradish peroxidase (Abliance), enhanced chemi-luminescence (ECL) substrates, and ECL-Hyperfilm (GE Healthcare, Buckinghamshire, United Kingdom) were used for visualization. CEA protein bands were quantified by using ImageJ software (NIH, Bethesda, MD).

For determination of apical and basolateral distribution of endogenous transmembrane proteins, gp114 and gp135 MDCK/CEA cells grown on Transwell filters were first treated (or not) with Con A, and then selectively biotinylated by using Sulfo-NHS-SS-biotin (Pierce Biotechnology, Inc., Rockford, IL) in PBS for 30 min at +4°C. After quenching with 100 mM glycine-PBS, cells were lysed with cold RIPA buffer and biotinylated proteins were collected with NeutrAvidin-agarose (Pierce Biotechnology) according to the manufacturer's instructions. Samples were analyzed by SDS-PAGE and immunoblotting with gp114 [a dog homologue of human CEACAM (19)] and gp135 [a dog homologue of human podocalyxin (43)] with specific monoclonal antibodies (a kind gift from Dr. Aki Manninen, Oulu, Finland) as described earlier. Confocal Z-stack imaging, when appropriate, was used for detecting apically localized proteins by using the protocol described earlier.

Solubilization of CEA by using cold TX-100 detergent solution

MDCK/CEA cells grown in six-well plates were treated (or not) for 24 h with either 10 mM NH₄Cl or 50 ng/mL

Con A. Cells were divided into Triton X-100 soluble and -insoluble fractions as previously described in (8, 52). Briefly, cells were lysed for 20 min on ice by using TNE/TX-100 buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100). TX-100 soluble fractions were collected by centrifugation, and the supernatants were adjusted to 0.1% SDS and precipitated with trichloroacetic acid. Cell pellets were lysed in 2 $\times$ SDS sample buffer. CEA content in the samples was determined by SDS-PAGE and immunoblotting with CEA-specific antibody, as described earlier.

Blue Native PAGE

MDCK and CaCo-2 cells were treated (or not) with 50 ng/mL Con A (24 h), washed sequentially with PBS and 20 mM Tris (pH 7.5), and scraped from the dishes before pelleting by centrifugation. Cell pellets were then lysed on ice for 30 min in TNE/TX-100 buffer (25 mM Tris pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100) and cleared by centrifugation (15,000 g, +4°C, 15 min) to get the TX-100 soluble fraction. Insoluble cell remnants (TX-100 insoluble fraction) were further lysed on ice for 30 min in TNS/TX-100 buffer (20 mM Tris pH 7.5, 100 mM NaCl, 0.4% SDS, 0.2% Triton X-100). The fractions were mixed with a Tris/Glycerol native sample buffer and loaded onto a Mini-Protean TGX 4%–15% gel (BioRad, Hercules, CA). The gel was run in Tris/Glycine buffer supplemented with 0.02% Coomassie Brilliant Blue G-250 until the samples had migrated 1–2 cm into the gel, then without the dye at 20 mA for 2 h. The samples were then transferred onto the 0.2 μ m PVDF membrane (BioRad) for 2 h at 200 mA in a Transfer Buffer (25 mM Tris–192 mM in glycine buffer, pH 8.3). The membrane was quenched by using 5% nonfat milk in TBS-Tween (50 mM Tris–150 mM NaCl–0.02% Tween 20, pH 7.6) supplemented with 0.1% BSA overnight at +4°C. The blot was thereafter incubated with monoclonal COL-1 anti-CEA antibody (1:500 in TBS-Tween +0.1% BSA) for 2 h at RT, washed 3 $\times$ 10 min with TBS-Tween before incubation with the goat anti-mouse HRP 1:10,000 (Abliance, Compiègne, France) in TBS-Tween +0.1% BSA for 1 h at RT. After final washings (4 $\times$ 15 min in TBS-Tween), ECL reagent (BioRad) was added and the filter was photographed by using the GelDoc instrument (BioRad).

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Author Disclosure Statement

All the authors declare that there are no commercial associations that might create a conflict of interest.

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Abbreviations Used

BFA = brefeldin A
BGN = benzyl-N-acetyl- α -galactosaminide
BN-PAGE = Blue Native polyacrylamide gel electrophoresis
BSA = bovine serum albumin
CEA = carcinoembryonic antigen
COL-1 = anti-CEA antibody
Con A = concanamycin A
CSP = castanospermine
DAB = 3,3'-diaminobenzidine
DMEM = Dulbecco's modified Eagle medium
DMJ = deoxymannojirimycin
DTT = dithiothreitol
ECL = enhanced chemi-luminescence
EGFP = enhanced green fluorescent protein
EGFP-GPI = GPI-anchored enhanced green fluorescent protein
GPI = glycosylphosphatidylinositol
M β CD = methyl- β -cyclodextrin
MDCK = Madin-Darby canine kidney
NH₄Cl = ammonium chloride
PBS = phosphate buffered saline
pH-G = pH of the Golgi lumen
PI-PLC = phosphatidylinositol-specific phospholipase C
ROS = reactive oxygen species
SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis
SW = swainsonine
TX-100 = Triton X-100