Published in final edited form as:

Traffic. 2006 November ; 7(11): . doi:10.1111/j.1600-0854.2006.00482.x.

Regulatory Dissociation of Tctex-1 Light Chain from Dynein Complex Is Essential for the Apical Delivery of Rhodopsin

Ting-Yu Yeh¹, Diego Peretti¹, Jen-Zen Chuang^{1,2}, Enrique Rodriguez-Boulan^{1,2}, and Ching-Hwa Sung^{1,2,*}

¹Department of Ophthalmology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA

²Department of Cell and Developmental Biology, Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10021, USA

Abstract

Post-Golgi to apical surface delivery in polarized epithelial cells requires the cytoplasmic dynein motor complex. However, the nature of dynein–cargo interactions and their underlying regulation are largely unknown. Previous studies have shown that the apical surface targeting of rhodopsin requires the dynein light chain, Tctex-1, which binds directly to both dynein intermediate chain (IC) and rhodopsin. In this report, we show that the S82E mutant of Tctex-1, which mimics Tctex-1 phosphorylated at serine 82, has a reduced affinity for dynein IC but not for rhodopsin. Velocity sedimentation experiments further suggest that S82E is not incorporated into the dynein complex. The dominant-negative effect of S82E causes rhodopsin mislocalization in polarized Madin-Darby canine kidney (MDCK) cells. The S82A mutant, which mimics dephosphorylated Tctex-1, can be incorporated into dynein complex but is impaired in its release. Expression of S82A also causes disruption of the apical localization of rhodopsin in MDCK cells. Taken together, these results suggest that the dynein complex disassembles to release cargo due to the specific phosphorylation of Tctex-1 at the S82 residue and that this process is critical for the apical delivery of membrane cargoes.

Keywords

apical transport; cytoplasmic dynein; rhodopsin; Tctex-1

The cytoplasmic dynein motor complex is involved in post-Golgi transport to the apical surface in polarized epithelial cells (1–4). However, the mechanisms by which dyneins control the binding and release of their cargoes are poorly understood. According to the most widely accepted model, dynein–cargo interaction is controlled through binding to dynactin complex. Dynactin subunit p150^{Glued} binds to dynein intermediate chain (IC), and the dynactin subunit Arp1 binds to membrane vesicles through its interaction with the membrane cytoskeletal protein spectrin (5–9). A recent study showed that phosphorylation of IC at serine 84 decreased its binding affinity for p150^{Glued}, indicating that this may serve as a mechanism for uncoupling dynein complex from dynactin complex and, hence, from cargo (10). However, the cargo binding of dynein could also be mediated by direct protein–protein interaction between cargo receptors and specific dynein accessory proteins. For

example, Tctex-1 [i.e. DYNLT1 (11)], a 14-kDa dynein light chain, links rhodopsin-bearing vesicles onto dynein motor complexes through its direct interaction with rhodopsin's cytoplasmic C-terminal tail and IC (12). Dynactin is not required for the dynein binding of rhodopsin in *in vitro* binding assays. At present, the molecular steps regulating dynactin-independent cargo binding to dynein have not been studied. More interestingly, it is also unclear whether the dynein motor complex is able to disassemble and reassemble itself for recycling once it completes its transportation role. Although a recent study suggested that dynein could serve as an anchor for delivering messenger RNA cargo to apical surfaces in fly blastoderm embryonic cells (13), the details of dynein delivery of membranous cargoes to cell surface have not been studied. In this study, we studied this question by Tctex-1/dynein-mediated apical transport of rhodopsin in MDCK cells as a model system.

Results and Discussion

The serine 82 residue of Tctex-1 is critical for binding to dynein IC but not to rhodopsin cargo

Rhodopsin is predominantly targeted to apical surfaces when expressed in polarized MDCK cells (14). Previous studies suggested that Tctex-1-mediated dynein activity is critically involved in this vectorial translocation of rhodopsin. This finding is supported by the fact that overexpression of non-rhodopsin-binding Tctex-1 homologue rp3 [i.e. DYNLT3 (11)], which displaces Tctex-1 from the dynein complex and induces Tctex-1 degradation, resulted in a non-polar distribution of rhodopsin in MDCK cells (2). To further investigate the molecular mechanism regulating Tctex-1/dynein-mediated apical targeting of rhodopsin, we searched for dominant-negative Tctex-1 mutant(s) that could bind to IC but not to the cargo rhodopsin. To this end, we hypothesized that the phosphorylation of Tctex-1 could regulate its interaction with IC, so we sought to identify putative phosphorylation sites within the region where Tctex-1 binds to IC (aa 55–95) (15). Consensus sequences of five putative phosphorylation sites (T55, S82, S88, S92 and T94) were identified using a group-based phosphorylation scoring method (http://973-proteinweb.ustc.edu.cn/gps/gps_web/). Phosphorylated Tctex-1 mimics were subsequently generated by mutating each corresponding putative phosphorylated serine/threonine residue to glutamic acid (E). The ability of these mutants to bind to rhodopsin's C-terminus and IC was then screened by two-hybrid and coimmunoprecipitation analyses.

These experiments led us to identify an interesting phosphomimic mutant S82E, which bound to rhodopsin but not to IC. The dephosphomimic counterpart, S82A, like the wild-type (WT) Tctex-1, bound to both rhodopsin and IC. As shown in Figure 1, anti-rhodopsin antibody (Ab) was able to coimmunoprecipitate roughly equal amounts of Flag-tagged, WT, S82E and S82A Tctex-1 from lysates of human embryonic kidney (HEK) cells overexpressing these proteins (Figure 1A). Yeast two-hybrid analysis showed that the C-terminal 39 residues of rhodopsin bound indistinguishably to WT, S82E and S82A Tctex-1 (Figure 1B). In contrast, Tctex-1 homologue rp3 did not bind to rhodopsin (Figure 1B), consistent with previous studies (12). Glutathione S-transferase (GST) fusion proteins containing WT, S82E or S82A, but not GST alone, pulled down similar amounts of maltose-binding protein (MBP) fusion protein containing rhodopsin's C-terminal 39 amino acids (Figure 1C). Finally, anti-IC Ab was used to immunoprecipitate HEK cell lysates containing overexpressed Tctex-1 variants. Both endogenous Tctex-1 and transfected Flag-WT and Flag-S82A Tctex-1, but not Flag-S82E Tctex-1, were coimmunoprecipitated with endogenous IC (Figure 1D). The reciprocal immunoprecipitation experiments using anti-Flag Ab also showed that IC was present in the immunoprecipitates of Flag-WT and Flag-S82A Tctex-1 but not in Flag-S82E Tctex-1 (data not shown). The other Tctex-1 mutants tested were not chosen for further study because either they had no effect on binding to either IC or rhodopsin, or they had lost the ability to bind to both molecules (Table S1).

The apical targeting of rhodopsin is perturbed by the expression of Tctex-1 S82 mutants

To study the role of S82 phosphorylation in Tctex-1-mediated dynein transport of rhodopsin, we produced MDCK lines inducibly expressing Flag-WT, Flag-S82A or Flag-S82E Tctex-1 under the Tet-off control system. We first used immunostaining (data not shown) and immunoblotting to verify that the uniform expression of these Tctex-1 proteins could be induced by the removal of doxycycline from the medium (Figure 2A). We found that the level of endogenous Tctex-1, but not p150^{glued} (data not shown), IC, or tubulin (Figure 2A), was reduced on Flag-WT, Flag-S82A or Flag-S82E Tctex-1 induction. The expression levels of two other dynein light chains – rp3 and LC8 [i.e. DYNLL1 (11)] – were not affected by the overexpression of either WT or S82 mutant Tctex-1 (Figures S1 and S2). Consistent with the transient transfection results, Flag-WT and Flag-S82A Tctex-1, but not Flag-S82E Tctex-1, were coimmunoprecipitated by the endogenous IC in lysates obtained from the stable lines (Figure 2B). The incorporation of these Tctex-1 proteins into dynein complexes was further examined by velocity density gradient sedimentation. Immunoblotting of the gradient fractions showed that IC and dynactin p150^{glued} sediment at ~19–20S (16,17), suggesting that the integrity of dynein complexes was not affected by the overexpression of either WT or S82 mutant Tctex-1. A portion of Flag-WT and Flag-S82A, along with a trace level of remaining endogenous Tctex-1 (Figure S2), cofractionated with IC, consistent with their incorporation into dynein complex. By contrast, Flag-S82E Tctex-1 was exclusively distributed in the lighter fractions and was not detectable in the 19–20S fractions, consistent with its inability to bind to IC. Finally, overexpression of WT, S82A, or S82E Tctex-1 had no effect on the perinuclear localization of the Golgi apparatus, indicated by the Golgi marker GOS-28 immunolabeling (Figure 2D–F); this result further suggests that the global functions of the dynein motor were preserved in these cells.

To study the rhodopsin's polarity, MDCK cells cultured under uninduced (Dox+) or induced (Dox–) conditions were plated onto Transwell filters to polarize the cells. The MDCK monolayers were then infected with adenovirus encoding rhodopsin for 1 day prior to immunolabeling. As expected, confocal analysis of immunolabeled filters revealed that rhodopsin was predominantly distributed on the apical surfaces in all stable lines under uninduced conditions (Figure 3A). Under induced conditions, rhodopsin was also apically localized in MDCK cells overexpressing WT Tctex-1, but it was mislocalized in cells expressing either S82A or S82E mutant Tctex-1 (Figure 3A). Rhodopsin in S82E-expressing cells appeared to be randomly distributed on both the apical and the lateral surfaces, a distribution that is strikingly similar to its expression pattern seen in rp3-expressing cells (2). MDCK cells overexpressing S82A also had severe rhodopsin mislocalization; interestingly, rhodopsin was distributed primarily to the basolateral surface in these cells.

Other apical markers examined, including the endogenous glycoprotein 135 (gp135) and the adenovirally transduced p75, remained at their apical localization in cells overexpressing mutant Tctex-1 (Figure 3B). Furthermore, the basolateral distribution of Na, K-adenosine triphosphatase (ATPase) and E-cadherin was not affected by WT Tctex-1 and S82 mutant Tctex-1 (Figure 3C). Finally, we sought to rule out the possibility that the mistargeting of rhodopsin in S82 mutants was due to the loss of tight junction structure and/or function. Thus, we performed ZO-1 immunolabeling, paracellular permeability and transepithelial electrical resistance (TER) assays in these cell lines. T23/MDCK cells treated by calcium depletion to perturb tight junction structure/function were used as controls in these assays. Our results showed that all cell lines displayed normal ZO-1 staining that demarcated the cell–cell junction at the apical side of lateral junctions (data not shown). In addition, all three cell lines showed normal and indistinguishable diffusion rates of fluorescein isothiocyanate (FITC)–dextran and TER regardless of the presence or absence of doxycycline (Figure 3D). These results collectively suggested that the apical delivery of rhodopsin was perturbed in a specific manner in MDCK cells expressing S82A and S82E mutants.

Rhodopsin is directly targeted to the apical surface in polarized MDCK cells

Previous studies using a biochemical surface-targeting assay showed that rhodopsin is directly targeted from the *trans* Golgi network (TGN) to the apical surface (14). Recently, however, Polishchuk et al. (18) suggested that some proteins previously believed to be directly apically targeted might actually be routed to the basolateral surface prior to their final apical destination, namely, through the transcytosis pathway. They suggested that domain-selective treatment with tannic acid, a membrane-impermeable fixative that instantly blocks local membrane fusion, provides an alternative approach to sensitively determine the protein transport route in polarized MDCK cells.

To eliminate the possibility that rhodopsin was transported to the apical surfaces through the transcytosis pathway and that rhodopsin mislocalization in S82 mutant cells was due to deregulation of the transcytosis pathway, we reexamined the route of rhodopsin in polarized MDCK cells. To this end, we generated a plasmid encoding a rhodopsin–green fluorescent protein (GFP) fusion that contains full-length rhodopsin, followed by the GFP moiety and an additional eight residues of rhodopsin's C-terminus (Figure 4A). This rhodopsin–GFP fusion folds correctly (19,20) and is capable of binding Tctex-1 (data not shown). On microinjection into MDCK cells, the rhodopsin–GFP was first allowed time to be synthesized (37°C, 1 h), then blocked in the TGN (20°C, 2 h) and finally released from the TGN (32°C) (Figure 4B). Our pilot experiments suggested that 120-min and 90-min incubations at 32°C allowed an almost complete TGN to surface delivery in subconfluent and polarized cells, respectively (Figure 4B). When polarized MDCK monolayers were injected, rhodopsin–GFP was predominantly targeted to the apical surfaces (Figure 4B).

To block the surface delivery in a domain-selective manner, tannic acid was added to either the apical or the basolateral chamber during the 32°C release. At all three time-points tested (45, 90 and 120 min), the apical addition of tannic acid caused an abnormal accumulation of rhodopsin at the subapical cytoplasm and at the lateral surfaces (Figure 4C). The addition of tannic acid to the basolateral compartment had no detectable effect on the apical surface targeting of rhodopsin–GFP (Figure 4C) although the surface delivery of basolateral marker GFP-neural cell adhesion molecule (NCAM) (21) was significantly blocked when it was expressed and treated in a similar manner (Figure S3). These results verified previous observations (14), suggesting that rhodopsin is targeted directly from the TGN to the apical surface. They further ruled out the possibility that the mislocalization of rhodopsin seen in S82A cells was a consequence of perturbation at the transcytosis step.

S82 phosphorylation of Tctex-1 plays a role in its uncoupling from the dynein complex

Our biochemical studies suggested that phosphorylation of the S82 residue may serve as a mechanism for regulating dissociation of Tctex-1 from the dynein complex. We thus hypothesized that the change of S82 to alanine suppressed its release from dynein; the mislocalization of rhodopsin indicated that the dynein disassembly is essential for the apical surface delivery of rhodopsin. To test this, we studied the kinetic incorporation of Tctex-1 into dynein complex using MDCK lines expressing similar levels of Flag-WT or Flag-S82A Tctex-1 (Figure S4). These MDCK cells were pulse labeled with [³⁵S]-methionine followed by a chase. Equal amounts of cell lysates were subjected to immunoprecipitation for 'total Tctex-1' (Figure 5A) and 'dynein-bound Tctex-1' using anti-Flag Ab and anti-IC Ab, respectively. The fraction of newly synthesized Tctex-1 incorporated into the dynein complex was estimated to be the ratio of the amount of IC-bound Tctex-1 relative to total remaining Tctex-1 at a given time-point (Figure 5B). Although the total levels of newly synthesized WT and S82A Tctex-1 were not significantly different after the 2-h chase (Figure 5A), their incorporation into dynein complexes was remarkably different at all time-points assayed. For example, after the 2-h chase time-point, ~50% of the newly synthesized

Flag-WT Tctex-1 remained detectable in the dynein complex. In contrast, only ~6% of Flag-S82A was found in the dynein complex. The poor ability of newly synthesized S82A Tctex-1 to be incorporated into dynein was not likely due to its inability to bind IC because the coimmunoprecipitation experiments suggested that WT and S82A Tctex-1 exhibited a similar ability to bind to IC under steady-state conditions (Figures 1D and 2B). Instead, the pulse–chase experiment results supported the idea that the previously incorporated mutant protein failed to be released and, hence, prohibited turnover.

To test the phosphorylation of Tctex-1 at the S82 residue in MDCK cells, we analyzed Flag-WT and Flag-S82A expressed in MDCK stable lines by two-dimensional gel electrophoresis and immunoblotting. As shown in Figure 5C (top panel), immunoprecipitated Flag-WT was detected as two distinct species migrating near 14 kDa, with estimated pIs of 4.57 and 4.64. In contrast, the Flag-S82A Tctex-1 immunoprecipitate was detected as a single predominant species with pI ~4.64; the lower pI (i.e. phosphorylated) species was greatly diminished. These results indicated that when expressed in MDCK cells, WT Tctex-1, but not S82A, could be phosphorylated by the endogenous kinase. In addition, consistent with a previous study (22), our results showed that only at the higher pI, dephosphorylated Tctex-1 was detected in the dynein complex immunoprecipitated by the anti-IC Ab (Figure 5C, bottom panel). Taken together, these results support the idea that S82 could serve as a site of Tctex-1 phosphorylation *in vivo*; specific phosphorylation at the S82 of Tctex-1 involves its dissociation from dynein complex. Although the consensus sequence search suggested that S82 may serve as a phosphorylation site for mitogen-activated protein kinase kinase kinase, the specific kinase that phosphorylated S82 in MDCK cells has not been determined.

Our data showing mislocalization of rhodopsin in Tctex1-S82A-expressing MDCK cells support the idea that the regulated dissociation of dynein from light chain Tctex-1, and hence from the cargo, is required for the correct membrane targeting of rhodopsin. We speculate that this uncoupling is critical for the rhodopsin-bearing cargo to navigate through the subapical terminal web using distinct translocation machinery and/or for the docking/fusion of rhodopsin cargo onto plasma membrane once dynein completes its transportation role. Interference with this regulation leads to an altered delivery pathway and protein mislocalization. However, how the rhodopsin cargo that fails to reach the apical surface is delivered to the basolateral surface instead is unclear. Interestingly, rhodopsin was also found on the basolateral surface when its apical delivery is blocked by the apically added tannic acid (Figure 4C). Dynein disassembly might be important for the recycling of the motor. However, a shortage of motor molecules is unlikely to fully account for the severe depolarization of rhodopsin seen in S82A cells. Last, the mislocalization of rhodopsin in S82E-expressing MDCK cells is consistent with the predicted dominant-negative effect of this mutant, which binds to rhodopsin but not to dynein. These findings add further support to the idea that the Tctex-1-mediated rhodopsin translocation in MDCK cells occurs through a dynein-dependent pathway. This contrasts with a recently described dynein-independent role for Tctex-1 in actin remodeling and neurite outgrowth (23).

Materials and Methods

Reagents

Unless otherwise specified, all chemical and tissue culture reagents were purchased from Sigma (St Louis, MO, USA) and Invitrogen (Carlsbad, CA, USA), respectively. Antibodies we used were dynein IC monoclonal antibody (mAb) 74.1, anti-ZO-1 rat Ab (Chemicon, Temecula, CA, USA), Flag M2 mAb, E-cadherin rat Ab (Sigma), p150^{Glued} mAb (BD Bioscience, Palo Alto, CA, USA), antirhodopsin N-terminus B6-30 mAb (gift of Dr P. Hargrave), antirhodopsin C-terminus mAb 1D4 (gift of Dr R. Molday), Golgi SNARE (GOS)-28 mAb (gift of Dr W. Hong), gp135 mAb (gift of Dr G.K. Ojakian), p75 mAb (gift

Dr M. Chao), Na,K-ATPase mAb (gift of Dr M. Caplan) and LC8 rabbit Ab [gift of Dr S. Jaffrey (24)]. Alexa dye 488 and 568 conjugated secondary antibodies were obtained from Molecular Probes (Eugene, OR, USA). The S82A and S82E mutants of Tctex-1 were generated by site-directed mutagenesis of Flag-Tctex-1 (in pRK5) using the QuickChange Kit (Stratagene, La Jolla, CA, USA). All mutations were confirmed by automated sequencing. The *EagI/XbaI* fragment of Tctex-1 in pRK5 plasmid was subsequently transferred into pBI-enhanced green fluorescent protein (EGFP) (BD Bioscience) to generate pTRE-WT, pTRE-S82A and pTRE-S82E. For two-hybrid analysis, Tctex-1 sequences were amplified from the pRK5 vector (5'-GGAATTCGAATGGAAGACTACCAGGCCG-3' and 5'-CCGCTCGAGTCAGATGGACAGGCCGAAG-3') by polymerase chain reaction, digested with *EcoRI/XhoI* and inserted into pACTII vector (BD Bioscience). Tctex-1 sequences in pACTII plasmid were further transferred into GST-5X-1 plasmid to generate constructs encoding GST-Tctex-1 fusion proteins. Eukaryotic expression plasmids encoding human rhodopsin (25,26), MBP fusion encoding rhodopsin C-terminal 39 amino acids (MBP-rho39Tr), yeast two-hybrid plasmid pDB-rho39Tr, pTRE-WT (2,12) and GFP-NCAM (21) were previously described. The expression construct encoding rhodopsin-GFP fusion was produced based on the suggestion by Jin et al. [(19); Figure 4A]; the details of this construct are available upon request.

Two-hybrid, pull-down, immunoprecipitation and velocity density gradient sedimentation assays

Yeast two-hybrid and X-gal filter assays were carried out as previously described (12). Control experiments showed that none of the pACTII plasmids containing Tctex-1 sequences gave rise to autoactivation. The procedures used for pull-down analysis, immunoprecipitation and velocity density gradient sedimentation were essentially as previously described (12,27).

Cell culture, transfection, virus infection and immunocytochemistry

To generate MDCK stable lines inducibly expressing WT or mutant Tctex-1, T23 cells (i.e. MDCK cell lines stably expressing tTA (28); gift from Dr K. Mostov) were cotransfected with pTK-Hyg plasmid and pTRE plasmids containing various Tctex-1 sequences. Hygromycin-resistant clones were selected as previously described (2). These stable lines were routinely maintained in the presence of 100 ng/mL doxycycline. To induce gene expression, cells were washed, plated in the absence of doxycycline for 2 days and then plated on Transwell filters at high density for an additional 3–5 days. Monolayers were infected with adenoviruses encoding human rhodopsin (2) or p75 (a gift of Dr M. Chao) for 24 h prior to the immunolabeling. Immunolabeled filters were acquired on a Leica TCS SP2 microscope with a HC× PL APO 63×/1.4 Oil CS Blue objective using Leica Confocal Software (Nussloch, Germany). Experiments carried out in multiple independent lines expressing the same Tctex-1 variant generated similar results.

Kinetics of Tctex-1 incorporation in dynein

MDCK monolayers inducibly expressing WT or S82A Tctex-1 were pulse labeled and chased as described previously (25). Equal amounts of cell lysates, harvested at different chase time-points, were immunoprecipitated by anti-IC and anti-Flag Abs. Immunoprecipitates were separated by SDS-PAGE and radiolabeled Tctex-1 was quantified by PhosphorImager. The data were analyzed by Student's *t*-test.

Two-dimensional gel electrophoresis

Lysates of MDCK cells inducibly overexpressing Flag-tagged WT and S82A Tctex-1 were immunoprecipitated by anti-Flag M2 agarose (Sigma) and anti-IC mAb. The immunoprecipitates were washed as usual (29) and resuspended in rehydration buffer (BioRad, Hercules, CA, USA) prior to being subjected to isoelectric focusing electrophoresis (pH 4–7) and 15% SDS–polyacrylamide gel according to the manufacturer's instructions.

Transepithelial electrical resistance and paracellular permeability assay

The TER of cells grown in 12-mm Transwell filters was measured using an EVOM™ epithelial voltohmmeter (World Precision Instruments, Sarasota, FL), as previously described (30). Paracellular flux was assayed as described (30,31). The apical chamber contained 200 μ L DMEM with 0.2 mg/mL FITC-conjugated 10-kDa anionic dextran (Molecular Probes), and the basolateral chamber was filled with 600 μ L DMEM without tracer. Three hours later, the basolateral compartment medium was collected, and the tracer amounts were quantified by the SpectraMax Gemini spectrofluorometer (excitation, 485 nm; emission, 538 nm; Molecular Devices, Sunnyvale, CA, USA). As a control, calcium depletion was carried out, as described (32), to perturb tight junctions by incubating polarized T23 monolayers with S-MEM plus 10% fetal calf serum for 16 h prior to the assay.

Microinjection and tannic acid experiments

Microinjection of MDCK cells was essentially carried out as described (33). Briefly, DNA (20 μ g/mL) was injected into cells, either subconfluent cultures on a coverslip or 4-day monolayers on a 24-mm filter. Then, the cells were incubated at 37°C for 1 h and 20°C for 2 h with the addition of cycloheximide (100 μ g/mL) and then released at 32°C. Tannic acid (0.5%; Sigma) was added to either the apical or the basal chamber in serum-free medium during the last 10 min during the 20°C incubation and throughout the 32°C incubation (18).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are indebted to our colleagues for providing reagents (Drs K. Mostov, M. V. Chao, P. Hargrave, R. Molday, W. Hong, G. K. Ojakian and M. Caplan) and for discussion (Drs M. Zhang, A. Musch and D. Cohen). We also thank Dr P. Alfinito for initial characterization of Tctex-1 phosphorylation and Dr W. Liu for advice on two-dimensional gel analysis. This work was supported by National Institutes of Health grant EY11307, Research to Prevent Blindness, Hirsch Foundation and Steinbach Foundation (to C.-H. S.) and NIH grant GM 34107 (to E. R.-B.).

References

1. Lafont F, Burkhardt JK, Simons K. Involvement of microtubule motors in basolateral and apical transport in kidney cells. *Nature*. 1994; 372:801–803. [PubMed: 7997271]
2. Tai AW, Chuang J-Z, Sung C-H. Cytoplasmic dynein regulation by subunit heterogeneity and its role in apical transport. *J Cell Biol*. 2001; 153:1499–1509. [PubMed: 11425878]
3. Fath KR, Trimbur GM, Burgess DR. Molecular motors are differentially distributed on Golgi membranes from polarized epithelial cells. *J Cell Biol*. 1994; 126:661–675. [PubMed: 8045931]
4. Wang Y, Jerdeva G, Yarber FA, da Costa SR, Xie J, Qian L, Rose CM, Mazurek C, Kasahara N, Mircheff AK, Hamm-Alvarez SF. Cytoplasmic dynein participates in apically targeted stimulated secretory traffic in primary rabbit lacrimal acinar epithelial cells. *J Cell Sci*. 2003; 116:2051–2065. [PubMed: 12679381]

5. Waterman-Storer CM, Karki S, Holzbaur EL. The p150Glued component of the dynactin complex binds to both microtubules and the actin-related protein centractin (Arp-1). *Proc Natl Acad Sci U S A*. 1995; 92:1634–1638. [PubMed: 7878030]
6. Muresan V, Stankewich MC, Steffen W, Morrow JS, Holzbaur EL, Schnapp BJ. Dynactin-dependent, dynein-driven vesicle transport in the absence of membrane proteins: a role for spectrin and acidic phospholipids. *Mol Cell*. 2001; 7:173–183. [PubMed: 11172722]
7. Waterman-Storer CM, Karki SB, Kuznetsov SA, Tabb JS, Weiss DG, Langford GM, Holzbaur EL. The interaction between cytoplasmic dynein and dynactin is required for fast axonal transport. *Proc Natl Acad Sci U S A*. 1997; 94:12180–12185. [PubMed: 9342383]
8. Holleran EA, Tokito MK, Karki S, Holzbaur EL. Centractin (ARPI) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. *J Cell Biol*. 1996; 135:1815–1829. [PubMed: 8991093]
9. Holleran EA, Ligon LA, Tokito M, Stankewich MC, Morrow JS, Holzbaur EL. beta III spectrin binds to the Arp1 subunit of dynactin. *J Biol Chem*. 2001; 276:36598–36605. [PubMed: 11461920]
10. Vaughan PS, Leszyk JD, Vaughan KT. Cytoplasmic dynein intermediate chain phosphorylation regulates binding to dynactin. *J Biol Chem*. 2001; 276:26171–26179. [PubMed: 11340075]
11. Pfister KK, Fisher EM, Gibbons IR, Hays TS, Holzbaur EL, McIntosh JR, Porter ME, Schroer TA, Vaughan KT, Witman GB, King SM, Vallee RB. Cytoplasmic dynein nomenclature. *J Cell Biol*. 2005; 171:411–413. [PubMed: 16260502]
12. Tai AW, Chuang J-Z, Bode C, Wolfrum U, Sung C-H. Rhodopsin's carboxy-terminal cytoplasmic tail acts as a membrane receptor for cytoplasmic dynein by binding to the dynein light chain Tctex-1. *Cell*. 1999; 97:877–887. [PubMed: 10399916]
13. Delanoue R, Davis I. Dynein anchors its mRNA cargo after apical transport in the *Drosophila* blastoderm embryo. *Cell*. 2005; 122:97–106. [PubMed: 16009136]
14. Chuang J-Z, Sung C-H. The cytoplasmic tail of rhodopsin acts as a novel apical sorting signal in polarized MDCK cells. *J Cell Biol*. 1998; 142:1245–1256. [PubMed: 9732285]
15. Mok YK, Lo KW, Zhang M. Structure of Tctex-1 and its interaction with cytoplasmic dynein intermediate chain. *J Biol Chem*. 2001; 276:14067–14074. [PubMed: 11148215]
16. Paschal BM, Shpetner HS, Vallee RB. MAP 1C is a microtubule-activated ATPase which translocates microtubules in vitro and has dynein-like properties. *J Cell Biol*. 1987; 105:1273–1282. [PubMed: 2958482]
17. Schroer TA, Sheetz MP. Two activators of microtubule-based vesicle transport. *J Cell Biol*. 1991; 115:1309–1318. [PubMed: 1835460]
18. Polishchuk R, Di Pentima A, Lippincott-Schwartz J. Delivery of raft-associated, GPI-anchored proteins to the apical surface of polarized MDCK cells by a transcytotic pathway. *Nat Cell Biol*. 2004; 6:297–307. [PubMed: 15048124]
19. Jin S, McKee TD, Oprian DD. An improved rhodopsin/EGFP fusion protein for use in the generation of transgenic *Xenopus laevis*. *FEBS Lett*. 2003; 542:142–146. [PubMed: 12729914]
20. Jin S, Cornwall MC, Oprian DD. Opsin activation as a cause of congenital night blindness. *Nat Neurosci*. 2003; 6:731–735. [PubMed: 12778053]
21. Musch A, Cohen D, Kreitzer G, Rodriguez-Boulton E. cdc42 regulates the exit of apical and basolateral proteins from the trans-Golgi network. *EMBO J*. 2001; 20:2171–2179. [PubMed: 11331583]
22. King SM, Barbarese E, Dillman JF 3rd, Benashski SE, Do KT, Patel-King RS, Pfister KK. Cytoplasmic dynein contains a family of differentially expressed light chains. *Biochemistry*. 1998; 37:15033–15041. [PubMed: 9790665]
23. Chuang JZ, Yeh TY, Bollati F, Conde C, Canavosio F, Caceres A, Sung CH. The dynein light chain Tctex-1 has a dynein-independent role in actin remodeling during neurite outgrowth. *Dev Cell*. 2005; 9:75–86. [PubMed: 15992542]
24. Jaffrey SR, Snyder SH. PIN: an associated protein inhibitor of neuronal nitric oxide synthase. *Science*. 1996; 274:774–777. [PubMed: 8864115]
25. Sung C-H, Schneider BG, Agarwal N, Papermaster DS, Nathans J. Functional heterogeneity of mutant rhodopsins responsible for autosomal dominant retinitis pigmentosa. *Proc Natl Acad Sci U S A*. 1991; 88:8840–8844. [PubMed: 1924344]

26. Sung C-H, Davenport CM, Nathans J. Rhodopsin mutations responsible for autosomal dominant retinitis pigmentosa: clustering of functional classes along the polypeptide chain. *J Biol Chem.* 1993; 268:26645–26649. [PubMed: 8253795]
27. Yeh TY, Chuang JZ, Sung CH. Dynein light chain rp3 acts as a nuclear matrix-associated transcriptional modulator in a dynein-independent pathway. *J Cell Sci.* 2005; 118:3431–3443. [PubMed: 16079286]
28. Barth AI, Pollack AL, Altschuler V, Mostov KE, Nelson WJ. NH2-terminal deletion of b-catenin results in stable colocalization of mutant-catenin with adenomatous polyposis coli protein and altered MDCK cell adhesion. *J Cell Biol.* 1997; 136:693–706. [PubMed: 9024698]
29. Tai AW, Chuang J-Z, Sung C-H. Localization of Tctex-1, a cytoplasmic dynein light chain, to the Golgi apparatus and evidence for dynein complex heterogeneity. *J Biol Chem.* 1998; 273:19639–19649. [PubMed: 9677391]
30. Jou TS, Schneeberger EE, Nelson WJ. Structural and functional regulation of tight junctions by RhoA and Rac1 small GTPases. *J Cell Biol.* 1998; 142:101–115. [PubMed: 9660866]
31. Balda MS, Whitney JA, Flores C, Gonzalez S, Cereijido M, Matter K. Functional dissociation of paracellular permeability and transepithelial electrical resistance and disruption of the apical-basolateral intramembrane diffusion barrier by expression of a mutant tight junction membrane protein. *J Cell Biol.* 1996; 134:1031–1049. [PubMed: 8769425]
32. Cohen D, Brennwald PJ, Rodriguez-Boulán E, Musch A. Mammalian PAR-1 determines epithelial lumen polarity by organizing the microtubule cytoskeleton. *J Cell Biol.* 2004; 164:717–727. [PubMed: 14981097]
33. Kreitzer G, Marmorstein A, Okamoto P, Vallee R, Rodriguez-Boulán E. Kinesin and dynamin are required for post-Golgi transport of a plasma-membrane protein. *Nat Cell Biol.* 2000; 2:125–127. [PubMed: 10655593]

Figure 1. S82 residue of Tctex-1 is involved in binding to dynein but not to rhodopsin

A) Lysates of 293 cells, transiently transfected with rhodopsin and Flag-tagged Tctex-1 variants, were subjected to immunoprecipitation (IP) using anti-rhodopsin Ab. The total input (left panel) and the rhodopsin immunoprecipitates (right panel) were separated on SDS-PAGE and immunoblotted (IB) with anti-rhodopsin and anti-Flag Abs. Note that rhodopsin expressed in tissue culture is heterogeneously glycosylated and prone to form higher orders of oligomers under denaturing conditions *in vitro* (e.g. SDS-sample buffer) and displays a 'smear' pattern on SDS-PAGE/immunoblotting (25). The brackets indicate the heterogeneous species of monomeric and oligomerized rhodopsins. Arrow points to the immunoglobulin (Ig) heavy chain (IgG). B) X-gal filter assay of yeast transformants cotransformed with the bait construct, pDB-Rho39Tr (rhodopsin C-terminus fused to the GAL4-DB), and each of the prey constructs, pAD-Tctex-1, pAD-S82A, pAD-S82E or pAD-rp3. C) Various GST fusion proteins immobilized on glutathione beads were incubated with MBP-Rho39Tr. Proteins eluted with glutathione were separated by SDS-PAGE followed by Coomassie Blue staining (top) or immunoblotting with mAb 1D4, which recognizes rhodopsin's C-terminus (bottom). D) Lysates of 293 cells transiently transfected with Flag-Tctex-1 variants or untransfected 293 cells (Mock) were subjected to direct immunoblotting (input) or immunoprecipitation using anti-IC mAb. The IC immunoprecipitates were subsequently immunoblotted with anti-IC and anti-Tctex-1 Abs. Anti-Tctex-1 Ab recognized both exogenously (exo) introduced Flag-Tctex-1 and the endogenous (endo) Tctex-1.

Figure 2. Characterization of MDCK cells inducibly overexpressing S82 mutants

A) Total cell lysates prepared from uninduced (Dox+) and induced (Dox-) cell lines, as indicated, were separated by SDS-PAGE and immunoblotted for tubulin (TUB), IC and Tctex-1. Anti-Tctex-1 Ab recognized both overexpressed Flag-Tctex-1 and endogenous Tctex-1. B) Total lysates or anti-IC immunoprecipitates (IP) obtained from MDCK cells expressing WT, S82A and S82E were immunoblotted (IB) with the indicated Abs. C) High-speed supernatants prepared from homogenates of induced MDCK cell lines were separated by velocity sedimentation on 5–20% sucrose gradients. Aliquots from all fractions were separated on SDS-PAGE and IB with the indicated Abs. Standards of 3.5S, 11.3S, and 19S are indicated. D–F) Confocal images of GOS-28 immunostaining (green) in cells overexpressing wt (D), S82A (E) and S82E (F). Blue: 4,6-diamidino-2-phenylindole (DAPI). Bar = 20 μ m.

Figure 3. Specific inhibition of rhodopsin's apical polarity in MDCK cells expressing S82 mutants

A–C) MDCK monolayers were left uninduced (DOX+) or induced (DOX–) to express WT or S82 mutants prior to infection with rhodopsin adenovirus (A) and p75 adenovirus (B). X–Z scanned confocal images of filters labeled with anti-rhodopsin Ab (green in A), anti-p75 (red in B) and anti-gp135 (green in B). Uninfected filters double labeled with anti-Na,K-ATPase (green) and E-cadherin (red) are shown in (C). Blue: 4,6-diamidino-2-phenylindole (DAPI). Bars = 10 μm. D) The rate of paracellular permeability and the TER of uninduced or induced MDCK monolayers grown on filters are shown. The control T23/MDCK monolayers were placed in the low Ca²⁺ medium for 16 h prior to the analysis. All values represent means ± SD of three independent experiments. Asterisks indicate significant difference ($p < 0.05$) in ANOVA. SD, standard deviation.

Figure 4. The apical transport of rhodopsin in polarized MDCK cells is through a direct pathway and not by transcytosis

A) Schematic diagram of the rhodopsin-GFP reporter. B) Confocal images of microinjected subconfluent cells after 20°C cold-block and after 120-min 32°C release (top panels; x-y views); microinjected polarized cells after 20°C cold-block and after 90-min 32°C release (bottom panels; x-z views). Arrows point to the rhodopsin-GFP accumulated at the TGN. Dotted lines demarcate the cell margins and/or the filters. Bars = 5 μm. C) Four-day-old fully polarized MDCK cells were injected with plasmid encoding rhodopsin-GFP, followed by the TGN block and release. Tannic acid (TA) was added to either the apical or the basal chamber, and the incubation time for 32°C is indicated. Filters were double labeled with ZO-1 (red) to reveal the position of apical junction. Bar = 10 μm.

Figure 5. S82 phosphorylation of Tctex-1 and its effect in dynein incorporation (A–B) and analysis of Tctex-1 mutant phosphorylation

A) MDCK monolayers expressing WT and S82A were pulse labeled with [35 S]-cysteine/methionine, followed by a chase. Cells harvested at indicated time-points were equally aliquoted for immunoprecipitation (IP) using anti-Flag and anti-IC Abs. Immunoprecipitates were separated on SDS-PAGE, and the [35 S]-incorporated Tctex-1 was quantified by PhosphorImager. Tctex-1 was measured by Tctex-1 radioactivity at each time-point relative to starting levels. B) Kinetics of newly synthesized Tctex-1 incorporation into dynein complex is represented by the percentage of IC-bound Tctex-1 within the total pool of Tctex-1 at each time-point. Specifically, the percentages represent the radioactivity of Tctex-1 coimmunoprecipitated by IC Ab relative to the radioactivity of total Tctex-1 immunoprecipitated by anti-Flag Ab at each chase time-point. Bars represent standard deviations. C) Anti-Flag (top panel) and anti-IC (bottom panel) immunoprecipitates of MDCK stable lines expressing Flag-WT and S82A mutant were subjected to two-dimensional gel electrophoresis and immunoblotted with anti-Tctex-1 Ab.