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Biochemical and Biophysical Research Communications 351 (2006) 106–112
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EFA6 regulates endosomal trafficking and affects early endosomes in polarized MDCK cells

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Received 17 September 2006
Available online 12 October 2006

Abstract

The small-GTPase family of ADP ribosylation factors (ARFs) recruit coat proteins to promote vesicle budding. ARFs are activated by an association with sec7-containing exchange factors which load them with GTP. In epithelial cells, the small GTPase ARF6 operates within the endocytic system and has been shown to associate with ARNO to promote apical endocytosis and early to late endosomal trafficking. EFA6 has been shown to stimulate tight-junction formation and maintenance. Here, we show that in polarized epithelial MDCK cells, EFA6 is localized to early endosomes, causes their dramatic enlargement, and promotes basolateral targeting of IgA, which is normally targeted to the apical PM. These results suggest that the physiological function of ARF6 within the endocytic system is regulated by the exchange factor it associates with.

Keywords: EFA6; ARNO; ARF6; MDCK cells; Epithelia; Early endosome; Membrane traffic

ADP ribosylation factors (ARFs) are a family of Ras-related small GTPases. To date, six isoforms have been identified in mammals that can be classified into three classes based on their structural similarities: Class I (ARF1–3), Class II (ARF4–5), and Class III (ARF6) [1]. ARF6 is the most divergent member of the ARF family; it regulates the actin cytoskeleton and plays a role in membrane-transport at the level of the plasma membrane (PM) [2]. In rat kidneys, ARF6 has been detected in the brush border at the apical PM of epithelial cells [3], and subcellular fractionation suggests that it could be present on apical early endosomes (EEs) [4]. We have shown that in polarized epithelial MDCK cells, ARF6 is localized to the apical PM where it regulates actin-dynamin-clathrin-dependent endocytosis [5,6]. Overexpression of ARF6 in nonpolarized MDCK cells has been found to lead to membrane ruffles and actin rearrangement, and to affect the turnover of adherens junctions [7,8].

Like all small GTPases, the ARF family members alternate between GTP-bound active and GDP-bound inactive forms by associating with guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs facilitate the exchange of the bound GDP with GTP, whereas GAPs inactivate ARFs by promoting the intrinsic hydrolysis of the bound GTP to GDP [9,10]. All ARF GEFs identified to date contain a Sec7 domain, which is essential for the guanine exchange activity, and can be classified into several groups based on structural and functional differences: the Gea/Gnom/GBF family, Sec7/BIG family, ARNO/cytohesin/GRP family, EFA6 family, and ARF-GEP100 [9–11]. Of these, EFA6, cytohesin-1, ARNO, GRP1, and ARF-GEP100 have been shown to be able to facilitate exchanges in ARF6 [11–15].

Ectopic expression of EFA6 (forms A and B) in nonpolarized cells (TRVb-1 and HeLa) induces the formation of actin-based membrane ruffles and microspikes on the PM. EFA6A perturbs the trafficking of transferrin [12,15] and both EFA6 forms localize to the apical domain of polarized MDCK cells and participate in tight-junction biogenesis [16]. We have shown that ARF6 and the exchange

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0006-291X/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2006.10.024

factor ARNO localize to and regulate endocytosis at the apical surface. Moreover, coexpression of ARNO with the wild-type form of ARF6 results in synergistic stimulation of apical endocytosis, whereas expression of a mutant ARF6-GDP-bound form inhibits ARNO-dependent stimulation of apical endocytosis [6,12,15,17]. Together, both EFA6 and ARNO localize to the apical domain of polarized MDCK cells: whereas ARNO participates in clathrin-mediated endocytosis at the apical PM, EFA6 is involved in tight-junction formation following E-cadherin engagement. To date, there has been no direct evidence linking EFA6 to any membrane-transport event in this epithelial domain, nor has it been linked to a specific ARF within the apical domain.

Here, we show that in polarized MDCK cells, EFA6 is localized to the EE compartment, where its ectopic expression results in EE enlargement and the accumulation of internalized IgA from either apical or basolateral PMs. In addition, while not affecting the rate of endocytosis at either PM, it affects transcytosis by supporting IgA transport toward the basolateral PM.

Experimental procedures

Materials

Chemicals were purchased from Sigma–Aldrich (Rehovot, Israel) unless otherwise indicated. Growth media were from Biological Industries (Beit Haemek, Israel). All fluorescent secondary antibodies were from Molecular Probes (Eugene, OR). Anti-MP30 was kindly provided by Peter Walter (UCSF, San Francisco, CA), mouse monoclonal antibody against vesicular stomatitis virus glycoprotein (VSV-G) epitope (clone P5D4) was from Boehringer Mannheim (Mannheim, Germany), sheep anti-human secretory IgA was from Cappel Laboratories (Cochranville, PA), and rat monoclonal antibody against ZO1 was obtained from Chemicon International (Temecula, CA). All fluorescent secondary antibodies were from Molecular Probes. Secondary antibodies conjugated to horseradish peroxidase (HRP) were from Jackson Immunoresearch (West Grove, PA).

Methods

Construction of recombinant adenovirus. All recombinant DNA steps were performed using standard techniques. EFA6, including its N-terminal VSV-G tag, was excised from pGEM1 and inserted into adenoviral vector pADtet 7 using *Eco*RI and *Xba*I [18].

Cell culture and adenoviral production. MDCK cells were grown as previously described [6]. Production of adenovirus in HEK293-cre recombinase-expressing cells and use of adenovirus in MDCK cells were as previously described [18,6]. Protein levels were regulated by the concentration of doxycycline (Dx), the amount of virus, and the length of time following infection and/or Dx removal. Cells infected by recombinant adenovirus were incubated for 18 h, to express the recombinant EFA6. We verified all heterologous expression by means of immunoblot assay. Controls in all experiments included cells that were (i) not infected, (ii) infected but expression of EFA6 was fully repressed by 20 ng/ml Dx, or (iii) infected with a control virus encoding β-galactosidase. These exhibited complete loss of EFA6-dependent EE enlargement, of effects in membrane-transport assays, and of specific signals in immunofluorescence.

Immunofluorescence of MDCK cells. These procedures were performed as previously described [6]. Images were taken using a NIKON TE-2000S (Melville, NY) inverted fluorescence microscope with a plan Apo 60× objective lens (Nikon), equipped with a *Z* stepper and a Hammamatzo CCD ORCAII camera (Hammamatzo, Tucson, AZ). Images were all deconvolved with *SimplePCI* software (Improvision, Coventry, UK). All images were compiled using Adobe Photoshop and/or Canvas software ACD Systems International Inc. (Victoria, British Columbia, Canada) and are representative of the original data.

IgA transcytosis assay in MDCK cells. These procedures were performed as previously described [19]. In Brief, ^{125}I-IgA was bound for 60 min at either the apical (5×10^6 cpm) or basolateral (3×10^6 cpm) at 4 °C; unbound ^{125}I-IgA was removed by extensive washing with ice-cold media. Cells were warmed up to 37 °C to enable endocytosis and the loading of labeled IgA into the EE. Cells were then cooled down by replacing the media with ice-cold media. ^{125}I-IgA remaining on either PM was removed by incubating the cells with 25 μg/ml trypsin for 10 min. The trypsin step was repeated twice. Excess trypsin was washed away and replaced with 125 μg/ml soybean trypsin inhibitor for an additional 10 min. The cells were then rewarmed to 37 °C and at the indicated time points, medium was collected from both the apical and basolateral PMs, and replaced with fresh medium. At the conclusion of the experiment, all media were quantitated for radioactivity and results were used to calculate the percentage of IgA transcytosed or recycled back to the original membrane.

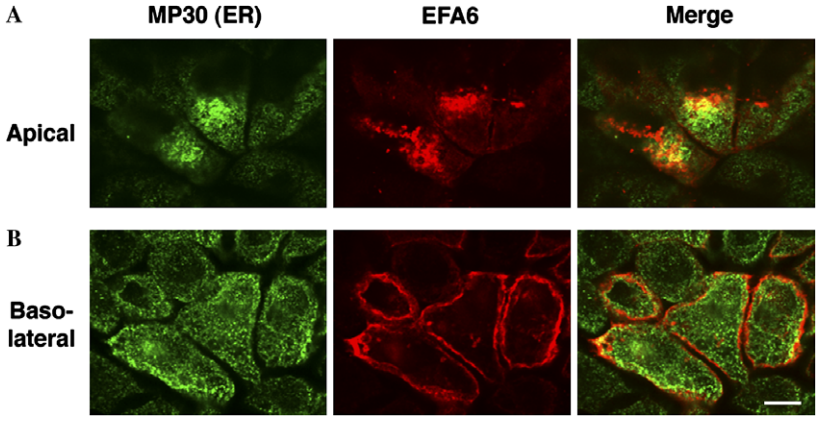

Fig. 1. EFA6 is localized to endosomal compartments throughout the cell. MDCK tet off cells were grown on a permeable support (corning transwells) for 3 days, and subsequently infected with EFA6 adenovirus, incubated for 18 h for expression, and processed for immunofluorescence with anti-ER marker MP30 (green) and anti-VSV-G tag P5D4 (red) antibodies. Apical (A) and basolateral (B) sections reveal intracellular punctate staining that does not colocalize with the ER marker and resembles endosomal staining. Bar 5 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

Results and discussion

EFA6 localizes to EEs and increases their size

To explore the role of the exchange factor EFA6 in polarized MDCK cells, we used rabbit antiserum raised against purified recombinant human EFA6 and found that it does not react with endogenous MDCK EFA6. The anti-human EFA6 most probably did not react with the canine EFA6 due to species differences. We therefore generated recombinant adenoviruses that harbor the wild-type form of EFA6 tagged at its N-terminus with VSV-G tag [12]. We infected Tet-MDCK II cells with various concentrations of recombinant adenovirus and defined low and medium levels of overexpression, as in previous studies with the exchange factor ARNO [17]. These expression levels were found to be nontoxic: they did not affect the polarity of MDCK cells and were high enough to affect membrane-transport events in a specific manner. In agreement with a previous observation that EFA6 does not activate ARF1 in vitro, immunofluorescence analysis revealed no colocalization of EFA6 with MP30 an endoplasmic reticulum (ER) marker [20]. This result precludes the possibility that in MDCK cells, EFA6 associates with ARF1. We therefore expected that EFA6 would localize to either the apical PM or apical endosomal compartment to affect trafficking regulated by ARF6. An immunofluorescence experiment in which the VSV-G-tagged EFA6 was costained with MP30 showed that it does not colocalize with the ER, nor does it affect its morphology (Fig. 1). In both basolateral and apical sections, EFA6 was seen as punctate staining, localized to the periphery of the cell near the PM. Within the basolateral region, EFA6 created a chain of puncta that surrounded the cell along the PM. In polarized MDCK cells, this region is commonly occupied by EEs. We therefore expressed EFA6 in MDCK cells and costained with EEA1, an EE marker. As shown in Fig. 2, in MDCK cells, EEA1 staining produced a small number of puncta in each microscopy section. Both low and medium EFA6 expression revealed complete colocalization with EEA1, indicating that EFA6 operates on EEs. In cells expressing a low level of EFA6, in addition to the punctate EEA1 staining we observed its diffuse cytoplasmic staining, suggesting that EFA6 caused increased expression of EEA1, which is required for EE fusion. Medium expression of EFA6 resulted in a substantial increase in EE structures, which were seen as a chain of puncta in the basolateral domain. The observation that EFA6 localizes to the EE and the massive increase in EE structures suggest that either EFA6 promotes budding of EE vesicles which participate in homotypic fusion to create enlarged EEs in the cell, or there is massive fragmentation of EEs that could not be observed under light microscope. The first possibility is supported by previous studies showing homotypic fusion of EEs regulated by Rab5-EEA1 and other polypeptides [21] indicating that alteration of fusion or in our case budding machinery may result in enlarged EE. The rab5-EEA1

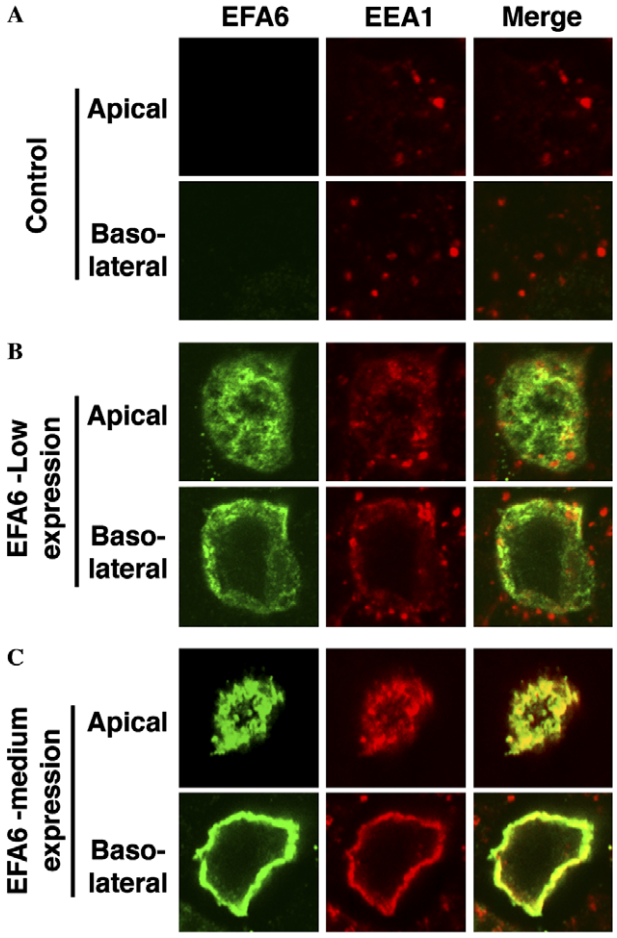

Fig. 2. EFA6 localizes to the early endosomal (EE) compartment and causes its enlargement. MDCK tet off cells were grown on a permeable support (corning transwells) for 3 days, and were subsequently left uninfected (control) (A), or infected with EFA6 adenovirus, incubated for 18 h for expression (B,C), and processed for immunofluorescence with anti-EE marker EEA1 (red) and anti-VSV-G tag P5D4 (green) antibodies. (A) Control cells show typical EE staining in MDCK cells. (B) Cells infected with a reduced amount of EFA6 adenovirus show that in addition to punctate endosomal staining, the EEA1 endosomal marker appears diffused in the cytoplasm. (C) Cells infected with a moderate amount of EFA6 adenovirus show an increased amount of punctate staining at the apical domain. In the basolateral domain, this increased punctate staining generates a "band" of EEs traced by EFA6 that is between the cytoplasm and lateral PM. EFA6 and EEA1 completely colocalize. Bar 2 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

complex has been shown to be critical for fusion of EE-derived vesicles (homotypic fusion) and for fusion of clathrin-coated vesicles arriving from the PM. In the same manner, EFA6 promotes vesicle budding from EEs. We speculate that EE vesicles undergo homotypic fusion because of a lack of fusion targets (recycling endosomes, common endosomes, or late endosomes), resulting in enlarged EEs. The phenomenon of increased EE size has been previously documented as a result of the expression of specific genes such as Rab5 and its effectors, or the treatment of cells with cytokines, hormones or drugs [22–26]. These previous observations of EE enlargement have been, in most cases,

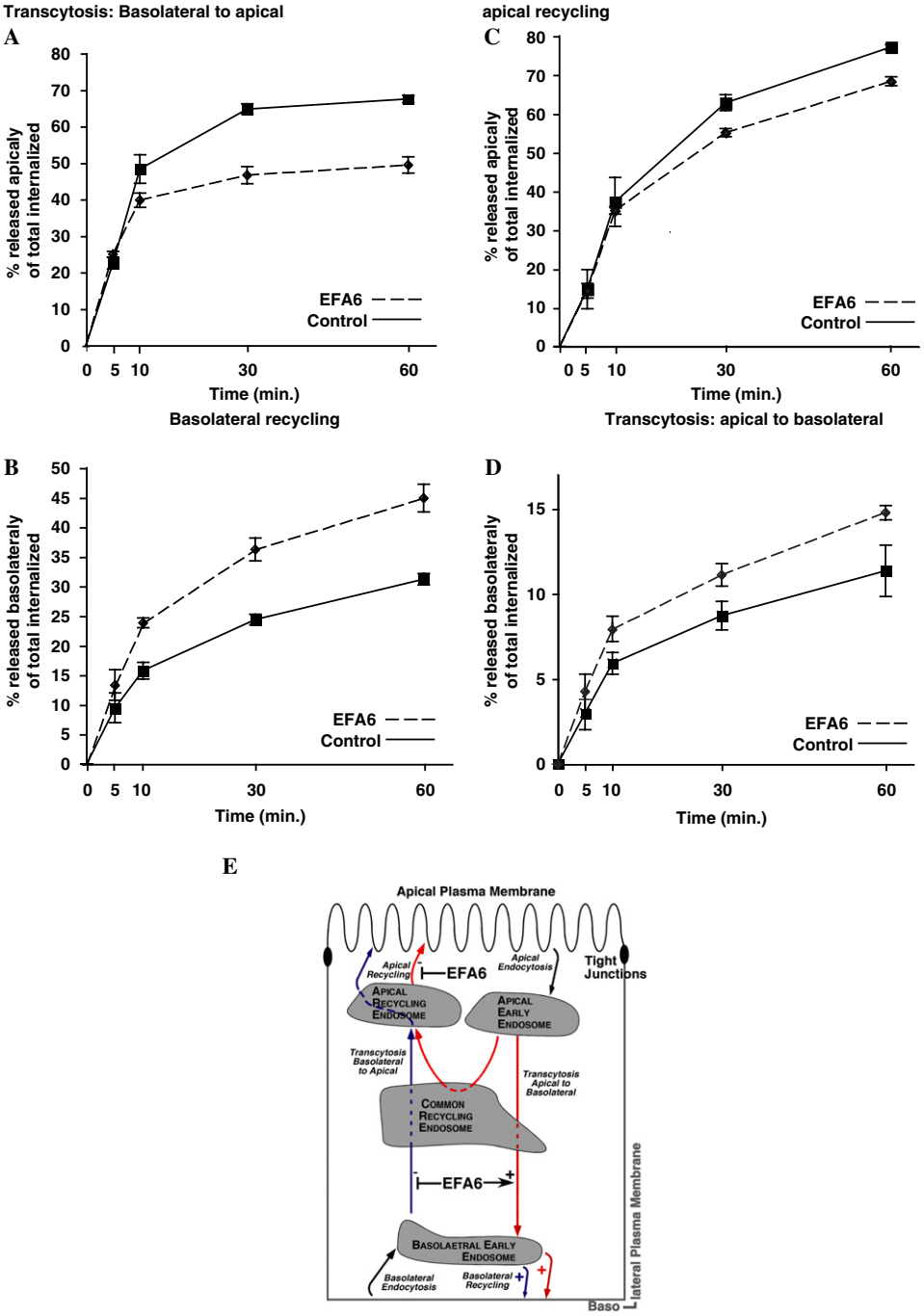

Fig. 3. Expression of EFA6 stimulates transcytosis from the apical domain toward the basolateral PM. MDCK tet off cells were grown as a confluent monolayer for 3 days on corning transwells. Control cells (uninfected) and cells expressing EFA6 were then incubated with ^{125}I-IgA for 60 min at 4 °C at either the basolateral (A,B) or apical (C,D) PM. Subsequently, unbound ^{125}I-IgA was extensively washed and cells were warmed for 5 min at 37 °C. ^{125}I-IgA remaining at either membrane was removed by trypsin treatment. Consequently, cells were rewarmed and media were collected from both apical and basolateral compartments at the indicated time points. The radioactivity was measured and used to calculate the amount of ^{125}I-IgA released to the apical (A,C) or basolateral (B,D) compartment. Control, unbroken line ■; EFA6, dashed line ♦. (E) Schematic diagram illustrating the transcytosis and recycling assay results. Red line describes EFA6-affected pathway for IgA loaded apically, blue line describes EFA6-affected pathway that was loaded from the basolateral PM. −/+ show pathways inhibited/stimulated by EFA6, respectively. (A,B) PM transcytosis was inhibited while recycling back to the basolateral PM was increased. When cells were loaded from the apical PM, transcytosis from the apical-to-basolateral PM was stimulated and apical recycling inhibited. Overall, EFA6 expression stimulates delivery toward the basolateral PM. Experiments done in triplicate. Representative experiment shown. The experiment was repeated four times. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

due to effects on the fusion machinery. Our observation of EFA6-dependent EE enlargement in MDCK epithelial cells being due to increase in budding events may be explained by stimulated budding from recycling endosomes, common endosomes or late endosomes. This would cause the specific endosomal compartment to be reduced in size in parallel to an increase in the EE compartment. However, we performed immunofluorescence staining of Rab7 (late endosomes) or Rab11 (recycling endosomes) in cells expressing EFA6 and found that expression of EFA6 does not alter their structure or their distribution (data not shown).

Expression of EFA6 results in increased apical to basolateral transcytosis and basolateral recycling

The effect of EFA6 expression on both apical and basolateral EEs and the well-established role of the EE in membrane-transport, together with its role in regulation of the transcytotic pathway, led us to assay EFA6's effect on membrane-transport in MDCK cells. Expression of EFA6 followed by endocytosis assay of IgA bound to IgA receptor from either the basolateral or apical PM showed no effect (data not shown). These results fall in with our observation that EFA6 is not localized to either the apical or basolateral PM. Moreover, we have previously shown that ARF6 localizes to and regulates clathrin-mediated endocytosis at the apical PM [5,6,17]. Additionally, coexpression of the wild-type forms of ARF6 together with EFA6 does not show a stimulatory effect on apical endocytosis (data not shown), while ARF6 coexpressed with ARNO revealed synergistic stimulation of apical receptor-mediated endocytosis [17]. Therefore, under these conditions, in polarized MDCK cells, ARF6 and EFA6 do not participate together in apical endocytosis. To further investigate membrane-transport steps that may be affected by EFA6, we performed a recycling and transcytosis assay from both PMs. We bound IgA to either the apical or basolateral PM, and internalized the IgA for 5 min, by a procedure known to cause loading of IgA into the EEs. Subsequently, IgA remaining on either PM was removed by trypsinization. We then followed the fate of the internalized IgA by quantitating the IgA released at either PM. In Fig. 3, we show that EFA6 expression inhibited transcytosis of IgA loaded from the basolateral transcytosing to the apical PM and concomitantly increased IgA recycling back to the basolateral PM (Fig. 3A and B). The fate of IgA loaded from the apical PM supports the results of IgA

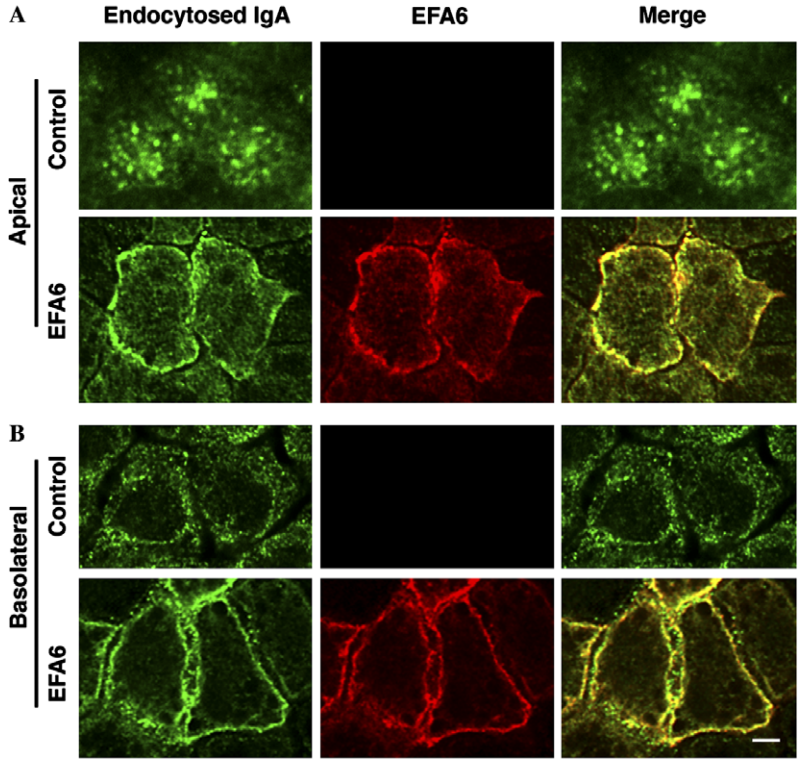

Fig. 4. Endocytosed IgA co-localizes to enlarged early endosomal (EE) compartment containing EFA6. MDCK tet off cells were grown on a permeable support (corning transwells) for 3 days, and were subsequently left uninfected (control), or infected with EFA6 adenovirus, incubated for 18 h for expression. Subsequently, cells were incubated with IgA at the apical (A), or basolateral (B), PM for 60 min at 4 °C. Unbound IgA was extensively washed and cells were warmed up for 5 min to enable endocytosis. Remaining IgA on either PM was removed by trypsinization. Cells were fixed and processed for immunofluorescence with anti-IgA (green) and anti-VSV-G tag P5D4 (red) antibodies. (A) In cells that internalize IgA from the apical PM, typical punctate IgA similar to early endosomal tracing is observed. When EFA6 is expressed, IgA is colocalized to EFA6 in a chain of EEs. (B) In cells that internalize IgA from the basolateral PM, punctate IgA is observed. When EFA6 is expressed, IgA is completely colocalized to EFA6 in a chain of EEs (compare to Fig. 2C). Bar 2 μM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

loaded from the basolateral PM. EFA6 expression stimulated apical-to-basolateral transcytosis and inhibited IgA recycling back to the apical PM (Fig. 3C and D). Taken together, in MDCK cells EFA6 supports IgA–IgA receptor complex-sorting vesicles destined to the basolateral PM (Fig. 3E). To further study the inhibitory effect of EFA6 on apical transport of IgA–IgA receptor complex, we performed an immunofluorescence experiment. We bound IgA to either the apical (Fig. 4A) or basolateral (Fig. 4B) PM. Following 5 min of warming, the remaining IgA bound to either PM was removed by trypsinization. Samples were further labeled for either EFA6 or IgA. Internalized IgA appeared in punctate staining that resembled EE staining (Fig. 2, control). In contrast, in cells expressing EFA6, IgA appeared completely colocalized with EFA6, independent of the PM it had been internalized from (apical or basolateral), and the staining resembled that of EEs traced by EEA1 (Fig. 2C). Interestingly, in cells expressing EFA6, where IgA was internalized from the apical PM, the brightest IgA section was shifted toward the basolateral side and looked like a basolateral section. This might be explained by EFA6's effect of shifting EE compartment transport toward the basolateral PM, which translocates the compartment's localization, resulting in IgA transport toward the basolateral PM (Fig. 3A–D).

The IgA–IgA receptor complex is one of the most well-studied and accepted models for transport across epithelial cells. Specifically, IgA stimulates IgA receptor to be sorted from basolateral EEs into transcytotic vesicles that fuse with apical early/recycling endosomes; it is subsequently transported to, and released at the apical PM [19,27]. Therefore, basolateral-to-apical transport is the default pathway for the IgA–IgA receptor complex. In contrast, we found that EFA6 expression causes transport in the opposite direction (Fig. 3E), i.e., apical to basolateral. This result suggests that altering the default route of the ligand-receptor complex may require specific recruitment of the EFA6 exchange factor to EE to stimulate budding of vesicles targeted toward the basolateral PM. The role of EFA6 is critical for the sorting of membrane proteins from the EE compartment, as members of the ARF family, in their GTP-bound state, together with phosphoinositides, initiate coat assembly by functioning as docking sites for the budding apparatus (clathrin, γ-adaptin homology-Golgi associated Arf-binding protein (GGA) coats), that in turn, binds directly or indirectly to sorting signals in transmembrane proteins. Thus modification of transmembrane receptors appears to determine their direction of transport by recruiting the appropriate accessory proteins. This recruitment is initiated by the appropriate ARF and its activation by the appropriate exchange factor [28,29]. A breakthrough in the mechanism by which ARF6 and ARNO are recruited from the cytosol to endosomes was recently made. Hurtado-Lorenzo and colleagues showed that ARF6 interacts with the c-subunit, and ARNO with the a2-isoform of V-ATPase on EEs, in an intra-endosomal acidification-dependent manner. Inhibition of endosomal acidification abrogates this interaction and membrane-transport between early and late endosomes [30]. Likewise, TWIK1, a potassium channel involved in epithelial potassium transport, localized to the subapical compartment in renal proximal tubules, has been shown to recruit EFA6 when already bound to ARF6 [31]. These recruitment mechanisms come in addition to the known recruitment of ARF6 and ARNO through its coiled-coil domain to receptor tails to stimulate receptor endocytosis [17,32,33]. We hypothesize that both exchange factors, EFA6 and ARNO, localize to either the apical PM or EE as the site at which they associate with and recruit ARF6 to stimulate a different membrane-trafficking pathway. While ARNO governs apical endocytosis, EFA6 recruits ARF6 for EE homotypic fusion as well as tight-junction formation and maintenance.

Acknowledgments

We thank Philippe Chavrier for EFA6 constructs and for critical reading of the manuscript. This research was supported by the Israel Science Foundation (Grant No. 1318/04) to Y.A., Y.A. is affiliated with the David R. Bloom Center for Pharmacy at the Hebrew University.

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