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Phosphatidylcholine passes through lateral tight junctions for paracellular transport to the apical side of the polarized intestinal tumor cell-line CaCo2

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ABSTRACT

Phosphatidylcholine (PC) is the most abundant phospholipid in intestinal mucus, indicative of a specific transport system across the mucosal epithelium to the intestinal lumen. To elucidate this transport mechanism, we employed a transwell tissue culture system with polarized CaCo2 cells. It was shown that PC could not substantially be internalized by the cells. However, after basal application of increasing PC concentrations, an apical transport of 47.1 ± 6.3 nmol h^{-1} mM PC^{-1} was observed. Equilibrium distribution studies with PC applied in equal concentrations to the basal and apical compartments showed a 1.5-fold accumulation on the expense of basal PC. Disruption of tight junctions (TJ) by acetaldehyde or PPARγ inhibitors or by treatment with siRNA to TJ proteins suppressed paracellular transport by at least 50%. Transport was specific for the choline containing the phospholipids PC, lysoPC and sphingomyelin. We showed that translocation is driven by an electrochemical gradient generated by apical accumulation of Cl^- and HCO_3^- through CFTR. Pretreatment with siRNA to mucin 3 which anchors in the apical plasma membrane of mucosal cells inhibited the final step of luminal PC secretion. PC accumulates in intestinal mucus using a paracellular, apically directed transport route across TJs.

1. Introduction

The intestinal epithelium is protected against bacterial invasion by a hydrophobic mucus layer [1]. This is of particular importance in the colon, which is exposed to a bacterial density of one trillion per gram of stool. The scaffold of mucus is composed of a family of highly glycosylated proteins, the mucins. These are secreted by goblet cells which are present throughout the intestine [2]. However, enterocytes also express mucins 3, 12, 13 and 17, which are transmembrane-anchored proteins located at the apical tight junction (TJ) [3]. Cystic fibrosis transmembrane conductance regulator (CFTR) localizes in its vicinity at the apical side of mucosal cells [4]. It needs to be evaluated whether the reported predominant appearance in intestinal crypts has functional implications or may be due to the proportional, structurally related higher density of apical membranes compared to the villi region. CFTR is responsible for the luminal Cl^- and bicarbonate secretion ensuring proper mucus function [5]. The hydrophobicitiy of the mucus is established by phosphatidylcholine (PC) [6]. PC is enriched in the mucus, i.e. >90% of the phospholipids within the mucus are PC and lysoPC (LPC) species [7], indicative of a specific transport route into this compartment. The release of PC together with mucins is unlikely, because goblet cells do not store phospholipids [8]. Hence, a separate translocation route of PC across the intestinal epithelial layer must exist accounting for the enrichment in mucus.

Monomeric transmembrane transport of a complex phospholipid such as PC has only been described for the ABC transporter ABCB4 (MDR3) so far [9–12]. This transporter is localized in the canalicular membrane of hepatocytes, but not in mucosal cells [13]. Otherwise, transmembrane transport of PC occurs only after hydrolysis to lysoPC and fatty acids via membrane carriers [14,15]. However, this facilitated transport occurs from the lumen across the apical microvillous membrane to the cytosol [16]. After reconstitution, PC – integrated into lipoproteins – passes only the basolateral, but not the apical plasma

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FCS, fetal calf serum; LPC, lysophosphatidylcholine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PLFF, plasma lipoprotein free fraction; PS, phosphatidylserine; RT-PCR, real time-polymerase chain reaction; SM, sphingomyelin; TER, transepithelial resistance; UC, ulcerative colitis; TJ, tight junction.

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membrane by exocytosis [16]. Thus, apical release of cellular PC being unlikely, there may be a translocation of systemic (extracellular) PC by a paracellular route across the lateral cell–cell adhesion structures, of which the tight junctions (TJ) are the most compact elements allowing fence and gate function. The latter control the paracellular passage of ions and solutes between cells [17]. Although a transport pathway for bulky surfactant like particles containing PC in association with intestinal alkaline phosphatase has already been suggested >20 years ago [18], it has not been considered anymore in later studies. It deemed to contradict the understanding of TJs, not allowing substrate transport [19].

Therefore, it was of great interest to reevaluate the mechanism underlying PC accumulation in mucus. For this purpose a transwell tissue culture system was employed in which transport of a substrate to the apical compartment can be selectively examined and modulated. For proof of concept, the human intestinal tumor cell line CaCo2 was chosen. In comparison to other available cell lines, CaCo2 is the best characterized intestinally differentiated cell line providing confluent growth and apical/basolateral polarization after 21 days in culture [20]. Most importantly, polarized CaCo2 cells establish a lateral TJ barrier which is suitable to examine the proposed paracellular transport route of PC [21].

Once apically released, PC can bind to negatively charged mucins with its positively charged choline head. The hydrophobic carbon chains then attract in an aqueous environment the fatty acid moieties of other PC molecules to form a phospholipid bilayer that conveys hydrophobicity to the mucus on the whole. The intestinal mucus layer thus acts as a seal to prevent bacterial penetration from the lumen [22].

The clinical significance of depleted mucus PC has become evident when the PC content was shown to be reduced by 70% in patients with the inflammatory bowel disease ulcerative colitis (UC) compared to those with Crohn's disease and healthy controls [23, 24]. Most importantly, this reduction was observed during periods of remission, indicating that it is intrinsic to UC and not caused by inflammation.

2. Materials and methods

2.1. Cell culture transport studies

CaCo2 cells (ATCC®) were seeded in 12-well collagen-coated transwell culture dishes (0.4 μm pore size) at 7.5×10^4 cells/well (corresponding to 80–100 μg protein/well) and cultured in Dulbecco's Modified Eagle's Medium containing 5% fetal calf serum (FCS) (Life Technologies, Carlsbad, CA, USA) for 3–30 days to allow apical/basolateral polarization and establishment of TJs, which was confirmed by measuring transepithelial resistance (TER) > 450 Ω [20]. The translocation of substrates applied to either the basal or apical side (1 ml) was determined in the adjacent compartment (1 ml) equilibrated in the same buffer for indicated times, in most cases for 1 h. [^{3}H]PC (0.05–10 mM), [^{14}C]lysophosphatidylcholine (LPC), [^{3}H]sphingomyelin (SM), [^{14}C]phosphatidylethanolamine (PE), [^{3}H]phosphatidylinositol (PI), [^{3}H]palmitate, and [^{3}H]oleate (all brought up with unlabeled ligand to 10 mM containing 100,000 cpm ml^{-1}) were used with an equimolar concentration of taurocholic acid (TC) in phosphate-buffered saline (PBS, pH 7.4). It was used as vehicle for solubilization of the lipids. [-^{14}C]inulin, [^{3}H]sucrose, [^{3}H]choline chloride, and [^{3}H]TC were used at 10 mM in PBS. Radiolabeled compounds were purchased from Perkin Elmer (Waltham, MA, USA). TJs were disrupted by an apical application of 150 μM acetaldehyde for 3 h [25], or the peroxisome proliferator-activated receptor γ inhibitors T0070907 (10 μM) or GW9002 (1 μM) for 1 h [26]. In addition, TJ disruption was achieved by knockdown experiments using siRNAs (78 pmol) [27] targeting claudin 1, 2, or 4, ZO-1, occludin, jam-1, kindlin 1 or 2. For analysis of the PC driving forces, 10 mM [^{3}H]PC equilibrated with 10 mM TC·PBS was always applied to the basal compartment. In a first set of experiments, the pH of the apical 10 mM TC·PBS medium was varied from 5 to 9. An equilibrium phase of 5 min was provided before the wells were placed in the lower [^{3}H]PC containing chambers, and apical transport was measured for 1 h.

In a second set of experiments, the 10 mM TC in the apical medium was applied in different buffers at 130 mM and pH 7.0 which generated a more positive charge in the medium (NH_4Cl, Na-thiocyanate and 10 mM urea in PBS) or a more negative charge ($NaHCO_3$, Na-glucomate and 10 mM sodium dodecylsulphate (SDS) in PBS). Apical transport from basally applied 10 mM [^{3}H] in 10 mM TC·PBS was determined over 1 h. In a third set, siRNA knockdown experiments were performed for functional analysis of CFTR as the outside negative charge generator; mucin 3 as the apical, enterocyte-bound, acceptor protein; and mucin 2 as negative control.

In a fourth set, the effect of increasing concentrations of taurocholate as PC solubilizer or mucins 1 and 2 as PC acceptor molecules in the upper compartment was examined. Thus, increasing concentrations of TC and mucins were apically added in PBS (1 ml), and apical translocation from basally provided 10 mM [^{3}H]PC in 10 mM TC·PBS was determined over 1 h.

2.2. SiRNA knockdown experiments

SiRNA transfection [27] was performed using Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The reaction mix containing 78 pmol siRNAs without FCS (1 ml) was applied to the apical side of polarized CaCo2 cells for 16 h at 37 °C. Thereafter the cells were washed and equilibrated with 10 mM TC·PBS in the upper compartment for the 1 h transport rate determination of 10 mM [^{3}H]PC in 10 mM TC·PBS from the basal compartment. The following sense and antisense probes (Sigma, St. Louis, MO, USA) were used: scrambled siRNA, s_5′-gaugggaccuggccaguga-3′[dT][dT] and a_5′-ucacuggccaggucccauc-3′[dT][dT]; claudin 1, s_5′-cagucaaugccagguacga-3′[dT][dT] and a_5′-ucguaccuggcauugacug-3′[dT][dT]; claudin 2, s_5′-gacacuaccacuggaucgu-3′[dT][dT] and a_5′-acgauccaguggugaguguc-3′[dT][dT]; claudin 4, s_5′-gaccaucugggagggccua-3′[dT][dT] and a_5′-uaggcccucccagaugguc-3′[dT][dT]; mucin 2, s_5′-gcaacauuaccgucugcaa-3′ [dT][dT] and a_5′-uugcagacgguaauguugc-3′ [dT][dT]; mucin 3, s_5′-ccaaacuacucuuacuaca-3′ [dT][dT] and a_5′-uguaguaagaguaguuugg-3′ [dT][dT]; and CFTR, s_5′-gaacacauaccuucga uau-3′ [dT][dT] and a_5′-auaucgaagguauguguuc-3′ [dT][dT]; ZO-1, s_5′-gagaugaacgggcuacgcu-3′ [dT][dT] and a_5′-agcguagcccguucaucuc-3′ [dT][dT]. Probes from other sources than Sigma: kindlin 1, s_5′-ggacauuacugauaucccu-3′ [dT][dT] and a_5′-agggauaucaguaaugucc-3′ [dT][dT]; kindlin 2, s_5′-gugugaauagaaauacugu-3′ [dT][dT] and a_5′-acaguauuucuauucacac-3′ [dT][dT] (both gifts from R. Faessler; MPI, Munich, Germany); occludin, s_5′-gagaugaacgggcuacgcu-3′[dT][dT] and a_5′-agcguagcccguucaucuc-3′[dT][dT] (sc 133,255, Santa Cruz, Heidelberg, Germany) and jam-1, s_5′-gagaugaacgggcuacgcu-3′[dT][dT] and a_5′-agcguagcccguucaucuc-3′[dT][dT] (orb-5911, Biorbyt, Cambridge, UK). The efficiency of siRNA knockdown was confirmed by Western blotting.

2.3. ABCB4 (MDR3) gene expression analysis by reverse transcription quantitative PCR (qPCR)

For quantification of ABCB4 in homogenate, 1 μg mRNA from CaCo2 and HepG2 cells were analyzed [28]. Following RT-PCR primers were used for ABCB4: s_5′-atcctcaccagaagactgctgcggt-3′, and a_5′-gcag catctgtggcaagtcttg-3′ (HP229029, Origine Technologies, Rockville, MD, USA) and for housekeeping β-actin, s_5′-aggatgcagaaggagatcact-3′ and a_5′-gggtgtaacgcaactaagtcatag-3′ (from our lab) [28].

2.4. Western blot antibodies

Cell homogenate samples with 30 μg protein were applied to the gel slots for electrophoretic separation and immunoblotting using a standard protocol [27].

Primary antibodies against the following human proteins were used: claudin 1 (9076), claudin 2 (9075), claudin 4 (1364) (all at 1:500; all from Acris Antibodies, Herford, Germany); ZO-1 (1:200; Abcam, Cambridge/MA, USA); kindlin 1 (4A5.14, 1:500; Millipore, Billerica, MA, USA); kindlin 2 (1:500; gift from R. Faessler, MPI, Munich, Germany); occludin (F-11/sc-133255) (1:200); mucin 2 (B 306.1/sc-59859) (1:500), mucin 3 (M 3.1/sc-7315) (1:500), CFTR (A-3/sc-376683) (1:200), ABCB4 (MDR 3) (P3 II-26/sc 58221) (1:200); all from Santa Cruz Biotechnology), jam-1 (orb-5911) (1:200; Biorbyt), β-actin (A5441) (1:10,000) Sigma.

2.5. Lipid binding capacity to mucins

In vitro, 1 mg of mucin 1 (499,643; Merck, Darmstadt, Germany), mucin 2 (M2378, Sigma), mucin 3 (M1778, Sigma) (hazy, light yellow solution in 10 mM TC·PBS) or – for comparison – fatty-acid free bovine serum albumin (A6003, Sigma) were separately incubated with 10 mM radiolabeled PC, LPC, SM, PE, cholesterol, palmitate or oleate in 10 mM TC PBS for 1 h. This was followed by immunoprecipitation with respective antibodies (from Santa Cruz Biotechnology, see Western blotting, for mucin 1 (C-Mu-1/sc-53376) and for albumin (H-126/sc-50535)) at a dilution of 1:200 for 16 h. After washing with PBS, the amount and composition of lipids bound to the specific mucins were determined by ESI-MS/MS.

2.6. Electrospray ionization tandem mass spectrometry (ESI-MS/MS)

Apically transported phospholipid species from basally applied mixtures of 1 mM PC, LPC, SM, PE, PS, and PI (Lipoid GmbH, Ludwigshafen, Germany) in 6 mM TC·PBS were quantified by ESI-MS/MS [29,30]. Lipids were extracted from the basal and apical compartment following an addition of 20 ng of the internal standards of PC (14:0/14:0), LPC (17:0), PE (12:0/12:0), and PI (17:0/17:0) (Avanti Polar Lipids, Alabaster, AL, USA). Lipid extracts were analyzed by direct flow injection into a Quattro Premier ESI-MS/MS instrument (Waters, Milford/MA, USA) with data acquired for 2 min [30]. Positive and negative ionization modes were used for different phospholipids according to established procedures [29].

2.7. Tissue culture imaging

PC movement across the CaCo2 cell monolayer was visualized by high-resolution microscopy [31,32]. Cultures were incubated for 16 h with 10 μM BODIPY® (581/591) PC (D3806; Molecular Probes, Eugene, OR, USA) applied at a 1:1 molar ratio with TC in PBS to the basal compartment. After washing in PBS, the membranes of the transwell culture systems were cut out and washed in PBS. Then 10 μM Oregon Green 488 was added for 60 s and the red fluorescent PC was detected with a SMLM-SIM combo microscope [32] using a Leica 100× objective oil immersion 140/70 HCX PL APO lens (Leica, Tokyo, Japan).

2.8. Statistical analysis

Statistical analysis was performed using Prism 4.0 software (GraphPad Software Inc., La Jolla, CA, USA). Differences between groups were evaluated using the Mann–Whitney *U* test. Multiple groups were compared by one-way ANOVA with a Dunnett's post-hoc test. Data are presented as means ± SD or medians with range, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Luminal phosphatidylcholine secretion occurs through TJs

We investigated the luminal secretion of [^{3}H]PC mixed in a 1:1 molar ratio with TC by applying it at 10 mM to the basal compartment of polarized CaCo2 cells (21 days cultured), which were established with a TER > 450 Ω. At 37 °C, apical secretion of [^{3}H]PC across polarized cells was linear over an examined time frame of 3 h, with a concentration-dependent accumulation rate of 47.1 ± 6.3 nmol h^{-1} mM PC^{-1}. In contrast, non-polarized CaCo2 cells (3 days-cultured) revealed a TER 160 ± 10 Ω and a reduced translocation rate of 20.8 ± 3.4 nmol h^{-1} mM PC^{-1} ($p < 0.001$).

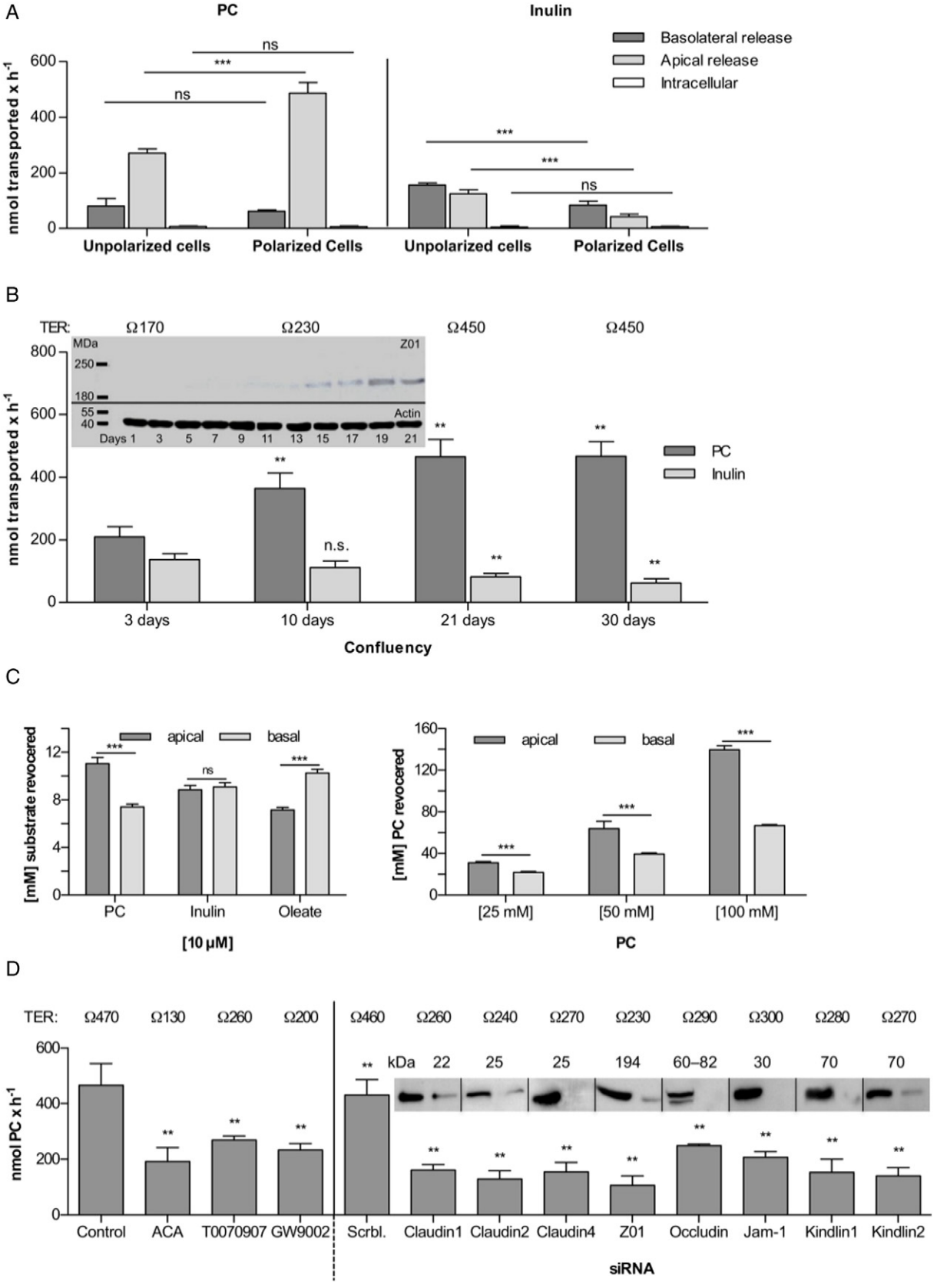
In contrast to the substantial amount of PC that appears at the apical compartment of the CaCo2 cells, there was only minute intracellular [^{3}H]PC detectable. Even when the pure radioactive tracer (100,000 cpm) of [^{3}H]PC (5 pmol) or of the PC precursor [^{3}H]choline chloride (8 pmol) were provided to the basal side of CaCo2 for 16 h, only 0.24 ± 0.12% and 0.18 ± 0.21% of the applied radioactivity were intracellularly recovered, respectively. It was comparable to the intracellular detection of the cell impermeable [^{14}C]inulin tracer with 0.16 ± 0.10%. It suggests that PC is not taken up or synthesized to such an amount that accounts for the observed transport to the apical compartment. Indeed, the PC transporter localized in the apical plasma membrane of hepatocytes, ABCB4 (MDR3), was not detectable in CaCo2 cells in contrast to the hepatocyte derived tumor cell line Hep G2 cells as proven by Western blotting and reverse transcription qPCR. The mRNA expression in relative units normalized to β-actin yielded 0.18 in CaCo2 cells and 114.83 in HepG2 cell. This is in agreement with earlier studies [11–13], although other investigators had reported a low level expression in CaCo2 cells [33,34].

The derived suggestion that there exists a paracellular movement of PC to the apical side was supported by an unaltered apical translocation capacity carried out at 25 °C (39.1 ± 2.1 nmol h^{-1} mM PC^{-1}), which would not be the case for a transcellular transport route. PC secretion occurred only in the apical direction, whereas apical release of inulin was reduced by >50% in polarized cells ($p < 0.001$) (Fig. 1A). With time of culturing, the apical translocation capacity of PC increased, while that of inulin decreased. This correlates with increased TJ formation determined by zonula occludens (ZO)-1 expression (Fig. 1B). Most convincingly, an equilibrium distribution study with PC, inulin, and oleate applied at equal concentrations to both the apical and basal side of polarized CaCo2 cells revealed a significant accumulation of PC and not of inulin in the apical compartment (Fig. 1C). In contrast, oleate accumulated within triglycerides in the basal compartment. TJ disruption triggered by treatment with acetaldehyde (ACA, 150 μM) [25] or a peroxisome proliferator-activated receptor γ inhibitor (T0070907, 10 μM or GW9002, 1 μM) [26] blocked PC accumulation in the apical side (Fig. 1D). On contrary, 150 μM ACA increased inulin secretion from 94 ± 7 to 207 ± 29 nmol h^{-1}. It is known that TJ complexes consist of constitutive proteins, e.g. claudin-1, -2, or -4, ZO-1, occludin and jam-1 [35], and are indirectly controlled by integrins regulated by kindlin 1 or 2 [36,37]. The knockdown of any of these proteins by siRNA resulted in disappearance of the respective protein, as demonstrated by Western blotting. The transepithelial resistance (TER) was in all cases reduced to ≤280 Ω, and PC translocation to the apical side dropped concomitantly (Fig. 1D, right). This indicates that the whole complex, but not an individual protein, mediates PC transport through the TJ.

We then visualized paracellular PC movement by incubating CaCo2 cells with 10 μM fluorescence-labeled PC for 16 h at 37 °C. This technique allows a live visualization of PC movement in the transwell culture system of CaCo2 cells. There was no intracellular staining of PC detectable. However, the three-dimensional high-resolution image (Fig. 2, left) showed small short spikes (16–33 pixels) between unpolarized cells, reflecting limited capacity for intercellular PC movement. In

contrast, the long wide rods (22–57 pixels) detected between polarized cells indicated an occupation of the paracellular space up to the apical TJ cell–cell adhesion complexes (Fig. 2, right).

In polarized cells, we observed a 2-fold increase in apical transport rates of phospholipids containing a positively charged choline head group – i.e., PC, lysophosphatidylcholine (LPC), and sphingomyelin (SM) – but not of other lipids, which had apical secretion rates of <1% (Fig. 3). This selectivity in the transport of choline-containing phospholipids across TJs was confirmed by ESI-MS/MS analysis. When we applied a phospholipid mixture of 1.0 mM PC,



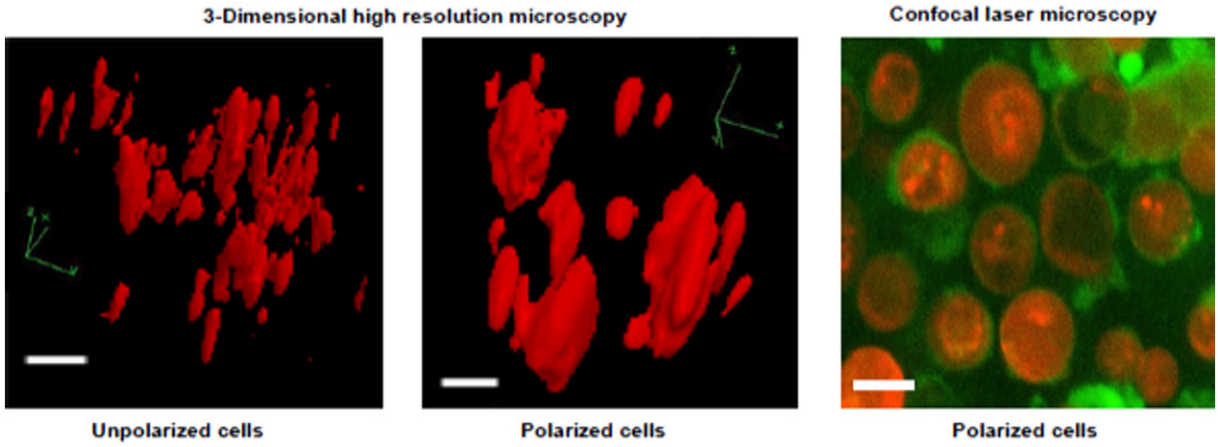


Fig. 2. Life visualization of paracellular PC movement observed in transwell Caco2 tissue culture systems as a function of cell polarization. High resolution microscopy after basal (top) exposure of 10 μM Bodipy PC for 16 h using 10 μM TC·PBS as vehicle. After washing in PBS 10 μM, Oregon Green 488 was added and fluorescent PC was detected in red color. The three-dimensional image shows small short spikes between unpolarized and long wide shaped rods between polarized cells indicating in polarized cells a full occupation of the paracellular space up to the apical TJ barrier. No intracellular staining of PC was detectable (bar = 1 μm).

LPC, SM, phosphatidylethanolamine (PE), phosphatidylinositol (PI), and phosphatidylserine (PS) to the basal compartment for 1 h, only the choline-containing phospholipids PC, LPC, and SM were apically translocated (Fig. 4).

For evaluation of the driving force for apical PC translocation, the pH and ionic milieu of the apical compartment were varied. PC translocation was optimal at pH 7.0 in a negatively charged apical environment. CFTR is known as the negative charge generator at apical epithelial cells by luminal secretion of Cl^- and bicarbonate [5] (Fig. 5). Knockdown of CFTR by siRNA blocked apical PC secretion (Fig. 6, right). Transmembrane-anchored, negatively charged mucins 3, 12, 13, and 17 [3] are apically located near CFTRs at the TJ barrier where they bind PC with high affinity. Here we tested the lipid binding capacity with different mucins. When mucins 1, 2 and 3 were exposed to a mixture of various lipids, only PC, SM and LPC were found to be bound to the mucins (Fig. 6). Accordingly, knockdown of mucosal plasma membrane bound mucin 3 reduced PC translocation to the luminal surface by 70% as compared to control siRNA (Fig. 5, right). This highlighted the role of mucin 3 in apical PC secretion. Knockdown of mucin 2, which is not produced by mucosal cells, consistently had no effect. Furthermore, an addition of TC or mucin 1 and 2 to the apical compartment as PC acceptor molecules mimicked the in vivo state in the ileum and increased translocation up to 8.1 ± 1.4% h^{-1} (Fig. 7).

4. Discussion

Here we demonstrated a paracellular transport route of PC across the TJ barrier of polarized CaCo2 cells.TJ assembly as a whole – and not pore-forming claudins – was responsible for apical PC translocation. Apical translocation was promoted by negative charge due to HCO_3^- and Cl^- accumulation through CFTR. At the luminal side, PC was shown to bind to mucin 3 anchored in the apical microvillus plasma membrane (Fig. 8). Thus, it is postulated that PC as a lipophilic substance traverses the TJ. It is not incorporated into the mucosal plasma membrane of enterocytes, i.e. its outer leaflet, where transport to the apical side is prevented by TJs. This route of monomeric PC translocation across the TJ contradicts the current molecular understanding of TJs, allowing ion but not substrate transport. The handling along this protein cascade is specific for the neutral zwitterionic phospholipids PC, LPC, and SM, which all contain a positively charged choline headgroup that is attracted to the negatively loaded mucin scaffold. In contrast, PE – which also contains a positive headgroup – was not transported, possibly due to its relatively low hydration, and thus higher capacity to form a compact hydration shield which results in a lower electrostatic potential [38,39]. Despite the principle transport capacity for SM, in vivo only PC and to a small degree LPC appear in mucus [40]. One explanation refers to the available sources of phospholipids for luminal secretion, as they are all mainly transported in the blood together with other lipid classes integrated into lipoproteins. Lipoproteins are unable to cross the vascular endothelium themselves; however, they exist in equilibrium with a plasma lipoprotein-free fraction (PLFF) that exclusively contains PC and LPC [41]. PLFF indeed traverses the vascular endothelial barrier, thus providing PC and LPC in a soluble form to the TJ barrier. After passage across the TJ barrier, PC is bound to mucin 3 and from there shifts to secretory mucin 2. The entire process of apical PC secretion serves to establish a luminally faced phospholipid bilayer that acts as a hydrophobic shield to prevent bacterial invasion (Fig. 8). According to the described translocation process, the density of PC is assumed to be highest in the inner layer of the mucus (bound to mucin 3) which is known to be impermeable to bacteria. Furthermore it is hypothesized that the outer layer of the mucus contains PC mainly bound to mucin 2. Driven by colonic motility, the outer layer can move distally to the rectum and serves as first line of defense. Only bacterial species with membrane bound ecto-phospholipases as pathogenetic factor may invade this layer.

The observation that PC translocates across intestinal epithelial cells by a paracellular pathway adds another PC transport mechanism to the

Fig. 1. PC transport through TJs examined in transwell tissue culture systems of CaCo2 cells. (A) Apical vs. basal transport of PC and inulin applied in the compartment positioned oppositely at concentrations of 10 mM over 1 h as a function of cell polarization. (B) Apical transport of 10 mM PC and inulin from the basal compartment over 1 h as a function of culture time of CaCo2 cells. The Western blot shown in inset illustrates the expression of Z01 as TJ marker over time of culture. TER in Ω was indicated in relation to number of days in culture. (C) Apical–basal equilibrium distribution of 10 mM PC, inulin and oleate in polarized CaCo2 transwell tissue culture systems after application of an equal concentration to both compartments for 1 h. (D) Apical PC release after basal application of 10 mM PC for 1 h following TJ disruption by ACA and peroxisome proliferator-activated receptor γ inhibitors (left), or (si)RNA knockdown of claudins 1, 2, 4, Z01, occludin, jam-1, kindlins 1 and 2 involved in TJ formation (right). Scrbl, control scrambled siRNA. Illustrated are apical PC transport rates, TER in Ω and reduction of the respective protein expression (left scrbl. control, right siRNA treated cells). Means ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant ($n = 6$).

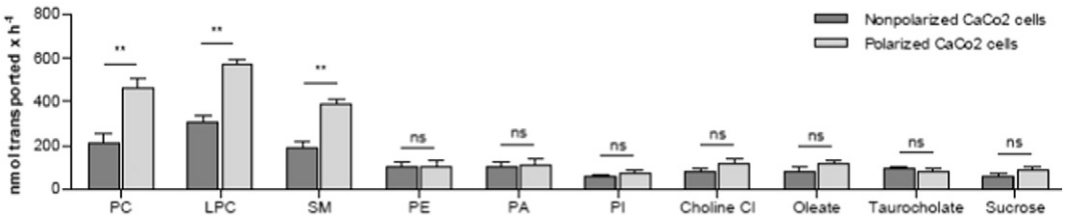


Fig. 3. Specificity of apical PC translocation observed in transwell CaCo2 tissue culture systems as a function of cell polarization. Enhanced apical release of the choline-containing phospholipid, PC, LPC and SM in comparison to PE, PA and PI and other substrates after basal application of indicated substrates (10 mM) for 1 h. Means ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant ($n = 6$).

known ABCB4 (MDR3) dependent PC carrier localized in the canalicular membrane of hepatocytes [9,42].

It is likely that also other epithelial surfaces with a PC-mucin assembly carry the feature of such a TJ mediated transport system. For example, this could be relevant in the biliary epithelium. Apically translocated PC could feed membrane bound mucins to establish a hydrophobic protective barrier against the biliary lumen. From there PC is handled to secretory mucins produced by the peribiliary glands [43]. The PC in bile is coupled to bile acids and, thus, may not be as easily accessible to these mucins. It was indeed demonstrated in our preliminary experiments with the human cholangiocyte derived cell line MzCha1 [44] that a paracellular TJ mediated, apical transport of PC exists. The

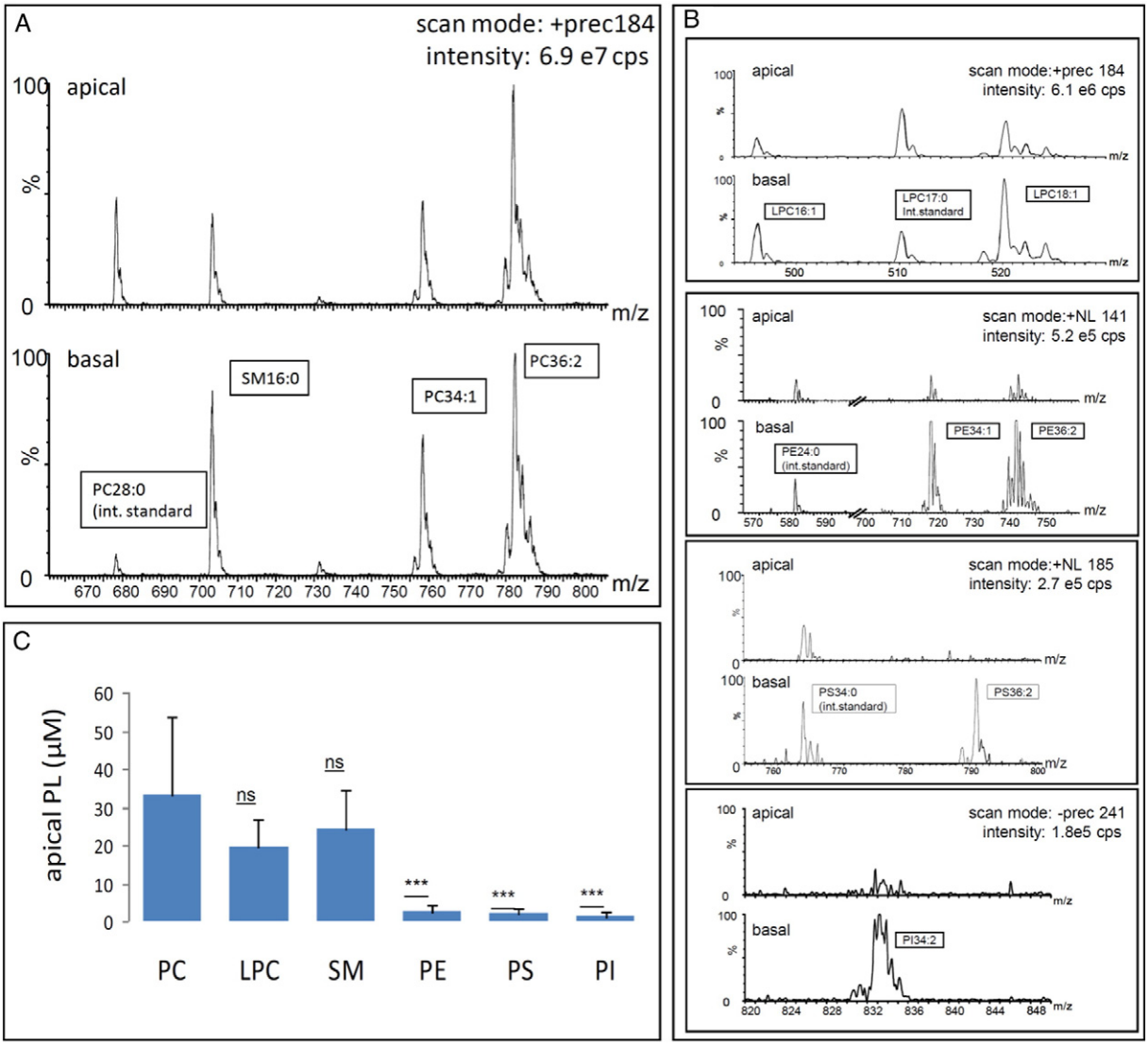


Fig. 4. ESI-MS/MS spectra of phospholipids apically transported from a basally applied mixture of 1 mM PC, LPC, SM, PE, PS, and PI in 6 mM TC·PBS for 1 h in a transwell, polarized CaCo2 tissue culture system. Phospholipids were monitored in positive (+) and negative (−) ion modes by precursor ion scans (prec184, prec241) and neutral loss scans (NL 141, NL 185). Internal standards of each PC (14:0/14:0), LPC (17:0), PE (12:0/12:0) and PI (17:0/17:0) at 20 ng were added as a reference to both compartments at the end of incubation. (A) Mass spectra of PC and SM in apical and basal compartments. (B) Mass spectra of LPC, PE, PS, and PI in apical and basal compartments. (C) Quantitative comparison of apically transported PC, PLC, SM, PE, PS and PI. Data represent means ± SD. $^{***}p < 0.001$; ns: not significant ($n = 3$).

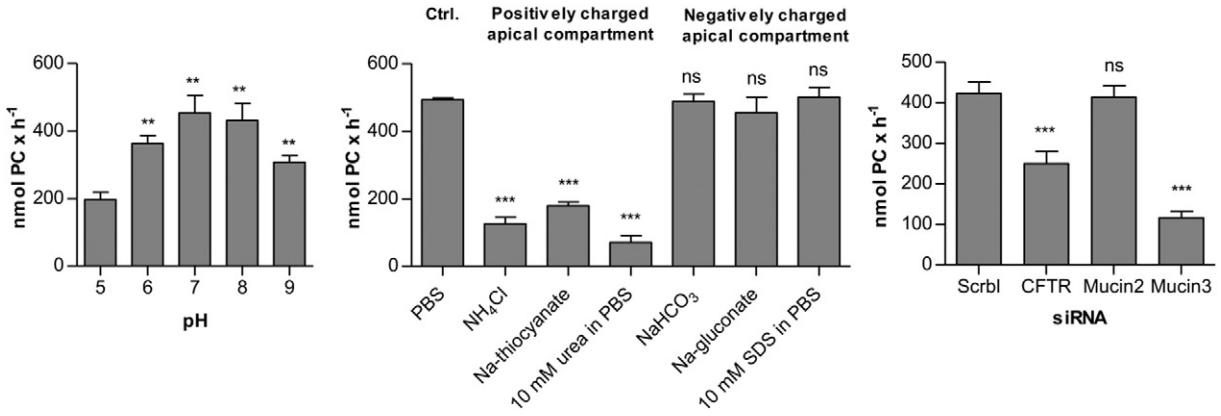


Fig. 5. Effect of pH, ions and mucins on apical translocation of 10 mM PC in 10 mM TC·PBS applied to the basal compartment of transwell polarized CaCo2 tissue culture system for 1 h. 1. Effect of pH 5–9 PBS in the apical compartment; 2. different apical isoosmotic ionic charge applications by exposure of NH_4Cl, Na-thiocyanate or urea generating positive medium charge, and $NAHCO_3$, Na-gluconate or SDS, generating negative medium charge; 3. knockdown of CFTR, mucin 2 or 3 by siRNA in comparison to control scrambled siRNA (scrbl), all applied for 16 h to the apical compartment. Means ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant ($n = 6$).

question arises now whether disturbance of this pathway results in a pathological condition. This is of particular interest for UC where an intrinsic low mucus PC content indicates a pathogenetic feature [23]. It will be the focus of further investigations employing mice with intestinal disruption of lateral TJ.

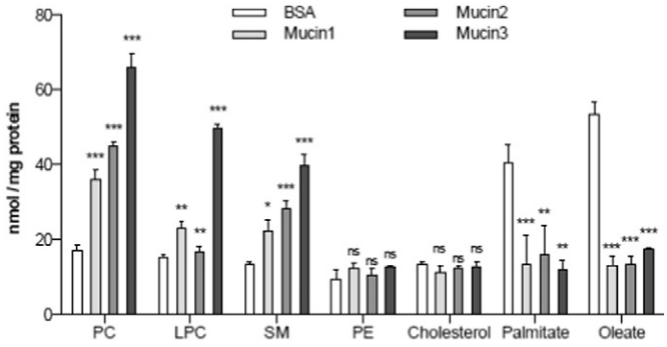


Fig. 6. Lipid-binding capacity of mucins 1, 2 and 3 versus albumin. In vitro, 1 mg of each protein was incubated in 1 ml 10 mM TC·PBS with 10 mM of various radiolabelled lipids for 1 h followed by immunoprecipitation with the respective antibody, washing of the pellets with PBS and determination of bound ligands. Significances are calculated between lipid binding to mucins versus albumin. Means ± SD. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$) ($n = 6$).

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Disclosures

None.

Competing interests

The adult children of Wolfgang Stremmel are shareholders of Lipid Therapeutics GmbH. All other authors have declared that no financial or other conflict of interest exists.

Author contributions

W.S. designed the study and wrote the manuscript, S.S. performed the kinetic experiments, H.G.-S. and A.G. were responsible for quantitative ESI-MS/MS phospholipid analysis, A.W. performed the statistical analyses and prepared the figures and M.B. carried out high-resolution microscopy experiments.

Transparency document

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

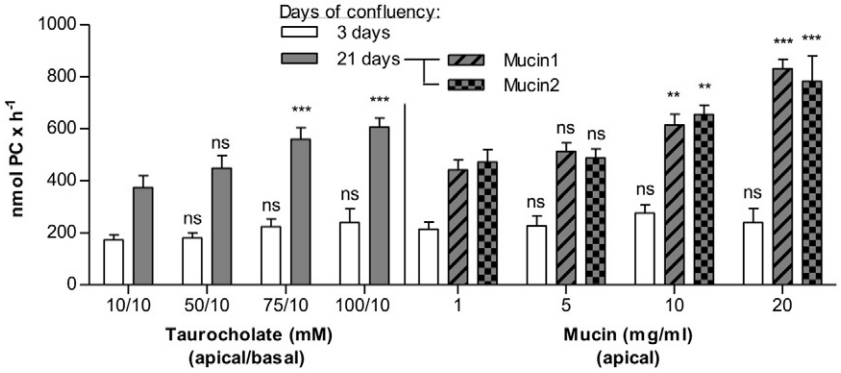

Fig. 7. Effect of increasing apical TC (10–100 mM) and mucins 1 and 2 (1–20 mg/ml) concentrations on apical PC translocation after basal application of 10 mM PC in 10 mM TC·PBS for 1 h in the transwell, polarized CaCo2 tissue culture system. Means ± SD. $^{**}p < 0.01$, $^{***}p < 0.001$, ns: not significant ($n = 6$).

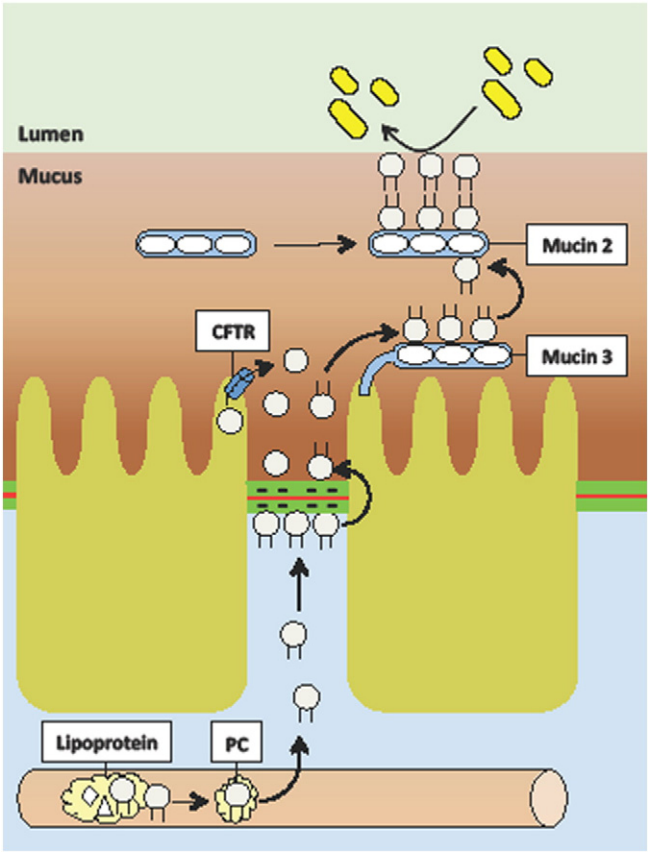

Fig. 8. Scheme illustrating the proposed paracellular TJ-mediated PC transport to the luminal side of intestinal epithelial cells. Originating from plasma lipoproteins and the segregated lipoprotein-free fraction, PC selectively accumulates in mucus via paracellular, tight junction (TJ)-dependent transport. Transport is driven by a negative electrical gradient, generated by CFTR mediated apical secretion of Cl^- and HCO_3^-. Consequently, PC binds to membrane-localized mucin 3 from where it shifts to secretory mucin 2.

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