Retromer maintains basolateral distribution of the type II TGF-β receptor via the recycling endosome

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ABSTRACT Transforming growth factor β (TGF-β) is critical for the development and maintenance of epithelial structures. Because receptor localization and trafficking affect the cellular and organismal response to TGF-β, the present study was designed to address how such homeostatic control is regulated. To that end, we identify a new role for the mammalian retromer complex in maintaining basolateral plasma membrane expression of the type II TGF-β receptor (TβRII). Retromer and TβRII associate in the presence or absence of TGF-β ligand. After retromer knockdown, although TβRII internalization and trafficking to a Rab5-positive compartment occur as in wild-type cells, receptor recycling is inhibited. This results in TβRII mislocalization from the basolateral to both the basolateral and apical plasma membranes independent of Golgi transit and the Rab11-positive apical recycling endosome. The data support a model in which, after initial basolateral TβRII delivery, steady-state polarized TβRII expression is maintained by retromer/TβRII binding and delivery to the common recycling endosome.

INTRODUCTION

Plasma membrane receptors are regulated, in part, through the action of cis regulatory motifs interfacing with the transport machinery. Because a number of diseases result from defects in the ability to sort or transport proteins to their appropriate cellular destination (Stein et al., 2002; Verges, 2007; Mellman and Nelson, 2008) and transforming growth factor β (TGF-β) regulates a variety of cellular processes critical for normal homeostasis (Roberts, 1992; Roberts and Wakefield, 2003; ten Dijke et al., 1996; Akhurst and Derynck, 2001; Elliott and Biobe, 2005), we initiated studies to define and characterize the mechanisms controlling the spatial distribution of the TGF-β receptor (TGF-βR) complex.

The response to TGF-β usually depends on the cell type involved, with effects as diverse as growth and growth inhibition (Moses and Serra, 1996; Serini and Gabbiani, 1999; Bissell, 2001; Yue and Mulder, 2001). In general, the majority of mammalian cells express three TGF-β-binding species, referred to as type I (TβRI), type II (TβRII), and type III (β-glycan) receptors. The pivotal role that TGF-β plays in modulating a number of biological activities makes it essential to identify the regulatory mechanisms through which appropriate type I and type II receptor expression is maintained.

Although there is an enormous body of literature documenting the biological relevance and signaling activity of the TGF-βR complex, there is a paucity of information on the trafficking itinerary of this receptor family and essentially no literature investigating this question in polarized epithelial cell models. To address that issue, we generated a chimeric receptor model consisting of the extracellular domain of the granulocyte-macrophage colony-stimulating factor (GM-CSF) α or β receptor fused to the transmembrane and cytoplasmic domains of TβRI or TβRII (Anders and Leof, 1996; Anders et al., 1997; see Methods and Materials, Cell culture, for discussion of chimeric receptors). This system provides a technically...
facile approach to evaluate the trafficking itinerary of various TGF-βR complexes. Using native as well as chimeric type I (αI) and type II (βII) TGF-βRs, we found that both receptors independently traffic to the basolateral surface in polarized Madin–Darby canine kidney (MDCK) and NMuMG cells adjacent to the zonula adherens complex (Murphy et al., 2004, 2007).

Although the targeting of many basolateral proteins is regulated by tyrosine- or dileucine-based motifs (Matter et al., 1992; Aroeti et al., 1993; Hunziker and Fumey, 1994; Stein et al., 2002; Rodriguez-Boulan et al., 2005; Mellman and Nelson, 2008), analysis of TβRII showed that a unique element (referred to as the LTA motif) between residues 529 and 538 was necessary and sufficient for basolateral delivery (Murphy et al., 2007). Because 1) the cellular activities/factors that maintain appropriate TGF-βR-directed membrane expression have not been identified and 2) these reflect fundamental questions that affect a number of TGF-βR-directed phenomena, as well as the homeostatic mechanisms by which cellular integrity is maintained, we used a terminal 84–amino acid cytoplasmic fragment (residues 484–567) as “bait” in a tandem-affinity tag pull down. Proteins were visualized by silver stain and identified by mass spectrometry. One protein of interest, due to its role in a variety of trafficking functions, was the retromer vacuolar protein sorting protein 26 (Vps26) subunit.

The retromer was initially identified in yeast as a multimeric protein complex that mediates intracellular sorting of Vps10, a receptor that transports vacuolar (i.e., lysosomal) enzymes (Seaman et al., 1998). It consists of two subcomplexes: Vps35, Vps29, and Vps26, which function in cargo selection, and Vps5 and Vps17, which are proposed to sense membrane curvature and induce the formation of tubules. Human orthologues have been found for each of the yeast Vps proteins (the one exception being Vps17), with sorting nexin-1 and/or -2 (SNX1, SNX2) functioning as the Vps5 homologue and Vps17 function in mammalian cells, being mediated by either SNX5 or 6 (Wassmer et al., 2007, 2009). Subsequently the retromer was found to perform an analogous activity in mammalian cells: the endosome-to-Golgi retrieval of the cation-independent mannose 6-phosphate receptor (CI-MPR; Arighi et al., 2004; Seaman, 2005). Retromer subunits have additional regulatory roles, including transcytosis of the polymeric immunoglobulin receptor, Wnt gradient formation, processing of the amyloid precursor protein, and, of most relevance to the present study, apoptotic cell clearance by phagocytic receptor recycling and Rab4-dependent β2 adrenergic receptor (β2AR) recycling (Verges et al., 2004; Verges, 2008; Seaman, 2005; Hierro et al., 2007; Chen et al., 2010; McGough and Cullen, 2011; Cullen and Korswagen, 2012; Temkin et al., 2011; Seaman, 2012).

Because the retromer had not been reported to differentially regulate apical versus basolateral delivery of any cargo in polarized epithelia, we investigated whether this TβRII/retromer association might indicate an additional role for this enigmatic complex. Surprisingly, although retromer knockdown has no significant effect on TGF-βR internalization, Smad2/3 phosphorylation, or initial basolateral targeting via the LTA motif, it provides an obligate function in the maintenance of TGF-βR basolateral membrane expression by promoting TβRII transit from the Rab5-positive early endosome to the common recycling endosome (CRE). In the absence of retromer, however, TβRII becomes mislocalized such that both apical and basolateral expression is observed.

RESULTS

Binding of type II TGF-βR to the mammalian retromer

Trafficking to the appropriate membrane domain is the initial event necessary for regulated epithelial cell growth (Drubin and Nelson, 1996) and is altered in a variety of disease states (Stein et al., 2002; Verges, 2007; Mellman and Nelson, 2008). Because the retromer Vps26 subunit was coprecipitated with a terminal 84–amino acid fragment of the type II TGF-βR (unpublished observations), to determine whether this novel interaction was specific and biologically important, we addressed the following questions. First, was the retromer/TβRII association ligand dependent and/or independent? Second, would retromer loss specifically prevent basolateral TβRII localization and/or TGF-βR signaling? Third, if TβRII trafficking was affected, what intracellular pathways and organelles were affected?

Retromer/TβRII association was examined in MDCK cells transiently transfected with epitope-tagged native TβRII or TβRII (Figure 1A), as well as an MDCK cell line (MD-1) expressing chimeric type I (αI) and type II (βII) TGF-βRs (Figure 1B; Anders and Leof, 1996; Anders et al., 1997; Mitchell et al., 2004; Murphy et al., 2004, 2007). Although both native and chimeric type II TGF-βRs could be coimmunoprecipitated with the retromer Vps26 subunit, this occurred independent of ligand, and no association was detected with TβRI or αI (Figure 1, A and B). Because retromer/type II TGF-βR binding was unexpected, we further verified this interaction by 1) demonstrating binding in another cell line, 2) documenting βII/retromer association regardless of the antibody order used for precipitation and blotting, and 3) showing that the complex could be coimmunoprecipitated with either Vps26 or Vps35 sera (Supplemental Figure S1). Finally, given that cargo binding is associated with Vps26, Vps29, and/or Vps35 retromer subunits, glutathione S-transferase (GST) fusion proteins demonstrated that in the absence of an intact retromer complex, βII binds Vps35 (Figure 1C).

Loss of retromer affects polarized TβRII localization independent of overall functional integrity, Smad phosphorylation, or TβRII localization

Identification of the retromer complex as a new interacting partner specific for TβRII generates a number of questions relating to TGF-βR regulation, trafficking, and/or signaling. To obtain information concerning the biological significance of TβRII/retromer binding, we knocked down the Vps35 subunit in MD-1 cells. Three independent clones from two short hairpin RNAs (shRNAs) were isolated and subsequently characterized (Figure 2A and Supplemental Figure S2). Consistent with previous publications (Arighi et al., 2004; Gullapalli et al., 2006), functional retromer loss was documented by observing decreased stability of the CI-MPR, as well as diminished levels of other retromer subunits (Figure 2B and Supplemental Figure S2B). Whereas CI-MPR levels depended on retromer expression (i.e., documenting effective retromer knockdown), similar to those reported for epidermal growth factor and transferrin receptors (Arighi et al., 2004), chimeric type I or type II TGF-βR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein expression was unaffected (Figure 2B). Most important, cellular polarity was independent of a functional retromer complex, as there was no apical-to-basal inulin flux in any of the retromer knockdown clones (Figure 2C). The inability of retromer knockdown to affect polarity per se in mammalian epithelial cells is in contrast to its role in the Drosophila follicle epithelium, where retromer controls epithelial cell polarity via the lysosomal degradation of the apical determinant Crumbs (Pocha et al., 2011).

In addition to regulating the trafficking itinerary of various cargo, retromer has been implicated in tumorigenesis through interaction with the Golgi-associated oncoprotein GOLPH3 and subsequent recycling of key components required for mTOR action (Scott et al., 2009). Because TGF-β signaling has a critical role in maintaining normal epithelial cell homeostasis, which is lost during tumor
findings document a novel role for the mammalian retromer in localizing TβRII to the basolateral plasma membrane in polarized epithelial cells.

**Maintenance of basolateral TβRII expression requires retromer-dependent endocytic trafficking**

Although the preceding data clearly document a requirement for the retromer in TβRII polarity, they do not address whether this reflects a “targeting” role to a defined membrane locale (i.e., analogous to the cis-acting LTA motif in TβRII; Murphy et al., 2007) and/or a “maintenance” activity by which retromer is critical to preserving steady-state basolateral TβRII protein. To investigate this issue directly, first, we Golgi blocked newly synthesized proteins in Transepithelial control MD-1 and retromer knockdown cells by 20°C treatment; second, we added a dilute trypsin/phosphate-buffered saline (PBS) solution to the apical and basal chambers during the last 30 min of the Golgi block to remove cell surface proteins; and third, we examined TβRII reexpression at the apical and/or basolateral plasma membranes over the next 3 h after transfer to 37°C. As progression (Elliott and Blobe, 2005; Derynck and Akhurst, 2007; Wu and Hill, 2009), we examined the effect of retromer loss on Smad activation. No appreciable difference was observed on Smad2 or Smad3 phosphorylation stimulated through either native (TGF-β) or chimeric (GM-CSF) receptor activation (Supplemental Figure S2, A and B).

In contrast to the lack of a role for retromer in Smad activation, retromer loss abrogated specific basolateral trafficking such that apical type II receptor expression was also observed. This was demonstrated for both native and chimeric receptors using confocal microscopy (Figure 2, D and E, and Supplemental Figure S2C), as well as by domain-specific membrane biotinylation (Figure 2F and Supplemental Figure S2D). Furthermore, consistent with previous data showing lack of retromer/TβRII binding and retromer knockdown having no effect on junctional integrity (Figures 1 and 2C), TβRII, α1, ZO-1, and E-cadherin localization were all unaffected by retromer loss (Figure 2, D–F, and Supplemental Figure S2, C and D). Because the localization of cytokine receptors to defined membrane domains is critical to appropriately respond to external cues, the present results indicate that retromer is necessary to maintain TβRII expression at the basolateral plasma membrane in polarized epithelial cells.
Internalization and subsequent intracellular trafficking of basolateral TβRIIs. The first of these issues was addressed (Figure 4) using two distinct methods. For the first approach, polarized MD-1 and retromer knockdown cells were basolaterally treated at 10°C with antibodies to the extracellular domain of the chimeric type II TGF-βR (βII), and their transcytosis to the apical membrane was monitored after 37°C chase (Thompson et al., 2007). Consistent with previous data documenting that TGF-βRs localize to the basolateral plasma membrane (Murphy et al., 2004, 2007), no appreciable apical βII staining was detected in wild-type MD-1 cultures. However, when βII transcytosis was examined in the absence of retromer, significant apical βII expression was observed (Figure 4, A and B). This finding was independently confirmed using cycloheximide to block de novo protein synthesis (∼95%; unpublished observations), apically treating with trypsin to remove cell surface receptors (time 0), and then examining by confocal microscopy for βII reexpression at the apical and basolateral plasma membrane domains over the next hour (Figure 4, C and D). As expected, control MD-1 cells showed basolateral βII expression at all times. In contrast to MD-1 cultures, shown in Figure 3A, although reexpression of TβRII at the plasma membrane became detectable within the first 40–60 min after trypsinization, it was initially localized to the basolateral membranes in both control and knockdown lines. In contrast to parental MD-1 cells, however, in the absence of retromer, apical TβRII localization also became apparent in the Vps35 knockdown cultures between 60 and 120 min. Because this was observed for both native and chimeric type II receptors (Figure 3, A and B, and C and D, respectively), the data indicate that although retromer does not direct membrane targeting of newly synthesized TGF-βRs, it has a fundamental and unique role in maintenance of type II TGF-βRs at the basolateral plasma membrane.

Given that the findings of Figure 3 were somewhat surprising, we further documented this conclusion and investigated the underlying mechanism. For retromer to provide a “maintenance” function, two important criteria would have to be fulfilled. First, apically localized TβRIIs would have to arise from preexisting basolateral receptors; and second, given that junctional integrity is maintained in the absence of retromer (Figure 2C), apical delivery would require the internalization and subsequent intracellular trafficking of basolateral TβRIIs. The first of these issues was addressed (Figure 4) using two distinct methods. For the first approach, polarized MD-1 and retromer knockdown cells were basolaterally treated at 10°C with antibodies to the extracellular domain of the chimeric type II TGF-βR (βII), and their transcytosis to the apical membrane was monitored after 37°C chase (Thompson et al., 2007). Consistent with previous data documenting that TGF-βRs localize to the basolateral plasma membrane (Murphy et al., 2004, 2007), no appreciable apical βII staining was detected in wild-type MD-1 cultures. However, when βII transcytosis was examined in the absence of retromer, significant apical βII expression was observed (Figure 4, A and B). This finding was independently confirmed using cycloheximide to block de novo protein synthesis (∼95%; unpublished observations), apically treating with trypsin to remove cell surface receptors (time 0), and then examining by confocal microscopy for βII reexpression at the apical and basolateral plasma membrane domains over the next hour (Figure 4, C and D). As expected, control MD-1 cells showed basolateral βII expression at all times. In contrast to MD-1 cultures,
Retromer retains basolateral TβRII

whereas transient trypsinization specifically removed all apically localized type II TGF-βRs from retromer knockdown cells (compare 0 min with control, no trypsin treatment), knockdown cells showed levels of new apical receptor expression approaching control by 60 min.

Figures 3 and 4 are consistent with the hypothesis that in the absence of retromer and new protein synthesis, basolaterally expressed type II TGF-βRs become relocalized to the apical membrane domain. Because this represents a unique role for the mammalian retromer, additional studies were performed to address the operative mechanism and pathway. For instance, if basolaterally expressed type II TGF-βRs (i.e., not intracellular or newly synthesized) provide the “receptor pool” that undergoes intracellular trafficking and mislocalization to the apical surface in the absence of retromer, such a process would require endocytic activity and depend on receptor internalization. This is directly examined in Figure 5, A and B. First, the apical membrane of Transwell polarized control and retromer knockdown MDCK cells was treated with a dilute trypsin solution as in Figure 3A to remove cell surface protein; second, cultures were then transfected with wild-type or dominant-negative green fluorescent protein (GFP)–dynamin II (K44A mutant; prevents the scission of endocytic vesicles); and third, apical βII expression was specifically examined in the GFP-positive transfected cells. As shown in Figure 5A and quantitated in Figure 5B, after removal of receptors from the apical surface, dominant-negative dynamin II (but not wild type) prevented subsequent basolateral-to-apical mislocalization of βII in retromer knockdown cells by ∼80%. Thus, in the absence of retromer, apical βII expression requires that basolateral receptors undergo dynamin-dependent internalization.

Because numerous endocytic pathways are contingent upon dynamin action (Doherty and McMahon, 2009) and TGF-βR internalization has been reported to use both clathrin- and caveolar-dependent mechanisms (Di Guglielmo et al., 2003; Mitchell et al., 2004), we extended this finding biochemically and determined the specific internalization machinery used for βII basolateral-to-apical mislocalization. As shown in Figure 5C (left), after trypsin removal of
clathrin-regulated endocytic response that is independent of ligand. Because previous work documented constitutive TβRII recycling dependent upon clathrin and Rab11 in nonpolarized monolayers (Di Guglielmo et al., 2003; Mitchell et al., 2004) and retromer has been implicated in regulating similar activity for phagocytic and β2-adrenergic receptors (Chen et al., 2010; Temkin et al., 2011), we examined whether a requirement for retromer in βII recycling might account for its apical mislocalization. Consistent with that hypothesis, when recycling assays were performed on MD-1 wild-type and retromer knockdown cells, the absence of retromer resulted in a ∼50–60% decrease in recycling (Figure 6, A and B).

Because recycling can be inhibited either before or after cargo internalization, we next investigated whether retromer acted at a defined site in type II TGF-βR trafficking. To initially address this question, we first performed studies using nonpolarized cultures. As shown in Figure 6, C–E, retromer knockdown had no effect on βII internalization to the Rab5-positive early endosome nor were receptors shunted to an alternative Rab4 recycling compartment. Apical proteins, inhibition of clathrin-dependent internalization with chlorpromazine (CPZ) prevented βII apical mislocalization to the same (or greater) degree as dominant-negative dynamin. In contrast, nystatin (Nys) inhibition of caveolar uptake was without effect, in that the reappearance of apically biotinylated type II TGF-βRs was detected with identical kinetics as seen in the absence of drug (control). That basolateral βII was unaffected by either apical trypsinization or CPZ/Nys (Figure 5C, right) further confirms junctional integrity and the absence of drug toxicity, respectively.

Specificity for clathrin and caveolar pathway inhibition by CPZ and Nys was determined using transferrin (Tfn) and lactosylceramide, respectively (unpublished observations).

Retromer regulates type II TGF-βR recycling downstream of Rab5
The preceding findings (Figures 2–5 and Supplemental Figure S2, C and D) show that maintenance of the type II TGF-βR at the basolateral plasma membrane in polarized epithelia requires a retromer- and clathrin-regulated endocytic response that is independent of ligand. Because previous work documented constitutive TβRII recycling dependent upon clathrin and Rab11 in nonpolarized monolayers (Di Guglielmo et al., 2003; Mitchell et al., 2004) and retromer has been implicated in regulating similar activity for phagocytic and β2-adrenergic receptors (Chen et al., 2010; Temkin et al., 2011), we examined whether a requirement for retromer in βII recycling might account for its apical mislocalization. Consistent with that hypothesis, when recycling assays were performed on MD-1 wild-type and retromer knockdown cells, the absence of retromer resulted in an ∼50–60% decrease in recycling (Figure 6, A and B).

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The preceding data identify a new role for the mammalian retromer in maintaining basolateral expression of the type II TGF-βR. Because retromer was initially characterized for its role in mediating retrograde endosome-to-Golgi trafficking and has been shown to regulate transport of Shiga toxin from the recycling endosome to the Golgi complex (Seaman, 2005; Lieu and Gleeson, 2010), we further investigated the pathways and organelles affected. Initial studies examined the role (if any) of retromer in retrograde Golgi transport by assessing the colocalization of internalized βII or CI-MPR membrane receptors with the trans-Golgi network (TGN) marker galactosyltransferase. In agreement with previous work (Arighi et al., 2004; Gomez and Billadeau, 2009), although retromer-dependent Golgi colocalization of internalized CI-MPR was observed, negligible βII/Golgi staining was detected in either monolayer (Figure 7, A and B) or polarized (Figure 7C) cultures.

Given that 1) Transwell polarized retromer knockdown cells show apical plasma membrane type II TGF-βR mislocalization (Figures 2–5 and Supplemental Figure S2, C and D) and 2) retromer is required for Rab11-dependent βII recycling after internalization to the Rab5-positive early endosome in nonpolarized MDCK cells (Figure 6), we investigated whether TβRII recycling in polarized cells both was retromer dependent and might reflect the operative pathway accounting for the apical mislocalization. Supportive of that hypothesis and analogous to what we observed in monolayer, the absence of retromer decreased βII recycling ~40–50% in polarized cultures yet had no effect on chimeric or native type II TGF-βR transit to the Rab5-positive basolateral early endosome (BEE; Figure 8, A–C). Because TβRII trafficking to a Rab5 compartment is retromer independent yet TβRII recycling is retromer dependent, this indicates that retromer functions downstream of Rab5.

Given that we previously reported a role for Rab11 in TβRII monolayer recycling (Mitchell et al., 2004) and Rab11 is believed to function primarily at the apical recycling endosome (ARE) in polarized epithelia (Fölsch et al., 2009; Golachowska et al., 2010), we next determined whether apical βII expression in retromer knockdown polarized cells reflected shunting to the ARE. Although there was no appreciable colocalization (Figure 8D, top) or cofractionation (Figure 8E, lane 3) of type II TGF-βRs and Rab11 in polarized wild-type MDCK cells (as expected, since this would reflect apical transit), contrary to our expectations, a similar lack of association was also seen in retromer knockdown cultures (Figure 8D, top) or cofractionation (Figure 8E, lane 3) of type II TGF-βRs and Rab11 in polarized wild-type MDCK cells (as expected, since this would reflect apical transit), contrary to our expectations, a similar lack of association was also seen in retromer knockdown cells (Figure 8D, top) or colocalization with Rab11 (Figure 8E, lane 3).

Similarly, in agreement with a recent study (Temkin et al., 2011), transferrin recycling via either the rapid Rab4 pathway or the Rab11 recycling endosome (Supplemental Figure S4), as well as Tfn receptor (TfnR) cofractionation with Rab4 (Figure 6E), was unaffected by retromer knockdown. In contrast to these findings, because TGF-βR recycling in monolayer cultures depends on retromer and Rab11 (Figure 6B; Mitchell et al., 2004), as expected, βII/Rab11 colocalization decreased ~40–50% in retromer knockdown cells (Figure 6, F and G). Thus, although initial type II TGF-βR trafficking to a Rab5-positive compartment is unaffected by the absence of retromer, subsequent transit to the Rab11-positive recycling endosome is reduced coincident with decreased recycling.

Apical type II TGF-βR mislocalization in retromer knockdown cells is independent of Golgi transit and the Rab11-positive apical recycling endosome

The preceding data identify a new role for the mammalian retromer in maintaining basolateral expression of the type II TGF-βR. Because retromer was initially characterized for its role in mediating retrograde endosome-to-Golgi trafficking and has been shown to regulate transport of Shiga toxin from the recycling endosome to the Golgi complex (Seaman, 2005; Lieu and Gleeson, 2010), we further investigated the pathways and organelles affected. Initial studies examined the role (if any) of retromer in retrograde Golgi transport endosome (ARE) in polarized epithelia (Fölsch et al., 2009; Golachowska et al., 2010), we next determined whether apical βII expression in retromer knockdown polarized cells reflected shunting to the ARE. Although there was no appreciable colocalization (Figure 8D, top) or cofractionation (Figure 8E, lane 3) of type II TGF-βRs and Rab11 in polarized wild-type MDCK cells (as expected, since this would reflect apical transit), contrary to our expectations, a similar lack of association was also seen in retromer knockdown cultures (Figure 8D, bottom; Figure 8E, lane 4) in spite of the apical mislocalization. The latter result in polarized cultures is contrasted by the expected retromer-dependent TβRII/Rab11 association observed in nonpolarized cells (Figure 8E, compare lanes 1 and 2). Thus, whereas apical type II TGF-βR expression is observed in retromer knockdown cells, it does not reflect trafficking through the Rab11-dependent ARE.

Whereas the previous data support a new role for retromer in the homeostatic control of the type II TGF-βR, an important question is whether this is a general or cargo-specific function. To initially address this issue, we further examined the effect of retromer loss on the TfnR in both nonpolarized and polarized epithelia, as it also is basolaterally expressed and undergoes constitutive clathrin-dependent recycling (Sheff et al., 1999; Grant and Donaldson, 2009). Consistent with our previous findings (Figure 6E and Supplemental Figure S4) and those of Temkin et al. (2011) showing an absence of
Although significant βII/TfnR colocalization was observed in MD-1 cells, this was diminished in knockdown cultures (Figure 8, F and G). Thus, after initial targeting to the basolateral plasma membrane in polarized epithelia, native and chimeric type II TGF-βRs, in contrast to TfnRs or the type I TGF-βR, use a retromer-dependent mechanism(s) for delivery to the recycling endosome in the maintenance of basolateral TβRII expression (Figure 9).

**DISCUSSION**

While the idea of recycling for TGF-β family receptors is a nearly 30-yr-old concept (Massagüé and Like, 1985; Sathre et al., 1991), defining the pathways and mechanisms controlling TGF-βR trafficking has significantly lagged from that reported for other plasma...

**FIGURE 6:** TGF-βR recycling to a Rab11-positive compartment in monolayer cultures is dependent upon the mammalian retromer. (A) Recycling of the chimeric type II TGF-βR in nonpolarized MD-1 (WT) and retromer knockdown 43-10 (KD) cells was determined as in Materials and Methods. Left, fluorescence; right, phase images. (B) Data represented as arbitrary units of fluorescence ± SD from 30 cells in each of three independent experiments. (C) MD-1 (WT) and 43-10 (KD) cells transiently transfected with GFP-Rab5 (green) or CFP-Rab4 (green) were fixed, permeabilized, and incubated with GM-CSFR-β antibody, followed by incubation with Cy3-conjugated secondary antibodies to detect total chimeric βII (red). (D) Percentage of receptor colocalization with GFP-Rab5 or CFP-Rab4 represented as the mean ± SEM of 30 cells from three independent experiments. (E) Microsomes were purified from nonpolarized MD-1 (WT) or 43-10 (KD) cells as described in Materials and Methods and specificity confirmed in Supplemental Figure S3. After immunoprecipitation of TβRII (lanes 1 and 2) or TfnR (lanes 3 and 4), cofractionation of the indicated proteins was determined by Western blotting. Before immunoprecipitation, wild-type and knockdown samples were first normalized by Western blotting to the TβRII or TfnR levels in the microsome lysate to allow direct comparison between conditions. (F) MD-1 (WT) and 43-10 (KD) cells transiently transfected with GFP-Rab11 were incubated with antibody to the chimeric type II TGF-βR and processed as in C. (G) Mean ± SEM colocalization of 75 cells from three independent experiments. Bar, 10 μm.
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expression was observed at both the apical and basolateral domains with no diminution in receptor ligand binding in retromer knockdown cells, we initially believed that retromer was providing a targeting function. This was not found to be the case, as when we performed kinetic analysis of TβRII membrane expression, the absence of retromer had no effect on basolateral TβRII delivery/expression during the first 60 min after release from a 20°C Golgi block (Figure 3). Apical TβRII expression did, however, become detectable over the next 30–60 min in the retromer knockdown cells, with steady-state mislocalization levels obtained within the next hour.

The fact that retromer provides "maintenance" rather than direct TβRII "trafficking" function(s) (Figure 3) is analogous to one role of the AP1B complex in basolateral sorting (Fölsch et al., 1999; Gan et al., 2002; Mellman and Nelson, 2008; Gonzalez and Rodriguez-Boulan, 2009; Gravotta et al., 2012). For instance, although initial studies indicated a targeting role of AP1B for a subset of basolaterally sorted proteins, including the low-density-lipoprotein receptor (Fölsch et al., 1999; Gan et al., 2002; Gonzalez and Rodriguez-Boulan, 2009; Gravotta et al., 2012), subsequent analyses recognized that this finding reflected a defect in postendocytic recycling. It should be noted, however, that this is not the case for membrane proteins (Grant and Donaldson, 2009; Hsu et al., 2012). Although more recent studies documented endocytic uptake being regulated via a primarily clathrin-dependent process (Anders et al., 1997; Doré et al., 2001; Yao et al., 2002; Mitchell et al., 2004) and defined roles identified for Rab11 and Dab2 in TβRII recycling (Penheiter et al., 2002, 2010; Di Guglielmo et al., 2003; Mitchell et al., 2004), there is a paucity of information on the operative sorting signals, coat proteins/adaptors, and/or how receptor trafficking is regulated in polarized epithelia. For instance, whereas native and chimeric type I and type II TGF-βRs traffic to, and signal from, the basolateral membrane in polarized epithelial cells (Murphy et al., 2004, 2007), the cellular activities directing and/or maintaining that response are unknown. To directly address that issue, we used a COOH-terminal fragment of the TβRII containing a motif required for appropriate basolateral expression in an attempt to identify interacting proteins important for TβRII trafficking. One such protein, the Vps26 subunit of the mammalian retromer, was identified and selected for further analysis.

Retromer/cargo binding can occur via Vps complex-dependent as well as -independent mechanisms (McGough and Cullen, 2011). Although TβRII/retromer communoprecipitation can be observed with antibodies to either the Vps26 or Vps35 subunits, no association with native or chimeric type I TGF-βRs was detected (Figure 1 and Supplemental Figure S1). Consistent with that finding are our data showing that retromer knockdown has no detectable effect on TβRII trafficking or Smad phosphorylation (Figure 2, D–F, and Supplemental Figure S2, A and B) yet is critical for appropriate membrane localization of TβRII in polarized epithelial cells (Figures 2–5 and Supplemental Figure S2, C and D). Because steady-state TβRII all cargo, as some traffic directly from the TGN to the recycling endosomes for initial basolateral or apical delivery (Ang et al., 2004; Gravotta et al., 2007), in a similar vein, although we initially expected that retromer/TβRII binding would be through the LTA motif (Murphy et al., 2007), this was not the case (unpublished observations). Given that the LTA motif, however, is necessary for TβRII basolateral targeting, in hindsight it would not be unexpected to find that TβRII basolateral "delivery" and subsequent "maintenance" (via a recycling mechanism; Figures 3–5) might be uniquely controlled. This is analogous to the complex interaction of the CI-MPR with Vps35 not requiring the YSKV sequence involved in CI-MPR internalization (Arighi et al., 2004). Moreover, 1) the LTA sequence (\textsuperscript{22}\textsuperscript{29}LTAxxVAxxF\textsuperscript{38}) does not fit the general retromer binding criteria of being highly hydrophobic and rich in aromatic amino acids, as seen for sortilin (FLV), CI-MPR (WLM), DMT1-II (YLL), or sorLA (FANSHY; Fjorback et al., 2012); and 2) most of the aforementioned motifs were identified for their role in endosome-to-Golgi/TGN trafficking. Given that we have now determined that the maintenance of basolaterally expressed TβRII is independent of Golgi transit (Figure 7), it will be of interest to determine how retromer interacts with the comparable region in TβRII.

Although a related role for retromer in β2AR endosome-to-plasma membrane trafficking has been reported (Temkin et al., 2011), in contrast to β2ARs, internalized type II TGF-βRs undergo apical missorting with no significant change in steady-state receptor levels (i.e., misrouting to a degradative compartment; Figure 2B). Furthermore, because TGF-βRs use a Rab11-regulated pathway and there are no recognizable PDZ domains in the TβRII (distinct from the Rab4 association and PDZ requirement for β2AR recycling;
contrasted by our previous determination that Rab4 had no identifiable role in TβRII recycling (Mitchell et al., 2004). As such, since we do not see colocalization (Figure 6, C and D) or cofractionation (Figures 6E and 8C) of chimeric or native type II TGF-βRs with Rab4, yet have documented both colocalization (Supplemental Figure S4A) and cofractionation (Figures 6E and 8C) of the transferrin receptor (i.e., positive control) with Rab4, this suggests 1) TβRIIs traffic via distinct population(s) of early endosomes devoid of Rab4, and/or 2) TβRIIs sufficiently segregate from Rab4 on the early endosome membrane such that they do not appear to colocalize or precipitate within the same microsome fragments. This latter point is consistent with the data of Sonnichsen et al. (2000), who proposed that endosomes are a mosaic of distinct domains defined by their composition of Rab proteins.

Mitchell et al., 2004; Penheiter et al., 2010), retromer differentially regulates TjRl and β2AR recycling. These differences, however, are not surprising, considering 1) the various roles these receptor families have, 2) the differing experimental models used, and, most important, 3) the myriad retromer functions that are just now being identified (Verges, 2008; McGough and Cullen, 2011; Cullen and Korswagen, 2012; Seaman, 2012).

It is of interest that we do not see any detectable colocalization of the type II TGF-βR and Rab4 (Figure 6, C and D), as Rab4 has been shown to colocalize with the early endosome in conjunction with other cargo, such as β2AR (Cao et al., 1999; Seachrist et al., 2000) or transferrin receptor (Sheff et al., 1999; Sonnichsen et al., 2000). In the majority of those studies, however, Rab4 is shown to functionally affect the trafficking of the cargo being studied. This is contrasted by our previous determination that Rab4 had no identifiable role in TβRII recycling (Mitchell et al., 2004). As such, since we do not see colocalization (Figure 6, C and D) or cofractionation (Figures 6E and 8C) of chimeric or native type II TGF-βRs with Rab4, yet have documented both colocalization (Supplemental Figure S4A) and cofractionation (Figures 6E and 8C) of the transferrin receptor (i.e., positive control) with Rab4, this suggests 1) TβRIIs traffic via distinct population(s) of early endosomes devoid of Rab4, and/or 2) TβRIIs sufficiently segregate from Rab4 on the early endosome membrane such that they do not appear to colocalize or precipitate within the same microsome fragments. This latter point is consistent with the data of Sonnichsen et al. (2000), who proposed that endosomes are a mosaic of distinct domains defined by their composition of Rab proteins.
Retromer retains basolateral TβRII domain. In nonpolarized cells this occurs via a Rab11-dependent process (Figure 6; Mitchell et al., 2004). In the absence of retromer, however, TβRIIs aberrantly sort in polarized cultures independent of Golgi transit and the Rab11-positive ARE (Figures 7, A–C, and 8, D and E) such that both basolateral and apical expression is observed. A model depicting these findings is presented in Figure 9. Ongoing studies focus on identifying and characterizing the retromer interacting motif(s), sorting nexin requirement(s) (Harterink et al., 2011), and accessory proteins to better understand and integrate retromer in maintaining TβRII polarity with its disparate effects on cargo such as basolateral-to-apical transcytosis of polymeric immunoglobulin A (Verges et al., 2004) and apical localization of Crumbs in Drosophila larvae (Pocha et al., 2011).

FIGURE 9: Model depicting role of retromer in maintaining steady-state basolateral expression of the type II TGF-βR.
Nonpolarized cells: TβRII and TfnR undergo constitutive clathrin-dependent internalization to the Rab5-positive early endosome (EE) and recycling via Rab11 (rapid TfnR recycling through Rab4 is not depicted). In the absence of retromer, whereas TfnR recycling and steady-state TβRII membrane levels are unaffected, TβRIIs are unable to enter the Rab11-positive compartment and show diminished recycling. Polarized cells: TβRII and TfnR are similarly internalized to the basolateral early endosome (BEE) and recycled through the common recycling endosome (CRE) to the basolateral plasma membrane. Entry of TβRII, but not TfnR, to the CRE depends on retromer such that retromer depletion results in TβRII mislocalization to both apical and basolateral membranes. In contrast to the apical-directed gp130 protein, apical TβRII trafficking occurs independent of the Rab11-positive apical recycling endosome (ARE). The ARE and CRE are depicted as distinct compartments for ease of presentation. The recycling endosome, however, may consist of a single endosome with multiple subdomains (Ang and Folsch, 2012). Given that retromer functions downstream of Rab5 and it is unknown whether apical and/or basolateral TβRII delivery in retromer knockdown cells occurs via distinct compartments/markers, TβRII membrane return is shown not originating from a defined locale.
**MATERIALS AND METHODS**

**Cell culture**

MDCK cells were maintained in DMEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; HyClone Laboratories, Logan, UT) at 37°C and 5% CO₂. For Transwell culture (12-mm Costar polycarbonate membranes), cells were plated at a density of 5 x 10⁴ cells/ml in 0.5 ml of culture medium. Fully polarized monolayers were achieved after 3 d (Murphy et al., 2004, 2007).

MD-1 cells represent a MDCK clone stably expressing chimeric type I and type II TGF-βRs (Murphy et al., 2004, 2007). The designations α and β refer to chimeric receptors expressing the extracellular ligand-binding domain of the GM-CSF α or β receptor coupled to the transmembrane and cytoplasmic domain of the TGF-β type I and type II receptors, respectively (Anders and Leof, 1996). Previous work documented that chimeric and native TGF-βRs have analogous signaling and trafficking activity regardless of the culture conditions or cell type tested (Anders and Leof, 1996; Anders et al., 1997; Yao et al., 2002; Mitchell et al., 2004; Murphy et al., 2004).

Plasmids (pLKO.1-puro) encoding shRNAs targeting human Vps35 were purchased from the Mayo Clinic RNA Interference Technology Resource (Rochester, MN). The production of lentivirus and the transduction of MD-1 cells were as described previously (Rahimi et al., 2009). The human Vps35 shRNA sequences CAGTGAGAAGAACAGAGCAGAT (#3; base pairs 2237–2257) and GCA- GATCTCTACGAACTTGTA (#4; base pairs 234–254) were used to generate clones 33-1/33-18 and 43-10, respectively. The 33 clones required two transfections of shRNA #3, and the 43 clone was sequentially transfected with #4 and then with #3 shRNA. Stable cell lines were maintained in the presence of 1.5 µg/ml puromycin.

Inulin flux was measured by plating 5 x 10⁴ cells/12-mm Transwell dish in 10% FBS/DMEM and allowing them to polarize over 3 d. Medium was removed and the apical and basal chambers washed 2× with serum-free DMEM containing 0.5 mM inulin (Sigma-Aldrich St. Louis, MO). After addition of fresh DMEM/inulin (to both chambers) and a 10-min incubation at 37°C, the apical medium is replaced with fresh DMEM supplemented with 0.2 µCi/ml [14C]inulin (PerkinElmer, Waltham, MA). Transwells are returned to 37°C and at the indicated times aliquots removed from the basal chamber and processed for liquid scintillation counting.

**Immunostaining and microscopy**

Transwell cultures were rinsed with filter sterilized HMEM+G (13.8 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], 13.7 mM NaCl, 5.4 mM KCl, 5.5 mM glucose, 2.0 mM glutamine, 0.44 mM KH₂PO₄, 0.18 mM Na₂HPO₄, 1× MEM vitamins, 1× MEM amino acids, pH 7.4; Singh et al., 2007) and incubated with primary antibody diluted in ice-cold HMEM+G at 10°C for 45 min. After washing with ice-cold PBS, the cultures were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. The cultures were then quenched with 50 mM NH₄Cl/PBS before incubating (room temperature, 45 min) with secondary antibodies (Cy3-labeled donkey anti-mouse immunoglobulin G [red; 715-165-150, Jackson ImmunoResearch Laboratories, West Grove, PA] or Alexa Fluor 488-labeled goat anti-rabbit IgG [green; A11008, Invitrogen, Carlsbad, CA]) and 4',6-diamidino-2-phenylindole (DAPI) diluted in 5% normal donkey serum (NDS)/1% BSA/PBS (blocking buffer). For costaining, cells were treated with blocking buffer at room temperature for 2 h and incubated with antibody diluted in blocking buffer at room temperature for 1 h. After PBS wash (5 x 5 min), cultures were incubated with secondary antibodies and DAPI diluted in blocking buffer. Slides were mounted using Vectashield (Vector Laboratories, Burlington, CA) and fluorescence internalization performed using an AX-70 Olympus microscope (Olympus, Tokyo, Japan) equipped with 60×/1.4 NA oil immersion objectives. Images were acquired at room temperature using a C4742-95-12NR (Hamamatsu, Hamamatsu, Japan) camera. Confocal images were acquired with a 100× objective (1.3 NA oil lens) on a Zeiss LSM 510 confocal system (Carl Zeiss, Jena, Germany). Individual figures were prepared using Photoshop CS (Adobe, San Jose, CA). Two-dimensional deconvolution of nearest neighbors was used for Figure 6, C and F, and Supplemental Figure S4. No three-dimensional reconstructions, surface or volume rendering, or gamma adjustments were performed. MetaMorph, version 7.3.2 (Molecular Devices, Sunnyvale, CA), was used for all quantitation.

**Direct recycling assay**

Recovering in nonpolarized and Transwell polarized cultures was based on a protocol by Fraile-Ramos et al. (2001). This was previously described in detail, in which an antibody recognizing the extracellular receptor domain is visualized through 1.5 cycles of recycling (Mitchell et al., 2004). Because the fluorescent secondary antibody binds only those receptors that return to the cell surface with attached primary antibody, intracellular fluorescence is observed only after an additional internalization event. For polarized cells, primary and secondary antibody was added to both apical and basal chambers.

**Immunoblotting and immunoprecipitation**

Cells were lysed in NP40 lysis buffer (20 mM HEPES, pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.2% NP-40) including protease inhibitor cocktail (Roche, Indianapolis, IN) on ice for 1 h. The lysate was clarified at 21,000 × g for 15 min. Equal protein was incubated with primary antibody at 4°C overnight with agitation and then with protein A– or G–agarose beads for 2 h. The beads were washed three times with lysis buffer and bound proteins recovered in 2× Laemmli sample buffer. Clarified lysate or immunopurified protein was resolved on SDS–PAGE and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA). Membranes were blocked with 5% nonfat milk in 10 mM Tris (pH 7.4)/0.1% Tween 20 (TBST). The membranes were incubated with antibodies diluted in blocking solution overnight at 4°C, washed with TBST, and incubated with horseradish peroxidase–conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature. After washing with TBST, membranes were incubated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL) and bands detected with an X-OMAT 2000A. GST pull down was performed as previously described (Yao et al., 2002).

**Microsome cofractionation**

A modification of the Qproteome Plasma Membrane Kit (37601; Qiagen, Valencia, CA) was developed that allows isolation of not only plasma membrane and plasma membrane–derived vesicles, but also of associated/cofractionated constituents such as Rab proteins. Briefly, cell pellets were collected (4 x 10⁶ mm plates or 6 x 24 mm Transwells per condition) and lysed by mechanical disruption through a 27 gauge needle (15x) in lysis buffer (125 mM HEPES, pH 7.5, 5% NP40, 750 mM NaCl, 50 mM MgCl₂, 5 mM EDTA, 10% glycerol, Roche protease inhibitor cocktail). Subsequent to centrifugation (20 min, 12,000 x g) to remove nuclei, large organelles, and cell debris, the resulting supernatant (consisting of 20- to 200-nm microsomes derived from the endoplasmic reticulum, Golgi, and plasma membrane) was then incubated (gentle rocking) for
Biotinylation of cell surface receptors
To detect biotinylated cell surface receptors, 4.5 × 10^5 MD-1 or ret- romer knockdown 43-10 cells were plated in 24 mm Transwell or six-well plates for 72 h with a medium (10% FBS/DMEM) change after 48 h. After three washes with ice-cold Hank's balanced salt solution (HBSS; Mediatech, Manassas, VA); freshly made sulfo-NHS-S5 biotin/HBSS (1 mg/ml; Thermo Scientific, Waltham, MA) was added to the six-well plate (1 ml) to assess total (T) labeling. Domain-specific receptor expression was determined by sulfo-NHS-S5 biotin/HBSS addition to the apical (0.5 ml) or basolateral (1 ml) surfaces of polarized Transwell cultures. Plates were rocked for 1 h at 4°C before washing with ice-cold 5 mM Tris/HBSS and lysis in 0.2 ml of modified RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 0.25% deoxycholate, 150 mM NaCl, 1 mM EDTA, 10 mM NaF) with protease inhibitors on ice for 1 h. Streptavidin–agarose (100 μl; 21331; Thermo Scientific) was mixed with 0.5–1 mg protein in 1 ml of total volume for 2 h at 4°C. The agarose was washed four times with lysis buffer (1 ml) and the biotin-bound proteins eluted by boiling for 10 min in 2× Laemml buffer. Samples were resolved on 10% SDS–PAGE and the target protein detected by Western blotting.

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