Retinal pigment epithelial cells exhibit unique expression and localization of plasma membrane syntaxins which may contribute to their trafficking phenotype

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Summary

The SNARE membrane fusion machinery controls the fusion of transport vesicles with the apical and basolateral plasma-membrane domains of epithelial cells and is implicated in the specificity of polarized trafficking. To test the hypothesis that differential expression and localization of SNAREs may be a mechanism that contributes to cell-type-specific polarity of different proteins, we studied the expression and distribution of plasma-membrane SNAREs in the retinal pigment epithelium (RPE), an epithelium in which the targeting and steady-state polarity of several plasma membrane proteins differs from most other epithelia. We show here that retinal pigment epithelial cells both in vitro and in vivo differ significantly from MDCK cells and other epithelial cells in their complement of expressed t-SNAREs that are known – or suggested – to be involved in plasma membrane trafficking. Retinal pigment epithelial cells lack expression of the normally apical-specific syntaxin 3. Instead, they express syntaxins 1A and 1B, which are normally restricted to neurons and neuroendocrine cells, on their apical plasma membrane. The polarity of syntaxin 2 is reversed in retinal pigment epithelial cells, and it localizes to a narrow band on the lateral plasma membrane adjacent to the tight junctions. In addition, syntaxin 4 and the v-SNARE endobrevin/VAMP-8 localize to this sub-tight junctional domain, which suggests that this is a region of preferred vesicle exocytosis. Altogether, these data suggest that the unique polarity of many retinal pigment epithelial proteins results from differential expression and distribution of SNAREs at the plasma membrane. We propose that regulation of the expression and subcellular localization of plasma membrane SNAREs may be a general mechanism that contributes to the establishment of distinct sorting phenotypes among epithelial cell types.

Key words: Epithelial polarity, Membrane traffic, SNARE

Introduction

Epithelial cells exhibit characteristic polarized distribution patterns of proteins and lipids on their apical and basolateral plasma-membrane domains (Mostov et al., 2000; Yeaman et al., 1999). This polarity is essential for proper epithelial function. Polarized protein transport to the plasma membrane of epithelial cells depends on highly specific vesicular membrane traffic pathways that recognize sorting signals encoded in the cargo proteins.

In higher metazoan organisms a large variety of epithelial cell types perform a multitude of different functions. The apical surfaces of these cell types can face dramatically different environments. Extreme examples are epithelial cells lining the stomach, colon, bile duct, urinary tract, and the retinal pigment epithelium (RPE). Correspondingly, different epithelial cell types require different sets of apical and basolateral surface proteins to perform their unique roles in the body.

In many cases, apical and basolateral sorting signals are recognized and interpreted identically between different epithelial cell types. In other cases, identical proteins can be sorted to different plasma-membrane domains in different epithelial cell types. One example is the Na^+/K^+-ATPase, which is localized to the basolateral domain in the vast majority of epithelial cells types. However, in a few epithelia, such as the RPE and the choroid plexus epithelium, the Na^+/K^+-ATPase localizes apically (Marmorstein, 2001; Marrs et al., 1995). Proteins can also be ultimately targeted to the same domain but the pathway that they take to reach this domain differs between cell types. For example, in the Madin Darby canine kidney (MDCK) cell line, newly synthesized influenza virus hemagglutinin (HA) is directly targeted from the Golgi to the apical plasma membrane (Matlin and Simons, 1984; Misek et al., 1984). By contrast, in the RPE it is first transported to the basolateral plasma membrane and subsequently endocytosed and transcytosed to the apical surface (Bonilha et al., 1997). It is currently unknown how the variability in sorting phenotypes between different epithelial cell types is accomplished.

The RPE has long been recognized to differ from many other epithelia in the polarity of a number of proteins (Marmorstein, 2001). A unique characteristic of RPE cells is that their apical plasma membrane is in contact with the extracellular matrix (the interphotoreceptor matrix) whereas almost all other epithelia face an apical lumen devoid of matrix. Other proteins that are sorted differently in RPE compared with most epithelia include the extracellular matrix metalloproteinase inducer (EMMPRIN; apical) (Marmorstein et al., 1998), N-CAM (apical) (Marmorstein et al., 1998), αvβ5 integrin (apical) (Finnemann et al., 1997), and possibly CFTR, which is thought to be basolateral in RPE cells but is apically localized in other epithelia (Gallemore et al., 1998). Furthermore, physiological experiments have demonstrated that Ca^{2+}-sensitive chloride channels, which are typically present on the apical membrane of epithelial cells, are basolaterally polarized in RPE cells (Gallemore et al., 1998).

It is unknown whether the establishment of different epithelial sorting phenotypes involves major changes in the machineries involved in polarized targeting or simply the rerouting of proteins into different sorting pathways. SNARE-mediated membrane fusion is the final step in all vesicle trafficking pathways (Chen and Scheller, 2001; Jahn and Sudhof, 1999). SNAREs belong to several related protein families (Weimbs et al., 1997; Weimbs et al., 1998), and different family members usually exhibit a distinct subcellular localization and function only in specific trafficking pathways. Only 'matching' combinations of v- and t-SNAREs lead to successful membrane fusion (McNew et al., 2000; Scales et al., 2000), suggesting that the SNARE machinery plays a role not only in the mechanics of the fusion process but also in its specificity. Members of the syntaxin family of t-SNAREs appear to play a central role since syntaxins can interact with virtually all other components implicated in SNARE-mediated membrane fusion. It is reasonable to assume that the presence of a given syntaxin on a membrane domain determines which classes of transport vesicles will be able to fuse with that domain. We hypothesized that a feasible mechanism contributing to the known variety of epithelial trafficking phenotypes is for different epithelial cell types to express different sets of plasma membrane syntaxins and/or to localize them to different domains.

The best characterized epithelial model system to date is the MDCK cell line. We have previously shown that in these cells the t-SNAREs syntaxin 3 and 4 are specifically localized at the apical or basolateral plasma membrane domains, respectively (Low et al., 1996). Syntaxin 3 is functionally involved in trafficking from the TGN to the apical plasma membrane and in apical recycling (Low et al., 1998a). Syntaxin 4 is involved in TGN-to-basolateral trafficking (Lafont et al., 1999). Two other syntaxins, syntaxin 2 and 11, are also expressed at the plasma membrane in MDCK cells but they are present in both domains and their function is unknown (Low et al., 1996; Low et al., 2000). In general, the localization of these t-SNAREs appears to be well conserved in a number of other epithelial cell lines and tissues such as Caco-2 cells, HepG2 cells, hepatocytes, kidney epithelium and intestinal epithelium (Delgrossi et al., 1997; Fujita et al., 1998; Galli et al., 1998; Lehtonen et al., 1999; Li et al., 2002; Low et al., 1998b; Riento et al., 1998). Syntaxin 3, however, has been found to deviate from its normal apical plasma membrane localization in two specialized epithelial cell types. It localizes to zymogen granules in pancreatic acinar cells (Gaisano et al., 1996) and to H^+/K^+-ATPase-containing tubulovesicles in non-stimulated gastric parietal cells (Peng et al., 1997). Moreover, syntaxin 2 localizes to the apical plasma-membrane domain in pancreatic acinar cells (Gaisano et al., 1996) in contrast to its non-polarized distribution in MDCK cells.

We hypothesized that changes in SNARE expression and/or subcellular localization may contribute to the differential sorting phenotypes of specialized epithelial cell types. To test this, we investigated the expression and localization of the plasma membrane SNARE machinery in RPE cells in vitro and in vivo. We report here that RPE cells in vitro and in vivo differ significantly from MDCK cells and other epithelial cells in the expressed complement of SNARE proteins as well as in their subcellular localization. Altogether, our results suggest that the differential expression and subcellular localization of SNAREs is used as a general mechanism contributing to the modulation of epithelial sorting phenotypes and is at least in part responsible for the unique distribution of plasma membrane proteins in the RPE.

Materials and Methods

Materials

Cell culture media were from the Lerner Research Institute Cell Culture Facility. Fetal bovine serum was from ICN Biomedicals (Costa Mesa, CA). Transwell polycarbonate cell culture filters were purchased from Corning Costar Corporation (Massachusetts, MA). Affinity-purified polyclonal antibodies against the cytoplasmic domains rat syntaxins 2, 3 and 4 and against an C-terminal peptide of human SNAP-23 have been described previously (Low et al., 2000; Low et al., 1998b). Rabbit antiserum was raised against a GST fusion protein of the cytoplasmic domain of rat endobrevin. The expression plasmid was a gift from Wanjin Hong (Institute for Molecular and Cell Biology, Singapore). The endobrevin antibody was affinity-purified against the immobilized cytoplasmic domain of endobrevin without GST. In addition, a similarly raised and affinity-purified rabbit antibody against endobrevin was a gift from Wanjin Hong. Affinity-purified polyclonal antibodies against rat syntaxins 1A, 1B, 2, 3 and 4 were gifts from Mark Bennett and Beatriz Quinones (UC Berkeley). A polyclonal antibody against a peptide from syntaxin 1B (peptide sequence identical in mouse, rat, cow, human) was from Synaptic Systems GmbH (Göttingen, Germany). A mouse monoclonal antibody against occludin was from Zymed Laboratories (South San Francisco, CA). A rat monoclonal antibody against ZO-1 was from Chemicon International (Temacula, CA). Secondary antibodies, cross-absorbed against multiple species and conjugated to FITC, Texas Red or Cy5 were from Jackson Immunoresearch (West Grove, PA).

Cell culture

RPE-J cells were from ATCC (CRL-2240) and cultured in Dulbecco's Modified Eagle's medium containing 4 mM glutamine, 1.5 g/l sodium bicarbonate, 4.5 g/l glucose and 1 mM sodium pyruvate. This media was further supplemented with 4% FBS, non-essential amino acids and penicillin and streptomycin. The cells were maintained at 32°C and 5% CO_2. For polarized monolayers, the cells were plated at 300,000 cells/cm^2 on Matrigel-coated Transwell Filters (Corning, Acton, MA) in culture medium supplemented with 2.5 nM retinoic acid and cultured for 1 week at 32°C. The cells were then transferred to a 40°C incubator for another week. The media was changed every 3 days.

Tissue and cell extracts, SDS-PAGE and immunoblotting

Total protein fractions of RPE-J cells were prepared by directly lysing cells from confluent dishes by boiling in SDS-PAGE sample buffer.

DNA was sheared by passing lysates through a 22G needle. Rat kidney and brain lysates were prepared by dissecting out the tissues and finely mincing with a razor blade. The tissues were then Dounce-homogenized in PBS containing 10 mM EDTA, protease inhibitors and PMSF. SDS was added to a final concentration of 2% and the lysate was passed through a 22G needle to shear the DNA, and the sample was boiled for 5 minutes. Proteins were separated on 10% or 15% SDS-polyacrylamide gels followed by transfer to PVDF membrane and incubation with the indicated antibodies. Bands were visualized by enhanced chemiluminescence.

RT-PCR

Total RNA was isolated from RPE-J cells, rat kidney or rat brain by using Trizol (GIBCO) according to the manufacturer's instructions. 5 μg of total RNA were used for reverse transcription in a 25 μl total volume using random hexamers as primers. For the detection of syntaxin 2 isoforms, 1 μl of the reverse-transcribed samples were used for 50 μl PCR reactions using primers and conditions as described previously (Quinones et al., 1999). The following primer pairs were designed to recognize rat syntaxin 3A; 5′-GCTGAGATGTTAG-ATAACATAG-3′ and 5′-TTCAGCCCAACGGACAATCCAA-3′ or syntaxin 3B; 5′-CAGGGAGCCATGATTGACCGTA-3′ and 5′-AAATATGCCCCCAATGGTAGAA-3′, utilizing the same PCR conditions as for syntaxin 2. 10 μl of each PCR reaction were separated on 2% agarose gels.

Immunolocalization

Sprague-Dawley rats were anesthetized by intraperitoneal administration of sodium pentobarbital (50 mg/kg) and perfused via the left ventricle with 4% paraformaldehyde in PBS containing 1 mM calcium and 1 mM magnesium for 20 minutes. The eyes were enucleated, the anterior segments removed, and the eyecups stored in the same fixative overnight at 4°C. Fixed eyecups were then dehydrated and embedded in paraffin. Immunostaining was carried out on 5 μm sections. After deparaffinization and rehydration to PBS, the sections were subjected to heat-mediated antigen retrieval by pressure cooking in 10 mM citric acid buffer, pH 6.0. The sections were blocked with 3% BSA, 2% Triton X-100 in PBS, incubated with the indicated antibodies overnight at 4°C. The reactions were visualized with fluorescein- and Texas-Red-labeled secondary antibodies (Jackson ImmunoResearch, West Grove, PA). SNARE-signals were amplified by incubating with Alexa 488-labeled rabbit anti-FITC antibody (Molecular Probes) after FITC-labeled secondary antibodies. Nuclei were stained with DAPI. The fluorescent staining was analyzed using a confocal laser scanning microscope (TCS-NT, Leica, Benseim, Germany). Immunostaining for syntaxins 1B, 2, 3, 4 and endobrevin was verified using two independently raised antibodies each of which resulted in identical staining patterns. Only one antibody was available against syntaxin 1A.

For immunostaining of cultured RPE-J cells, cells were either fixed in methanol at –20°C or with 4% paraformaldehyde in PBS, permeabilized with 0.025% (wt/vol) saponin Sigma (St. Louis, MO) in PBS and blocked with 3% BSA followed by sequential incubations with primary antibodies and FITC- and Texas-red-conjugated secondary antibodies. Signal amplification and imaging were performed as described above.

Results

SNARE protein expression pattern in RPE-J cells

To investigate the expression pattern in RPE cells of t- and v-SNAREs that are normally involved in plasma membrane fusion in other cell types we made use of a cell line derived from rat RPE. These cells, RPE-J, were immortalized by infection with a temperature-sensitive SV40 virus (Nabi et al., 1993). RPE-J cells polarize in culture and have many of the attributes of native RPE, such as the ability to phagocytose photoreceptor outer segments (Finnemann et al., 1997). Total lysates of polarized RPE-J cells were separated by SDS-PAGE and probed by western blot using a panel of SNARE-specific antibodies. For comparison, equal amounts of total homogenate from rat kidney or 1/10 of the amount of total rat brain homogenate were investigated side-by-side. Among the SNAREs that are expressed in MDCK and other epithelial cells, the expression levels of syntaxins 2, 4 and SNAP-23 are roughly comparable between RPE-J cells and kidney (Fig. 1A). By contrast, syntaxin 3 was undetectable in RPE-J cells. The v-SNAREs cellubrevin and endobrevin are found in both kidney and RPE-J cells.

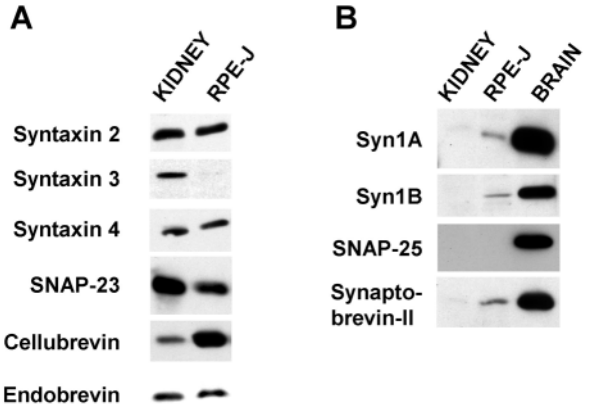

Fig. 1. Comparison of SNARE expression in RPE-J cells and rat kidney or brain by western blot analysis. Equal protein amounts of total homogenates of differentiated RPE-J cells, rat kidney or rat brain (1/10 of the protein) were separated by SDS-PAGE and analyzed by western blot with specific antibodies as indicated. Note that syntaxin 3 is virtually undetectable in RPE-J cells. By contrast, the expression levels of syntaxins 2 and 4 are roughly comparable between RPE-J and rat kidney. SNAP-23, cellubrevin and endobrevin are expressed in both kidney and RPE-J. RPE-J cells also express the 'neuron-specific' t-SNAREs syntaxin 1A and 1B but there is no detectable SNAP-25.

Next, we investigated the possible expression of SNAREs that are typically restricted to neurons and neuroendocrine cells. Brain tissue contains very large amounts of the t-SNAREs syntaxin 1A, syntaxin 1B, SNAP-25 and of the v-SNARE synaptobrevin-2, all of which are involved in calcium-regulated synaptic vesicle exocytosis. Surprisingly, RPE-J cells expressed significant quantities of syntaxins 1A, 1B and synaptobrevin-2 (Fig. 1B). However, no SNAP-25 was detectable even after prolonged exposure of the blot (data not shown). None of these SNAREs were detectable in kidney, with the exception of low amounts of synaptobrevin-2.

In summary, the SNARE expression pattern of RPE-J cells shows two unexpected features: the absence of the normally apical-specific syntaxin 3 and the presence of 'neuronal' SNAREs.

Expression of syntaxin 2 and 3 transcripts in RPE-J cells

Four syntaxin 2 isoforms derived from alternative RNA

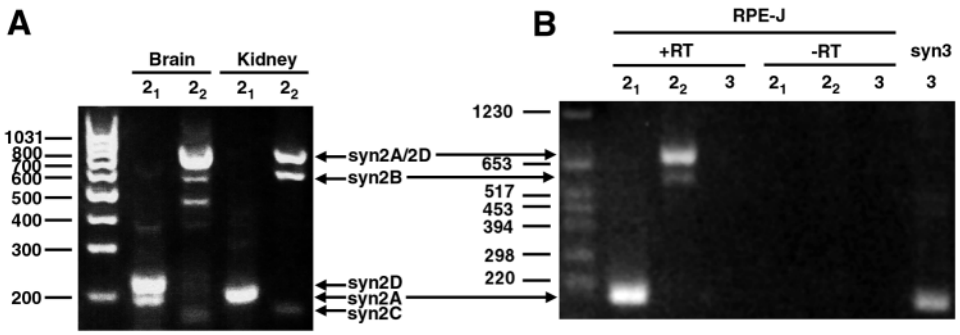

Fig. 2. RT-PCR analysis of syntaxin isoform expression in RPE-J cells. mRNA of syntaxin 3 and of different alternatively spliced isoforms of syntaxin 2 were amplified by RT-PCR. For syntaxin 2 isoform determination, two primer pairs were used that had been previously shown to distinguish between isoforms 2A, 2B, 2C and 2D (Quinones et al., 1999). Primer combination 2_1 generates the following PCR products: syntaxin 2A (200 bp), 2D (228 bp), no products for syntaxin 2B and 2C. Primer combination 2_2 generates the following PCR products: syntaxin 2A (700 bp), 2B (600 bp), 2C (170 bp), 2C 170 bp), 2D (728 bp). The respective positions of the different products are indicated by arrows. A shows that transcripts for syntaxin 2A, B and C can be detected in rat kidney. Rat brain expresses all four syntaxin 2 isoforms as previously reported. As shown in B, reaction products for syntaxins 2A and 2B were detected from RPE-J cells but not 2C, 2D. For the detection of syntaxin 3A transcripts, a single primer pair was used. B shows that syntaxin-3A-specific reaction product was detected in the positive control (lane 'syn3', rat syntaxin 3A cDNA as template), whereas no syntaxin 3A product could be detected from RPE-J cells. As a negative control, no products were detected in samples that were not reverse transcribed (-RT).

splicing have previously been identified in rat tissues. Syntaxins 2A and 2B are membrane anchored by hydrophobic domains, whereas syntaxin 2C and 2D lack hydrophobic domains and are only partially membrane bound (Quinones et al., 1999). All four syntaxin 2 isoforms differ only in their C-termini whereas the N-terminal bulk of their sequences are identical. Since our polyclonal syntaxin 2 antibodies react with all four isoforms (data not shown) and since the molecular weights of these isoforms are very similar, they can not be distinguished from each other by western blot analysis. In order to investigate which syntaxin 2 isoforms are expressed in RPE-J cells and to confirm the observed absence of syntaxin 3-expression, we analyzed total RNA by RT-PCR. We used two primer combinations that have previously been shown to distinguish between the four different rat syntaxin 2 isoforms (Quinones et al., 1999). Fig. 2A shows that all four syntaxin 2 isoforms could be detected in rat brain whereas rat kidney expressed syntaxins 2A, B and C as previously described. By contrast, in RPE-J cells, syntaxins 2A and a lesser amount of syntaxin 2B, but no transcripts for syntaxins 2C or 2D, could be detected (Fig. 2). No syntaxin 3 transcripts were detectable, confirming the lack of expression observed on the protein level.

SNARE localization in RPE-J cells

To determine the subcellular localization of the expressed t-SNAREs, RPE-J cells were cultured as polarized monolayers on Matrigel-coated polycarbonate filters, labeled with affinity-purified syntaxin antibodies and analyzed by confocal fluorescence microscopy. As expected, syntaxin 3 was undetectable (data not shown). Fig. 3 shows that both syntaxins 2 and 4 localize to the plasma membrane at the regions of cell-cell contact whereas no significant apical staining was detectable. Both syntaxins 2 and 4 colocalize with the basolateral marker EMMPRIN as well as with the tight junction protein ZO-1. Since RPE-J cells are typically flat (Marmorstein et al., 1998), it was not possible to determine by light microscopy whether syntaxin 2 and 4 are distributed all along the lateral membrane or whether they are concentrated close to the tight junctions. The expression levels of syntaxins 1A and 1B were below the detection limit.

In conclusion, syntaxin 4 localizes to the basolateral plasma membrane domain in RPE-J cells similar to all previously investigated epithelial cell types. However, syntaxin 2 localizes to the basolateral plasma membrane domain, which is in striking contrast to its apical localization in other epithelial cell types.

SNARE localization in RPE in situ

RPE-J cells and other cultured RPE cells have been found to differ in their protein-sorting phenotype from RPE cells in situ. Na^+/K^+-ATPase, N-CAM and EMMPRIN, which are apically polarized in RPE cells in the eye, are typically non-polar or basolateral in culture (Marmorstein, 2001). We therefore investigated SNARE expression and subcellular localization in rat retina. Sections of the posterior pole of rat eyes were fluorescently double-labeled using SNARE-specific antibodies and antibodies against various marker proteins and analyzed by confocal microscopy. Cryosections and paraffin sections yielded identical results. Fig. 4A shows that syntaxin 3 is abundantly expressed in photoreceptor cells but is not detectable above background in RPE cells, in agreement with our results on RPE-J cells.

As predicted from our studies on RPE-J cells, syntaxins 2, 4, 1A and 1B are all clearly detectable in RPE cells in situ. Syntaxins 2 and 4 exhibit a surprising subcellular localization. Syntaxin 2 is restricted to a narrow band that localizes closely with the tight junctional protein occludin. At higher magnification it was apparent that these two proteins do not exactly overlap but that syntaxin 2 localizes to a region of the lateral plasma membrane basal to the tight junctions (Fig. 4D). Since the tight junctions represent the border between the apical and basolateral plasma membrane domains in epithelial cells, syntaxin 2 is therefore a basolateral SNARE in RPE cells in contrast to its apical localization in other epithelial cells. This is in agreement with our results on RPE-J cells.

Syntaxin 4 also localizes to the same narrow band underneath the tight junctions. However, in contrast to syntaxin 2, it is also clearly present at the basal plasma membrane of RPE cells (Fig. 4E). Both syntaxin 1A (Fig. 4B) and syntaxin 1B (Fig. 4C) are localized throughout the apical plasma

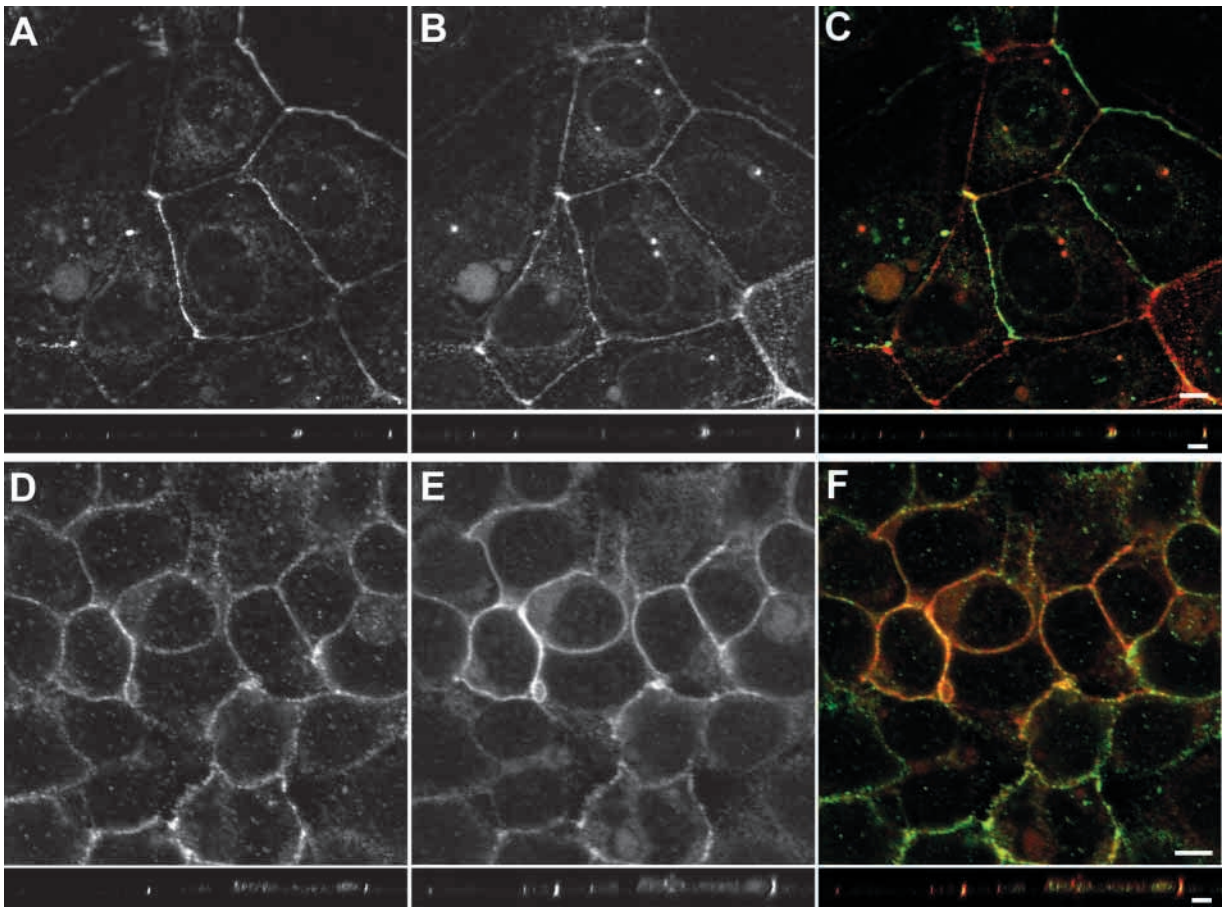

Fig. 3. Localization of syntaxins 2 and 4 in RPE-J cells. Polarized monolayers of RPE-J cells were cultured on Transwell filters, and syntaxin localizations were analyzed by double-label confocal immunofluorescence microscopy. Panels A-C and D-F, respectively, represent the same fields of xy confocal sections. Small panels underneath are xz confocal sections. A shows immunostaining for syntaxin 2, B for the tight junction protein ZO-1 and C the color-merged image. D shows immunostaining for syntaxin 4, E for the basolateral plasma membrane protein EMMPRIN and F the color-merged image. Note that syntaxin 2 and ZO-1 colocalize throughout most of the areas of cell-cell contact. However, the localization of ZO-1 at the tight junctions is more uniform. There are areas of intense syntaxin 2 staining, whereas others are nearly devoid of it. Syntaxin 4 and EMMPRIN both colocalize in a relatively uniform fashion at the areas of cell-cell contact. Bars, 5 μm.

membrane in RPE cells, which constitutes 75% of the total RPE plasma membrane owing to microvilli that interdigitate with the photoreceptor outer segments. The outer segments themselves are negative for both syntaxins.

Endobrevin is a v-SNARE that has been found to be most highly expressed in epithelial cells. It has been implicated in endosome fusion in non-epithelial cells (Antonin et al., 2000) but has also been shown to cycle through the apical plasma membrane in MDCK cells (Steegmaier et al., 2000). Fig. 4F shows that endobrevin was surprisingly also highly concentrated underneath the tight junctions in RPE cells similar to syntaxins 2 and 4. In addition, endobrevin localized to intracellular vesicles throughout the cytoplasm.

Immunostaining results for syntaxins 1B, 2, 3, 4 and endobrevin were confirmed using independently raised antibodies and yielded identical results (see Materials and Methods, data not shown).

Discussion

The retinal pigment epithelium is a prime example of an epithelial cell type exhibiting unique cell polarity and intracellular trafficking (Marmorstein, 2001). Other examples include the α- and β-intercalated cells of the renal connecting tubule (Al-Awqati et al., 1998), the choroid plexus epithelium, the Fischer rat thyroid (FRT) cell line and the renal LLC-PK_1 cell line. In these epithelial cells, the targeting of several plasma membrane proteins was found to differ from that of the MDCK cell line – the most frequently studied model system. So far, only in the case of LLC-PK_1 cells has a molecular cause of the different targeting phenotype been identified. This cell line lacks expression of the μ1B subunit of the epithelial-specific AP-1B adaptor, which results in the inability to recognize tyrosine-based basolateral targeting signals (Fölsch et al., 1999; Fölsch et al., 2001) in the endocytic pathway (Gan et al., 2002). Consequently, several normally basolateral proteins are apically mistargeted in LLC-PK_1 cells. It is, however, unclear whether the absence of μ1B expression represents a defect or may indeed be used by epithelial cell types in vivo to achieve a certain sorting phenotype. FRT cells are known to target most GPI-anchored proteins to the basolateral plasma membrane, whereas they are apically

targeted in MDCK and most other epithelial cells (Zurzolo et al., 1993). The relevance of the sorting phenotype in the FRT cell line is unclear, however, since GPI-anchored proteins are correctly apically targeted in primary thyroid epithelial cells (Kuliawat et al., 1995).

In the present work we tested our hypothesis that the expression of different sets of plasma membrane syntaxins and/or their localization to different domains is a mechanism contributing to the known variety of epithelial trafficking phenotypes. Our investigation of this possibility using RPE cells as an example of an epithelium with a polarity phenotype that diverges substantially from the prototypic MDCK cell is in agreement with this hypothesis. Fig. 5 shows a schematic summary of the results.

RPE cells both in vitro and in vivo lack expression of the normally apical-specific syntaxin 3. This may suggest that a trafficking pathway that normally involves syntaxin 3 is absent in this cell type. This finding is in excellent agreement with a known difference in the targeting of the influenza virus hemagglutinin (HA) in RPE cells. We have previously shown that syntaxin 3 functions in transport from the trans-Golgi network to the apical plasma membrane in MDCK cells (Low et al., 1998a) (route A in Fig. 5). This route is taken by newly synthesized HA in MDCK cells (Matlin and Simons, 1984; Misek et al., 1984), and indeed Lafont et al. could demonstrate that apical HA-trafficking in MDCK cells is syntaxin 3 dependent (Lafont et al., 1999). By contrast, newly synthesized HA is initially transported to the basolateral plasma membrane domain in RPE-J cells and in RPE cells in situ (Bonilha et al., 1997) (route B_1 or B_2) and is subsequently transcytosed to the apical domain (route T). HA therefore bypasses the direct TGN-to-apical route in this cell type. Importantly, our previous results indicated that syntaxin 3 is not involved in basolateral-to-apical transcytosis (route T) in MDCK cells, suggesting that this step requires another (unidentified) syntaxin. The absence of syntaxin 3 in RPE cells therefore agrees well with the itinerary of HA in this cell type, which avoids syntaxin-dependent trafficking routes.

A direct TGN-to-apical route does exist in RPE cells, however, and is taken by cargo proteins such as p75-NTR, VEGF-165, TGF-β, (Marmorstein et al., 2000; Marmorstein et al., 1998) and retinol binding protein and transthyreitin (Jaworowski et al., 1995). Since this occurs in the absence of syntaxin 3 we propose that an alternative route exists in RPE cells (route A_2) that utilizes a different syntaxin and may or may not exist in MDCK cells. Likely candidates are syntaxin 1A or 1B, which we found to be expressed at the apical plasma membrane in RPE cells. These two syntaxins are usually expressed in neurons and neuroendocrine cells and function in calcium-regulated exocytosis such as synaptic vesicle fusion with the presynaptic plasma membrane. An intriguing speculation is that apical vesicle fusion in RPE cells may be calcium regulated.

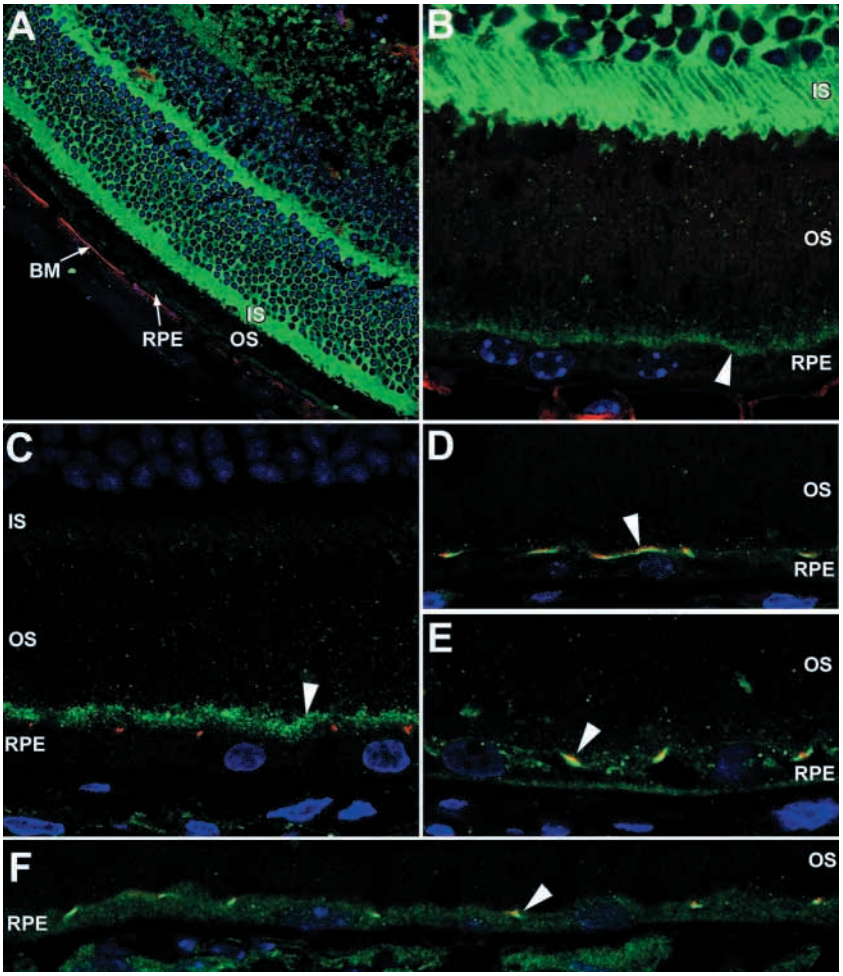

Fig. 4. Expression and subcellular localization of SNAREs in vivo. Rat eye tissue sections were double-labeled with specific antibodies and analyzed by confocal fluorescence microscopy. In all panels, the respective SNARE is in green, staining for the double-label is in red and the nuclear stain is in blue. (A) Syntaxin 3 (green), collagen-IV (red). Note that syntaxin 3 is expressed in the photoreceptor inner segments (IS) but is not detectable in the photoreceptor outer segments (OS) or the RPE cells. RPE cells are identified by the collagen-IV-positive basement membrane (BM). (B) Syntaxin 1A (green), collagen-IV (red). Note the expression of syntaxin 1A at the apical plasma membrane of RPE cells (arrowhead) that is in contact with the photoreceptor outer segments, which themselves are negative. (C) Syntaxin 1B (green), occludin (red). Note the syntaxin 1B signal on the apical plasma membrane of the RPE cells (arrowhead) above the tight junctions identified by occludin-staining. (D) Syntaxin 2 (green), occludin (red). Note that syntaxin 2 localizes to a narrow band just underneath the occludin-positive tight junctions. Syntaxin 2 does not overlap with occludin but is instead always towards the basolateral side of the cells, indicating that it localized to a region of the lateral plasma membrane that is adjacent to the tight junctions. (E) Syntaxin 4 (green), occludin (red). Similar to syntaxin 2, syntaxin 4 localizes to a narrow band underneath the occludin-positive tight junctions (arrowhead). In addition, syntaxin 4 staining is evident at the basal plasma membrane and in intracellular vesicles that are often perinuclear. (F) Endobrevin (green), occludin (red). Note that endobrevin localizes to narrow a band underneath the tight junctions (arrowhead) in addition to localizing in small vesicles that are diffusely distributed throughout the cytoplasm of RPE cells.

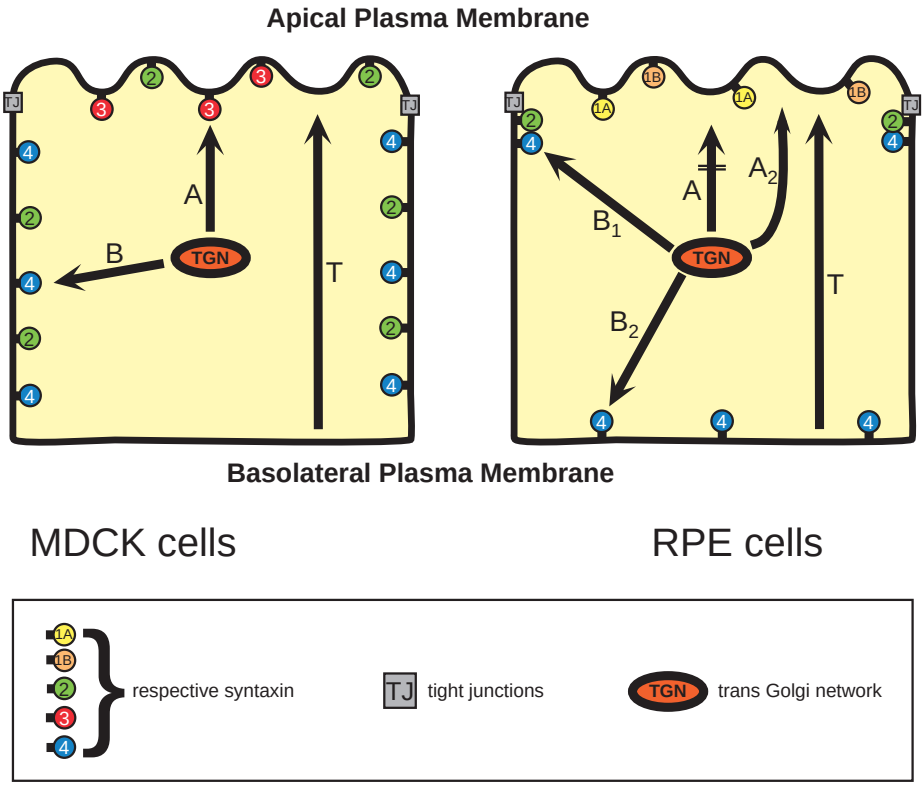

Fig. 5. Schematic models of syntaxin distribution and polarized trafficking pathways in MDCK and RPE cells. The subcellular localizations of syntaxins are indicated as reported previously in MDCK cells (Low et al., 1996) and in the present work in RPE cells. In MDCK cells, trafficking from the TGN to the apical plasma membrane (route A) was previously shown to involve syntaxin 3 (Low et al., 1998a), whereas trafficking from the TGN to the basolateral plasma membrane (route B) was shown to involve syntaxin 4 (Lafont et al., 1999). By contrast, basolateral-to-apical transcytosis is independent of syntaxin 3 in MDCK cells (Low et al., 1998a). The absence of syntaxin 3 in RPE cells suggests that route A does not exist in this cell type but that probably an alternative route designated A_2 exists. This alternative route may depend on syntaxin 1A and/or 1B, which are expressed in RPE cells and localize to the apical plasma membrane. In RPE cells, syntaxin 2 is exclusively localized to a region below the tight junctions, in contrast to its distribution along both plasma membrane domains in MDCK cells. Syntaxin 4 localizes all along the lateral membrane in MDCK cells, and post-Golgi transport vesicles carrying basolateral cargo can fuse along the entire lateral membrane (Kreitzer et al., submitted). By contrast, in RPE cells syntaxin 4 localizes to the same narrow band underneath the tight junctions as does syntaxin 2. In addition, syntaxin 4 localizes to the basal membrane. This suggests that syntaxin 4 may serve two different trafficking pathways that lead to either location (designated B_1 and B_2). These syntaxin-4-dependent routes may originate from the TGN and/or endosomes.

A surprising finding is that syntaxin 2 is localized to the basolateral plasma membrane domain in RPE cells. Of the four known syntaxin 2 splice isoforms, we found that only syntaxins 2A and 2B were expressed in RPE-J cells; these isoforms differ only in their C-terminal transmembrane domains (Quinones et al., 1999). In pancreatic acinar cells, syntaxin 2 localizes to the apical plasma membrane but it was not investigated which isoforms are expressed in these cells (Gaisano et al., 1996). Syntaxin 2 is therefore the first known syntaxin whose polarity can be reversed depending on the epithelial cell type. In MDCK cells, syntaxin 2A expressed by stable transfection localizes to both the apical and basolateral plasma membrane domains (Low et al., 1996). It is interesting to note that syntaxin 2A expressed by adenovirus-mediated transfection was found to be concentrated at the apical plasma membrane of MDCK cells whereas syntaxin 2B was more evenly distributed on both domains (Quinones et al., 1999). From these data it is unlikely that the reversed polarity of syntaxin 2 in RPE cells can be explained simply by the expression of alternative isoforms. Other mechanisms must be responsible for this differential targeting, for example, differential recognition of targeting signals within the amino-acid sequence of syntaxin 2. This mechanism remains to be identified. It also remains unknown which cargo proteins utilize a syntaxin-2-dependent fusion step. To date, syntaxin 2 has been implicated in only two fusion events, zymogen granule exocytosis in acinar cells (Hansen et al., 1999) and the fusion of the acrosome with the plasma membrane of spermatozoa (Katafuchi et al., 2000). Since the major contents of both zymogen granules and the acrosome are hydrolytic enzymes it is tempting to speculate that syntaxin 2 is used for the secretion of similar cargo in RPE and other epithelial cells.

Unexpectedly, we found that both syntaxins 2 and 4 localize to a narrow band just underneath the tight junctions in RPE cells in vivo. Confocal microscopy of double-stained sections showed that both syntaxins do not overlap with the tight junction protein occludin; instead they are immediately adjacent on the lateral membrane, indicating that both are involved in basolateral trafficking pathways. On the basis of the localization of other proteins implicated in vesicle fusion on the tight junctions of epithelial cells, it has been proposed that the tight junctions – or structures in close proximity – may be preferred sites of vesicle exocytosis. These proteins include rab8 (Huber et al., 1993), rab3b (Weber et al., 1994), rab13 (Zahraoui et al., 1994), the sec6/8 complex or exocyst (Grindstaff et al., 1998) and VAP-A (Lapierre et al., 1999). Direct evidence of vesicle fusion events at the tight junctions, however, has been lacking, and the fact that syntaxin 4 localizes all along the lateral membrane in MDCK and other epithelial cells seemed to be at odds with this hypothesis. Recently, fusion events of post-Golgi transport vesicles carrying GFP-tagged apical and basolateral marker proteins were monitored in polarized MDCK cells using time-lapse microscopy (G. Kreitzer, J. Schmoranzel, S.-H. Low, Y. Chen et al., unpublished). The results demonstrated that fusion of basolateral vesicles occurs all along the lateral plasma

membrane in agreement with the lateral localization of syntaxin 4. It is still possible that more specialized trafficking pathways may be directed towards the tight junctions in MDCK cells, but this remains to be investigated. Our striking finding that syntaxins 2 and 4 localize close to the tight junctions in RPE cells strongly suggests that a tight junctional 'fusion patch' indeed exists in this epithelial cell type and that exocytic pathways that depend on these syntaxins will be directed toward this site (route B_1 in Fig. 5). Syntaxin 4, but not syntaxin 2, was also present at the basal membrane in RPE cells, suggesting that basolateral fusion events occur either at the 'tight junctional fusion patch' or at the basal membrane (route B_2). It is possible that syntaxin 4 is not only involved in TGN-to-basolateral trafficking in epithelial cells but also in recycling pathways as could be deduced from its known involvement in GLUT-4 translocation from endosomes to the plasma membrane in adipocytes (Macaulay et al., 1997; Olson et al., 1997; Tellam et al., 1997). In this case, routes B_1 and B_2 may differ in that one of them originates from endosomes rather than the TGN.

In conclusion, we have shown that RPE cells differ in their complement of plasma membrane syntaxins and in the polarity of one syntaxin from other epithelial cell types. Furthermore, they exhibit the prominent localization of two syntaxins at a putative 'sub-tight junctional fusion patch'. These differences suggest that epithelial cell types can indeed differ in the molecular machineries controlling membrane trafficking. We consider it therefore unlikely that the known variability of epithelial trafficking phenotypes is solely due to re-routing of cargo proteins – for example, by differential recognition of sorting signals – into otherwise identical trafficking pathways. A more likely scenario may be that epithelial cells can regulate the presence/absence, preponderance and/or direction of entire trafficking pathways. This would include the expression and localization of plasma membrane syntaxins, which serve as 'end points' of all exocytic pathways. It is unlikely, however, that regulation of syntaxin expression/localization alone can accomplish the diversity of epithelial trafficking phenotypes. Membrane trafficking pathways consist of a succession of mechanisms – such as vesicle budding, transport along cytoskeletal elements etc. – many of which may need to be modulated depending on the epithelial cell type.

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