A new role for RINT-1 in SNARE complex assembly at the trans-Golgi network in coordination with the COG complex

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ABSTRACT Docking and fusion of transport vesicles/carriers with the target membrane involve a tethering factor–mediated initial contact followed by soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)–catalyzed membrane fusion. The multisubunit tethering CATCHR family complexes (Dsl1, COG, exocyst, and GARP complexes) share very low sequence homology among subunits despite likely evolving from a common ancestor and participate in fundamentally different membrane trafficking pathways. Yeast Tip20, as a subunit of the Dsl1 complex, has been implicated in retrograde transport from the Golgi apparatus to the endoplasmic reticulum. Our previous study showed that RINT-1, the mammalian counterpart of yeast Tip20, mediates the association of ZW10 (mammalian Dsl1) with endoplasmic reticulum–localized SNARE proteins. In the present study, we show that RINT-1 is also required for endosome-to-trans-Golgi network trafficking. RINT-1 uncomplexed with ZW10 interacts with the COG complex, another member of the CATCHR family complex, and regulates SNARE complex assembly at the trans-Golgi network. This additional role for RINT-1 may in part reflect adaptation to the demand for more diverse transport routes from endosomes to the trans-Golgi network in mammals compared with those in a unicellular organism, yeast. The present findings highlight a new role of RINT-1 in coordination with the COG complex.

INTRODUCTION

Eukaryotic cells contain an endomembrane system that consists of morphologically and functionally distinct organelles. Communication between organelles is mediated by coated vesicular/tubular carriers that are generated from the donor compartment, traffic to their destinations, lose their coat, and fuse with the acceptor compartment. Docking and fusion of transport carriers with the target membrane involve an initial contact mediated by Rab GTPases and tethering factors, followed by soluble N-ethylmaleimide–sensitive factor attachment protein receptor (SNARE)–catalyzed membrane fusion (Bonifacino and Glick, 2004).

Tethering factors are large proteins or protein complexes that not only facilitate long-range interactions between transport carriers and the acceptor membrane, but also coordinate SNARE complex assembly at the trans-Golgi network (Bonifacino and Glick, 2004).

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Abbreviations used: CI-MPR, cation-independent mannose 6-phosphate receptor; CTB, cholera toxin B; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; GFP, green fluorescent protein; GST, glutathione S-transferase; PBS, phosphate-buffered saline; siRNA, short interfering RNA; SNARE, N-ethylmaleimide–sensitive factor attachment protein receptor; TGN, trans-Golgi network.

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The Dsl1 complex consists of three subunits (Dsl1, Tip20, and Sec39/Dsl3) and participates in retrograde transport from the Golgi to the endoplasmic reticulum (ER; Schmitt, 2010). X-ray crystallographic analyses revealed that Dsl1 and Tip20 have common α-helical folds (Tripathi et al., 2009), whereas Sec39/Dsl3 lacks the shared fold (Ren et al., 2009). The Dsl1 complex resides on ER membranes and binds to and regulates the assembly of the ER SNAREs Sec20, Ufe1, and Use1/Slt1 (Kraynack et al., 2005; Ren et al., 2009; Diefenbacher et al., 2011; Meiringer et al., 2011). The structural model suggests that the Dsl1 complex forms a 20-nm-tall tower from the ER surface (Ren et al., 2009). Given that Dsl1 interacts with subunits of the COPI, the Dsl1 complex likely serves as an acceptor for Golgi-derived COPI-coated carriers (Andag et al., 2001; Andag and Schmitt, 2003; Reilly et al., 2001). Moreover, the Dsl1 complex may assist uncoating of COPI-coated carriers tethered on ER membranes (Zink et al., 2009).

The mammalian orthologue of the Dsl1 complex is composed of ZW10 (Ds11), RINT-1 (Tip20), and NAG (Sec39/Dsl3; Aoki et al., 2009). Like the Dsl1 complex (Meiringer et al., 2011), the ZW10 complex can associate with the ER SNAREs syntaxin 18 (Ufe1), BNIIP1 (Sec20), p31 (Use1/Slt1), and Sec22b (Sec22; Hatzuzawa et al., 2000; Hirose et al., 2004; Nakajima et al., 2004; Aoki et al., 2009). The protein–protein interactions among Dsl1 complex subunits and between Dsl1 complex and SNARE subunits are also well conserved between yeast and mammals (Sweet and Pelham, 1993; Hirose et al., 2004; Nakajima et al., 2004; Aoki et al., 2009; Ren et al., 2009; Tripathi et al., 2009; Uemura et al., 2009; Meiringer et al., 2011).

RINT-1 was originally discovered as a Rad50-interacting protein and implicated in cell cycle control (Xiao et al., 2001). Depletion of RINT-1 causes partial Golgi fragmentation (Arasaki et al., 2006; Sun et al., 2007), together with defects in mitosis, including centrosome amplification and chromosome loss (Lin et al., 2007). Rint-1 heterozygotes succumb to multiple tumor formation with haploinsufficiency (Lin et al., 2007). RINT-1 also interacts with Rb-related p130 and has been implicated in telomere length control (Kong et al., 2006). These results raise the possibility that RINT-1 plays additional roles by interacting with unidentified partners. Indeed, in yeast Tip20 mutants, crystal-like structures are formed in the nucleus by an unknown mechanism (Spang, 2012).

In the present study, we show that RINT-1 is required for endosome-to–trans-Golgi network (TGN) transport. Immunoprecipitation and binding studies revealed that RINT-1 interacts with TGN SNAREs and Cog1, a subunit of the octameric COG complex (Smith and Lupashin, 2008; Miller and Ungar, 2012), which also belongs to the CATCHR family complex (Yu and Hughson, 2010).

**RESULTS**

**Depletion of RINT-1 causes redistribution of TGN proteins**

We and others previously reported that depletion of RINT-1 causes partial Golgi fragmentation with some change in the staining pattern for ER-Golgi intermediate compartment (ERGIC)-53 (Arasaki et al., 2006; Lin et al., 2007; Sun et al., 2007), an ERGIC marker that maintains its steady-state localization by cycling between the ERGIC and ER (Appenzeller-Herzog and Hauri, 2006). RINT-1 depletion, however, does not significantly inhibit brefeldin A– or Sar1p mutant–induced retrograde transport from the Golgi to the ER (Arasaki et al., 2006). To further characterize the contribution of RINT-1 to retrograde transport to the ER, we examined the distribution of other ER-Golgi/ERGIC recycling proteins (Rer1 and KDEL receptor; Figure 1A, Supplemental Figure S1A). RINT-1 depletion by this siRNA, however, did not markedly disrupt the distribution of two ER-Golgi/ERGIC recycling proteins (Rer1 and KDEL receptor; Figure 1A,
Depletion of RINT-1 inhibits transport from endosomes to the TGN

Because the localization of TGN proteins is regulated by transport from endosomes to the TGN (Pfeffer, 2011), the most straightforward interpretation of the foregoing results is that RINT-1 is involved in endosome-to-TGN transport. To verify this possibility, we first measured the trafficking of cholera toxin B (CTB). CTB is endocytosed and transported to the ER via endosomes and the Golgi in endosome-to-TGN transport. To verify this possibility, we first measured the trafficking of cholera toxin B (CTB). CTB is endocytosed and transported to the ER via endosomes and the Golgi.
anti–RINT-1 antibody. Solubilized membrane fractions from 293T (Uetz et al., 2000). We first performed immunoprecipitation using an anti–RINT-1 antibody. Solubilized membrane fractions from 293T cells were immunoprecipitated with an anti-RINT-1 antibody and analyzed by immunoblotting. As shown in Figure 4A, endogenous Cog3 coprecipitated with an anti–RINT-1 antibody (lane 2). Of note, Vti1a and syntaxin 6 also coprecipitated with RINT-1. On the other hand, no coprecipitation of Cog3, Vti1a, or syntaxin 6 occurred with an anti-ZW10 antibody (lane 3). Cog1 coprecipitated with an anti–RINT-1 antibody with almost equal efficiency to Cog3 (Figure 4B), perhaps suggesting coprecipitation of the COG complex with RINT-1.

Next we sought to determine which COG subunit(s) interact with RINT-1. For this purpose, each of the eight COG subunits (carrying a FLAG tag) was coexpressed with GFP–RINT-1 and immunoprecipitated with anti-FLAG beads. As shown in Figure 4C, GFP–RINT-1 coprecipitated with FLAG-Cog1 (lane 1). Some GFP–RINT-1 coprecipitated with FLAG-Cog8 (lane 8) but not with other subunits (lanes 2–7). To confirm this interaction, we examined the interaction of endogenous RINT-1 with FLAG-Cog1. As shown in Supplemental Figure S6, endogenous RINT-1 also coprecipitated with FLAG-Cog1 (lane 1). In this experiment some RINT-1 also coprecipitated with FLAG-Cog4 and -Cog7. Given that these Cog subunits, when expressed, did not bind to GFP–RINT-1 (Figure 4C), they may indirectly bind to RINT-1. It is possible that expressed FLAG-Cog4 and -Cog7 are incorporated into the COG complex.

Consistent with the result that endogenous Cog1 or Cog3 was not precipitated with an anti-ZW10 antibody (Figure 4, A and B, lane 3), ZW10, NAG, or syntaxin 18 did not coprecipitate with FLAG-Cog1 (Supplemental Figure S6, lane 1). These results suggest that RINT-1 uncomplexed with ZW10 interacts with the COG complex.

Vps51-like domain of Cog1 is responsible for the association with the N-terminal, ZW10-interacting region of RINT-1
The fact that RINT-1 uncomplexed with ZW10 interacts with Cog1 suggested that the Cog1-binding site on RINT-1 overlaps with the binding site for ZW10. To test this, we expressed FLAG-Cog1 together with GFP fused to the N-terminal 264 amino acids of RINT-1, which was previously shown to bind to ZW10 (Arasaki et al., 2006; Supplemental Figure S7A) and conducted immunoprecipitation with anti-FLAG beads. As shown in Figure 4D, GFP–RINT-1 (amino acids 1–264) coprecipitated with FLAG-Cog1 (lane 2, middle), whereas no significant coprecipitation was observed.
for the fragment containing the central and C-terminal region (amino acids 265–792; lane 3, middle). The latter finding may partly explain why expression of this fragment failed to rescue the RINT-1 depletion effect on TGN protein localization (Supplemental Figure S9, second row) but was markedly different from ERGIC-53 staining (bottom). Because the GOG complex seems be evenly distributed among cisternae along the cis-/medial-Golgi structure, whereas its N-terminal, ZW10-interacting region causes Golgi fragmentation (Arasaki et al., 2006), this fragmented Cog3 pattern likely reflects its partial fragmentation (Supplemental Figure S9). Double staining revealed that the fragmented Cog3 staining almost completely overlapped with GM130 (cis-Golgi) staining (Supplemental Figure S9, second row) but was markedly different from ERGIC-53 staining (bottom). Because the GOG complex seems be evenly distributed among cisternae along the cis-to-trans direction (Vasile et al., 2006), this fragmented Cog3 pattern likely reflects its cis-/medial-Golgi localized pool. As in the case of TGN proteins.

Next we determined the RINT-1-binding site on Cog1. The SMART program (http://smart.embl-heidelberg.de/) predicts the presence of a Vps51-like domain (amino acids 12–93) in Cog1 (Supplemental Figure S7B). Vps51 is a subunit of the GARP complex, another member of the CATCHR family complexes (Bonifacino and Hierro, 2011). Of interest, the Pfam database (http://pfam.sanger.ac.uk/) indicates that the RINT-1 partner ZW10 is a member of the Vps51 clan. To determine whether the Vps51-like domain is required for the association with RINT-1, we expressed glutathione S-transferase (GST)–Cog1 full-length or GST-Cog1 lacking the N-terminal 93 amino acids (GST–Cog1Δ1-93) together with FLAG–RINT-1, pulled down with glutathione beads and analyzed by immunoblotting with an anti-FLAG antibody. As shown in Figure 4E, FLAG–RINT-1 was pulled down with the full-length Cog1 construct (lane 1, middle) but much less with GST–Cog1Δ1-93 (lane 2). Given that the COILS program (www.ch.embnet.org/software/COILS_form.html) predicts the presence of a putative coiled-coil region (amino acids 27–55) in the Vps51-like domain, we examined the interaction of RINT-1 with the N-terminal Vps51-like domain (Vps51: amino acids 12–93) and the putative coiled-coil region (CC: amino acids 27–55). As shown in Figure 4E, the Vps51-like domain bound to FLAG–RINT-1 (lane 3, middle), whereas no binding was observed between the putative coiled-coil region and FLAG–RINT-1 (lane 4).

We next examined whether GARP subunits interact with RINT-1. The GARP complex consists of Vps51/Ang2, Vps52, Vps53, and Vps54 (Bonifacino and Hierro, 2011). Each GARP subunit as a V5-tagged protein was expressed together with FLAG–RINT-1, and then cell lysates were immunoprecipitated with anti-FLAG beads. Although a very small amount of V5-tagged Vps52 coprecipitated with FLAG–RINT-1 (Supplemental Figure S8, lane 1), no interaction was observed between V5-Vps51/Ang2 and FLAG–RINT-1 (lane 4). These results suggest that the interaction of RINT-1 with Cog1 is specific.

**Overexpression of RINT-1 causes redistribution of Cog3**

To further characterize the relationship between RINT-1 and the COG complex, we examined the effect of overexpression and depletion of RINT-1 on the localization of the COG complex. Our previous data showed that overexpression of a RINT-1 full-length construct has no marked effect on the cis/medial-Golgi structure, whereas its N-terminal, ZW10-interacting region causes Golgi fragmentation (Arasaki et al., 2006). When FLAG–RINT-1 full-length construct was overexpressed, dispersal of Cog3, as well as that of TGN46 and γ-adaptin, was observed in many cells (Figure 5). Dispersal of TGN46 and γ-adaptin may be a consequence of the disruption of the TGN structure. Consistent with our previous result (Arasaki et al., 2006), on the other hand, cis-Golgi markers (GPP130, GM130, and β-COP) remained in the perinuclear region in a substantial fraction of RINT-1-overexpressing cells. These results confirm a link between RINT-1 and the COG complex, although the precise mechanism underlying the dispersal of Cog3 by the overexpression of FLAG–RINT-1 is unclear.

In contrast to overexpression, depletion of RINT-1 did not markedly affect Cog3 localization; it was principally localized in partially fragmented structures (Supplemental Figure S9). Double staining revealed that the fragmented Cog3 staining almost completely overlapped with GM130 (cis-Golgi) staining (Supplemental Figure S9, second row) but was markedly different from ERGIC-53 staining (bottom).

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**FIGURE 4:** Interaction of RINT-1 with Cog1. (A, B) Solubilized membrane fractions of 293T cells were incubated for 1 h with an antibody against RINT-1 (lane 2) or ZW10 (lane 3). Immunocomplexes were precipitated with protein G–Sepharose, subjected to SDS–PAGE, and then analyzed by immunoblotting with the indicated antibodies. Three percent input was also analyzed (lane 1). (C) Each of the FLAG-Cog subunits was coexpressed with GFP–RINT-1 in 293T cells. Cell lysates were immunoprecipitated with anti-FLAG beads, subjected to SDS–PAGE, and then analyzed by immunoblotting with antibodies against GFP (middle) and FLAG (bottom). Five percent input was also analyzed by immunoblotting with an anti-GFP antibody (top). (D) Each of the GFP-tagged RINT-1 constructs was coexpressed with FLAG–Cog1, and cell lysates were immunoprecipitated with anti-FLAG beads, subjected to SDS–PAGE, and analyzed by immunoblotting with antibodies against GFP (middle) and FLAG (bottom). Five percent input was also analyzed by immunoblotting with an anti-GFP antibody (top). (E) GST fused to each of the Cog1 constructs was coexpressed with FLAG–RINT-1, and cell lysates were pulled down with glutathione beads and analyzed by immunoblotting with antibodies against FLAG (middle) and GST (bottom). Five percent input was also analyzed by immunoblotting with an anti-FLAG antibody (top).
We then determined the RINT-1–interacting site on FLAG-syntaxin16 (lane 2, middle), whereas the remaining region was N-terminal 264–amino acid fragment of RINT-1 coprecipitated with syntaxin 16 was much lower than that of FLAG-Vti1a (lane 3 vs. lane 4), indicating that syntaxin 16 interacts with RINT-1. As shown in Figure 6D, the amounts of syntaxin 16, syntaxin 6, and VAMP4 coprecipitated with Vti1a were decreased by 67–81% compared with the control levels, suggesting that RINT-1 regulates the assembly of TGN SNARE proteins. The amount of Cog3 coprecipitated with Vti1a was also decreased by 80%, supporting the idea that RINT-1 is important for the connection between the TGN SNAREs and the COG complex. The fact that the steady-state level of Cog3 was decreased by 30% upon RINT-1 knockdown might imply that RINT-1 affects the stability of Cog3. The primary effect of RINT-1 depletion, however, is the defect in SNARE complex assembly (Bröcker et al., 2010; Brown and Pfeffer, 2010; Yu and Hughson, 2010). We next examined whether RINT-1 depletion affects TGN SNARE complex assembly. To this end, RINT-1–depleted cells were lysed, immunoprecipitated with an anti-Vti1a antibody, and analyzed by immunoblotting. As shown in Figure 6D, the amount of syntaxin 16, syntaxin 6, and VAMP4 coprecipitated with Vti1a were decreased by 67–81% compared with the control levels, suggesting that RINT-1 regulates the assembly of TGN SNARE proteins. The amount of Cog3 coprecipitated with Vti1a was also decreased by 80%, supporting the idea that RINT-1 is important for the connection between the TGN SNAREs and the COG complex. The fact that the steady-state level of Cog3 was decreased by 30% upon RINT-1 knockdown might imply that RINT-1 affects the stability of Cog3. The primary effect of RINT-1 depletion, however, is the defect in SNARE complex assembly (Bröcker et al., 2010; Brown and Pfeffer, 2010; Yu and Hughson, 2010). We next examined whether RINT-1 depletion affects TGN SNARE complex assembly. To this end, RINT-1–depleted cells were lysed, immunoprecipitated with an anti-Vti1a antibody, and analyzed by immunoblotting. As shown in Figure 6D, the amount of syntaxin 16, syntaxin 6, and VAMP4 coprecipitated with Vti1a were decreased by 67–81% compared with the control levels, suggesting that RINT-1 regulates.

**DISCUSSION**

The ZW10/Dsl1 and COG complexes, as well as the GARP and exocyst complexes, belong to the CATCHR family of multisubunit tethering complexes (Yu and Hughson, 2010). These family members were believed to function in distinct membrane trafficking pathways, although the COG and GARP complexes are involved in endosome-to-TGN trafficking (Bonifacino and Hierro, 2011; Miller and Ungar, 2012). Whereas CATCHR family proteins share subtle sequence homology (Whyte and Munro, 2002), emerging structural similarities provide strong evidence of a common evolutionary origin and may reflect a shared mechanism of action (Richardson et al., 2009; Tripathi et al., 2009; Pérez-Victoria et al., 2010a; Vasan et al., 2010). The present study reveals for the first time interplay between members of the CATCHR family proteins; RINT-1, a component of the ZW10/Dsl1 complex, regulates endosome-to-TGN transport by interacting with Cog3, one of the eight subunits of the COG complex.
FIGURE 6: RINT-1 modulates TGN SNARE complex assembly. (A) Lysates of cells expressing each of the FLAG-SNARE constructs were immunoprecipitated with anti-FLAG-beads (lanes 7–12) and analyzed by immunoblotting with antibodies against RINT-1 (top) and FLAG (bottom). Five percent input was also analyzed (lanes 1–6). (B) Interaction between RINT-1 and syntaxin 16. Lysates of cells expressing FLAG–syntaxin 16 and each of the GFP–RINT-1 constructs were immunoprecipitated with anti-FLAG beads and analyzed by immunoblotting with antibodies against GFP (middle) and FLAG (bottom). Five percent input was also analyzed by immunoblotting with an anti-GFP antibody (top). (C) SNARE domain is responsible for the interaction with RINT-1. Lysates of cells expressing each of the FLAG–syntaxin 16 constructs were immunoprecipitated with anti-FLAG beads and analyzed by immunoblotting against RINT-1 (middle) and FLAG (bottom). Five percent input was also analyzed by immunoblotting with an ant-RINT-1 antibody (top) (D) TGN SNARE complex assembly is abrogated in RINT-1–depleted cells. HeLa cells were mock transfected (lanes 1 and 3) or transfected with RINT-1 (1149) (lanes 2 and 4). At 72 h after transfection, cell lysates were immunoprecipitated with an anti-Vti1a antibody and analyzed by immunoblotting with the indicated antibodies (lanes 3 and 4). Five percent input was also analyzed (lanes 1 and 2). The intensities of immunostained bands were quantitated with ImageJ. The quantitative data represent the average of two independent experiments.
whose size and sequence are markedly different from those of yeast Cog1 (Chaterton et al., 1999; Ungar et al., 2002). The interaction between RINT-1 and Cog1 relies upon known structural similarities and protein–protein interactions of CATCHR family members. Both the N-terminal region of Cog1, which is responsible for binding to RINT-1, and an ER RINT-1 partner ZW10 have a Vps51-like structure. Moreover, RINT-1 bears strong resemblance to Cog4 (Richardson et al., 2009), which can directly interact with Cog1 in the mammalian COG complex (Loh and Hong, 2004).

Figure 7 depicts two distinct RINT-1–containing complexes, localized at the TGN (Figure 7A) and the ER (Figure 7B), respectively. There are similar and different protein–protein interactions in these complexes. Given that RINT-1/Tip20 interacts with ZW10/Dis1 (tether) through its N-terminal region in the ER complex (Kraynack et al., 2005; Arasaki et al., 2006; Diefenbacher et al., 2011; Meiringer et al., 2011), the N-terminal region of RINT-1 is responsible for the binding to Cog1 (tether) in the TGN complex. In terms of SNARE binding, however, RINT-1 uses its N-terminal region for syntaxin 16 (Qa-SNARE) binding, whereas the central and C-terminal regions of RINT-1/Tip20 are involved in the interaction with BNIP1/Tip20 (Qb-SNARE; Tripathi et al., 2009; Supplemental Figure S7A). Moreover, the SNARE domain of syntaxin 16 participates in the interaction with RINT-1 in the TGN complex, whereas the N-terminal regulatory domain of Sec20 is involved in the interaction with Tip20 in the ER complex (Ren et al., 2009).

In addition to a RINT-1–mediated link, there are links between tethers and SNAREs in the TGN and ER. In the TGN, the SNARE domain of syntaxin 6 interacts with the N-terminal region of Cog6 (Laufman et al., 2011). More recently, it was reported that the SNARE domains of syntaxin 16 and Vti1a bind to the N-terminal region of Cog4, although Vti1a also interacts with the C-terminal fragment of Cog4 (Laufman et al., 2013). These links may provide explanations for the coprecipitation of some Cog3 with Vti1a in the absence of RINT-1 (Figure 6D). Moreover, the GARP complex, consisting of Vps51, Vps52, Vps53, and Vps54 (Bonifacino and Hierro, 2011), can bind to TGN SNAREs. Vps51 (Ang2 in mammals) interacts with the N-terminal Habc region of Tlg2 (syntaxin 6 [Qa-SNARE] in mammals; Siniosoglou and Pelham, 2002; Conibear et al., 2003; Fridman-Sirks et al., 2006; Pérez-Victoria et al., 2010b), and the N-terminal coiled-coil domains of Vps53 and Vps54 of the mammalian complex interact with the SNARE motifs of syntaxin 6, syntaxin 16, and VAMP4 (Pérez-Victoria and Bonifacino, 2009). Of interest, the N-terminal regions of COG and GARP subunits seem to be commonly involved not only in the interactions with SNAREs (RINT-1 in the case of Cog1), but also in complex assembly (Ungar et al., 2005; Lees et al., 2010; Bonifacino and Hierro, 2011). In the ER, on the other hand, p31/Use1 (Qc-SNARE) binds to the N-terminal region of NAG, and the extreme N-terminal region of p31/Use1 is responsible for this interaction (Aoki et al., 2009; Ren et al., 2009).

Although RINT-1 plays an important role as a tether for ZW10 to syntaxin 18–containing SNARE complexes, it does not significantly contribute to SNARE complex assembly on the ER membrane (Arasaki et al., 2006). This is in contrast to the critical role of yeast Tip20 in ER SNARE complex assembly (Kraynack et al., 2005). The minor contribution of RINT-1 to ER SNARE complex assembly may be related to a unique mechanism of ER SNARE complex assembly. Association of syntaxin 18 with BNIP1 and p31/Use1 is dramatically induced by the v-SNARE Sec22b (Aoki et al., 2008), which may reduce the requirement of tethers such as RINT-1 in SNARE complex assembly. Under these circumstances, RINT-1 might have acquired, during the course of evolution, an additional function, that is, participation in endosome-to-TGN transport. Of interest, the sites of the RINT-1–syntaxin 16 interaction are different from those of the Tip20/RINT-1–Sec20/BNIP1 interaction. The central and C-terminal regions of Tip20/RINT-1 bind to the N-terminal region of Sec20/BNIP1 on the ER (Ren et al., 2009; Tripathi et al., 2009), whereas RINT-1 on the TGN binds to the SNARE domain of syntaxin 16 through its N-terminal region (Figure 7). Mammals have a greater complexity in their transport pathways from endosomes to the TGN than a unicellular organism, yeast (Bonifacino and Rojas, 2006). In addition to the increase in the number of transport devices (Kloeper et al., 2007), mammals might adopt a preexisting device to fulfill the requirement for diverse transport routes from endosome to the TGN. Moreover, mammals might endow RINT-1 with the ability to interact with Rad50 (Xiao et al., 2001) and Rab-related p130 (Kong et al., 2006).

In conclusion, the present findings highlight a new layer of complexity for the interactions of proteins responsible for the tethering of transport carriers with SNAREs. The CATCHR family COG complex interacts with coiled-coil tethers at the cis-side of the Golgi apparatus (Sohda et al., 2007, 2010) and with the CATCHR family tether RINT-1 at the TGN (this study). Taken together, these data indicate that CATCHR family members function in vesicle tethering by regulating SNARE complex assembly.

**MATERIALS AND METHODS**

**Antibodies and reagents**

Monoclonal antibodies against GM130, γ-adaptin, p230, syntaxin 6, EEA1, calnexin, Cog1, and Vti1a were purchased from BD Biosciences PharMingen (San Diego, CA). Polyclonal antibodies against TGN46 and Golgin-97 were obtained from Abcam (Cambridge, MA). Monoclonal and polyclonal antibodies against FLAG were purchased from Sigma-Aldrich (St. Louis, MO). Polyclonal antibodies against syntaxin 16 and VAMP4 were purchased from Synaptic Systems (Göttingen, Germany). Polyclonal antibodies against GPP130,
GFP, and GST and monoclonal antibodies against CI-MPR and Hsp47 were obtained from Covance (Princeton, NJ), Invitrogen (Carlsbad, CA), Santa Cruz Biotechnology (Santa Cruz, CA), Thermo Fisher Scientific (Waltham, MA), and Enzo Life Science (Farmingdale, NY), respectively. Polyclonal antibody against Cog3 was prepared as described (Sohda et al., 2007). Polyclonal antibodies against RINT-1, ZW10, NAG, syntaxin 18, and Ret1 were prepared in our laboratory (Hirose et al., 2004; Nakajima et al., 2004, Aoki et al., 2009). Glutathione Sepharose 4B was from GE Healthcare (Piscataway, NJ).

Cell culture
293T cells were grown in DMEM supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin, and 10% fetal calf serum. HeLa cells were cultured in Eagle’s MEM supplemented with the same materials.

Plasmids and transfection
The plasmids encoding FLAG–Cog1-8 were constructed previously (Sohda et al., 2007, 2010). The cDNAs encoding full-length or truncated mutants of RINT-1 were inserted into pEGFP-C3 vector so as to express proteins with an N-terminal GFP tag. The cDNAs encoding full-length syntaxin 16 and its truncated mutants, syntaxin 6, syntaxin 10, VAMP3, VAMP4, and Vti1a were inserted into pFLAG-CMV-6 (Sigma-Aldrich) so as to express proteins with an N-terminal FLAG tag. To express full-length or truncated Cog1 fragments as GST fusion proteins in mammalian cells, the cDNAs encoding Cog1 and its fragments were inserted into the Smal site of pEBG-Sma vector. The plasmids encoding GARP subunits were a generous gift from J. S. Bonifacino (National Institutes of Health, Bethesda, MD). Transfection was carried out using LipofectAMINE PLUS or LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s protocol. At 24 h after transfection, the cells were fixed and immunofluorescence analysis or lysed for immunoprecipitation or pull-down assays.

Immunofluorescence microscopy
For immunofluorescence microscopy, cells were fixed with 4% paraformaldehyde for 20 min at room temperature or ice-cold methanol at −20°C and observed with an Olympus Fluoview 300 or 1000 laser scanning microscope (Olympus, Tokyo, Japan).

Immunoprecipitation
For immunoprecipitation of endogenous RINT-1 or ZW10, −90% confluent 293T cells (two 15-cm dishes) were washed twice in phosphate-buffered saline (PBS) and then once in homogenization buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]–KOH, pH 7.2, 150 mM KCl, 2 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride). The cells were collected, suspended in 1 ml of homogenization buffer, and homogenized with 20 strokes in a Dounce homogenizer. The homogenate was centrifuged at 1000 × g for 10 min, and then the supernatant was centrifuged at 100,000 × g for 30 min to separate the cytosol and membrane fractions. The membrane pellet was solubilized in homogenization buffer containing 1% Triton X-100. Equal volumes of Triton X-100 extracts were incubated for 1 h with 2 μg of a polyclonal antibody against RINT-1 or ZW10. After incubation, 10 μl of protein G-Sepharose (GE Healthcare) was added, and the suspension was gently mixed for 2 h. The beads were thoroughly washed, and the attached proteins were eluted by SDS sample buffer, resolved by SDS–PAGE, and analyzed by immunoblotting.

For immunoprecipitation of expressed proteins, 293T cells expressing FLAG-tagged proteins were lysed in homogenization buffer containing 1% Triton X-100 and centrifuged at 17,000 × g for 10 min. The supernatants were immunoprecipitated with anti–FLAG M2 affinity gels (Sigma-Aldrich). After extensive washing of the beads, the bound proteins were eluted from the gels by adding SDS-sample buffer and analyzed by immunoblotting.

RNA interference
The RNA duplexes used for targeting RINT-1 (1149), ZW10 (102), and NAG (4160) were described previously (Hirose et al., 2004; Arasaki et al., 2006; Aoki et al., 2009). The RNA duplexes used for targeting RINT-1 (687) (5′-aagugauuuugaggaaauu-3′, which corresponds to positions 687–707 relative to the start codon) were purchased from Japan Bioservice (Asaka, Japan). Transfection was performed using Oligofectamine (Invitrogen) according to the manufacturer’s protocol.

CTB transport assay
At 48 h after transfection of siRNA, HeLa cells were incubated in OPTI-MEM containing 20 mM HEPES, pH 7.4, and 5 μg/ml Alexa Fluor 594–labeled CTB (Invitrogen) for 30 min at 4°C. The cells were then washed with PBS and incubated in a complete growth medium at 37°C. The cells were fixed, immunostained with an anti-EEA1 antibody, and analyzed by confocal microscopy.

Antibody uptake assay
At 48 h after transfection of siRNA, HeLa cells were transfected with a mammalian expression vector encoding FLAG-TGN38. At 24 h later, the cells were incubated at 37°C in medium containing 1.5 μg/ml anti–FLAG M2 monoclonal antibody for different time periods (3, 15, and 45 min). The cells were extensively washed with PBS, fixed, stained with a Texas red–labeled anti-mouse secondary antibody, and analyzed by confocal microscopy.

Two-hybrid assay
Two-hybrid analysis was carried out essentially according to the manufacturer’s protocol using pGBK7 vector for BNIPI and pACT2 vector for RINT-1 and its fragments. To detect β-galactosidase activity, filters were incubated at 30°C for 1 h.

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REFERENCES
Andarg U, Neumann T, Schmitt HD (2001). The coatomer-interacting protein Dsl1p is required for Golgi-to-endoplasmic reticulum retrieval in yeast. J Biol Chem 276, 39150–39160.

Andarg U, Schmitt HD (2003). Dsl1p, an essential component of the Golgi-endoplasmic reticulum retrieval system in yeast, uses the same sequence motif to interact with different subunits of the COPI vesicle coat. J Biol Chem 278, 51722–51734.
Aoki T, Ichimura S, Itah A, Kuramoto M, Shinkawa T, Isobe T, Tagaya M (2009). Identification of the neuroblastoma-amplified gene product as a component of the syntaxin 18 complex implicated in Golgi-to-endoplasmic reticulum retrograde transport. Mol Biol Cell 20, 2639–2649.

Aoki T, Kojima M, Tani K, Tagaya M (2008). Sec22b-dependent assembly of endoplasmic reticulum Q-SNARE proteins. Biochem J 410, 93–100.

Appenzeller-Herzog C, Hauni HP (2006). The ER-Golgi intermediate compartment (ERGIC): in search of its identity and function. J Cell Sci 119, 2173–2183.

Arasaki K, Taniguchi M, Tani K, Tagaya M (2006). RINT-T regulates the localization and entry of ZW10 to the syntaxin 18 complex. Mol Biol Cell 17, 2780-2786.

Bonifacino JS, Glick BS (2004). The mechanisms of vesicle budding and fusion. Cell 116, 153–166.

Bonifacino JS, Hierro A (2011). Transport according to GARP: receiving retrograde cargo at the trans-Golgi network. Trends Cell Biol 21, 159–167.

Bonifacino JS, Rojas R (2006). Retrograde transport from endosomes to the trans-Golgi network. Nat Rev Mol Cell Biol 7, 568–579.

Bröcker C, Engelbrecht-Vandré S, Ungermann C (2010). Multisubunit tethering complexes and their role in membrane fusion. Curr Biol 20, R943–R952.

Brown FC, Pfeffer SR (2008). An update on transport vesicle tethering. Mol Membr Biol 25, 475–461.

Carr CM, Rizo J (2010). At the junction of SNARE and SM protein function. Curr Opin Cell Biol 22, 486–495.

Chatterton JE, Hirsch D, Schwartz JJ, Bickel PE, Rosenberg RD, Lodish HF, Brown FC, Pfeffer SR (2010). An update on transport vesicle tethering. Mol Biol Cell 21, 961–976.

Conibear E, Cleck JN, Stevens TH (2003). Vps51p mediates the association of the GARP (Vps52/S3/S4) complex with the late Golgi t-SNARE Tlg1p. Mol Biol Cell 14, 1615–1623.

Diefenbacher M, Thorsteinsdottir H, Spang A (2011). The Dsl1 tethering complex actively participates in soluble NSF (N-ethylmaleimide-sensitive factor) attachment protein receptor (SNARE) complex assembly at the endoplasmic reticulum in Saccharomyces cerevisiae. J Biol Chem 286, 25027–25038.

Dulubova I, Yamauchi T, Gao Y, Min SW, Huryeva I, Súdholc TC, Rizo J (2002). How Tlg2p/syntaxin 6 and positively regulates endosome-to-TGN retrograde transport involves two SNARE complexes and a Rab6 isoform. J Cell Biol 156, 653–664.

Meiringer CT, Rehmeyer R, Auffarth K, Wilson J, Perz A, Barlowe C, Schmitt FM, Krieger M (2005). Genetic analysis of the subunit organization and function of the COG complex: studies of COG5- and COG7-deficient mammalian cells. J Cell Biol 28, 3273–32745.

Pérez-Victoria FJ, Abascal-Palacios G, Tascón I, Kajava A, Magadán JG, Piro EOP, Bonifacino JS, Hierro A (2010a). Structural basis for the wobbly mouse neurodegenerative disorder caused by mutation in the Vps54 subunit of the GARP complex. Proc Natl Acad Sci USA 107, 12860–12865.

Pérez-Victoria FJ, Bonifacino JS (2009). Dual roles of the mammalian GARP complex in tethering and SNARE complex assembly at the trans-Golgi network. Mol Cell Biol 29, 5251–5263.

Pérez-Victoria FJ, Schindler C, Magadán JG, Mardones GA, Delevoye C, Romao M, Raposo G, Bonifacino JS (2010b). Ang2/Fat-free is a conserved subunit of the Golgi-associated retrograde protein complex. Mol Biol Cell 21, 3386–3395.

Pfeffer SR (2011). Entry at the trans-face of the Golgi. Cold Spring Harb Perspect Biol 3, a005272.

Reaves B, Horn M, Banting G (1993). TGN38/41 recycles between the cell surface and the TGN: brefeldin A affects its rate of return to the TGN. Mol Biol Cell 4, 93–105.

Reilly BA, Kraynack BA, VanRheenen SM, Waters MG (2001). Golgi-to-endoplasmic reticulum (ER) retrograde transport in yeast requires Dsl1p, a component of the ER-target site that interacts with a COP1 coat subunit. Mol Biol Cell 12, 3783–3796.

Ren Y, Yip CK, Tripathi A, Huie D, Jeffrey PD, Walz T, Hughson FM (2009). A structure-based mechanism for vesicle capture by the multisubunit tethering complex Dsl1. Cell 139, 1119–1129.

Richardson BC, Smith RD, Ungar D, Nakamura A, Jeffrey PD, Lupashin VV, Hughson FM (2009). Structural basis for a human glycosylation disorder caused by mutation of the COG4 gene. Proc Natl Acad Sci USA 106, 13329–13334.

Sandvig K, van Deurs B (2002). Membrane traffic exploited by protein toxins. Annu Rev Cell Dev Biol 18, 1–24.

Schmitt HD (2005). Dsl1p/Zw10: common mechanisms behind tethering vesicles and microtubules. Trends Cell Biol 20, 257–268.

Sinnosoglou S, Pelham HR (2002). Vps15p links the VFT complex to the trans-Golgi network. Mol Biol Cell 13, 923–936.

Smith RD, Lupashin VV (2008). Role of the conserved oligomeric Golgi (COG) complex in protein glycosylation. Carbohydr Res 343, 2034–2031.

Sohda M, Misumi Y, Yamamoto A, Nakamura N, Ogata S, Sakisaka S, Hirose S, Ikehara Y, Oda K (2010). Interaction of Golgin-84 with the COG complex mediates the intra-Golgi retrograde transport. Traffic 11, 1552–1566.

Sohda M, Misumi Y, Yoshimura S, Nakamura N, Fusano T, Ogata S, Sakisaka S, Ikehara Y (2007). The interaction of two tethering factors, p115 and COG complex, is required for Golgi integrity. Traffic 8, 270–284.

Spang A (2012). The Dsl1 complex: the smallest but not the least CATCHR. Trends Cell Biol 22, 908–913.

Sun Y, Shestakova A, Hunt L, Sehgal S, Lupashin V, Storrie B (2007). Rab6 regulates both ZW10/RINT-T and conserved oligomeric Golgi... Molecular Biology of the Cell
complex-dependent Golgi trafficking and homeostasis. Mol Biol Cell 18, 4129–4142.

Sweet DJ, Pelham HR (1993). The TIP1 gene of Saccharomyces cerevisiae encodes an 80 kDa cytoplasmic protein that interacts with the cytoplasmic domain of Sec20p. EMBO J 12, 2831–2840.

Tripathi A, Ren Y, Jeffrey PD, Hughson FM (2009). Structural characterization of Tip20p and Dsl1p, subunits of the Dsl1p vesicle tethering complex. Nat Struct Mol Biol 16, 114–123.

Uemura T, Sato T, Aoki T, Yamamoto A, Okada T, Hirai R, Harada R, Mori K, Tagaya M, Harada A (2009). p31 deficiency influences endoplasmic reticulum tubular morphology and cell survival. Mol Cell Biol 29, 1869–1881.

Uetz P et al. (2000). A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature 403, 623–627.

Ungar D, Oka T, Brittle EE, Vasile E, Lupashin VV, Chaterton JE, Heuser JE, Krieger M, Waters MG (2002). Characterization of a mammalian Golgi-localized protein complex, COG, that is required for normal Golgi morphology and function. J Cell Biol 157, 405–415.

Ungar D, Oka T, Vasile E, Krieger M, Hughson FM (2005). Subunit map of the conserved oligomeric Golgi complex. J Biol Chem 280, 32729–32735.

Vasan N, Hutagalung A, Novick P, Reinisch KM (2010). Structure of a C-terminal fragment of its Vps53 subunit suggests similarity of Golgi-associated retrograde protein (GARP) complex to a family of tethering complexes. Proc Natl Acad Sci USA 107, 14176–14181.

Vasile E, Oka T, Ericsson M, Nakamura N, Krieger M (2006). IntraGolgi distribution of the conserved oligomeric Golgi (COG) complex. Exp Cell Res 312, 3132–3141.

Whyte JR, Munro S (2002). Vesicle tethering complexes in membrane traffic. J Cell Sci 115, 2627–2637.

Xiao J, Liu CC, Chen PL, Lee WH (2001). RINT-1, a novel Rad50-interacting protein, participates in radiation-induced G2/M checkpoint control. J Biol Chem 276, 6105–6111.

Yu IM, Hughson FM (2010). Tethering factors as organizers of intracellular vesicular traffic. Annu Rev Cell Dev Biol 26, 137–156.

Zink S, Wenzel D, Wurm CA, Schmitt HD (2009). A link between ER tethering and COP-I vesicle uncoating. Dev Cell 17, 403–416.