RINT-1 Regulates the Localization and Entry of ZW10 to the Syntaxin 18 Complex^D

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Submitted October 24, 2005; Revised March 17, 2006; Accepted March 20, 2006

Monitoring Editor: Benjamin Glick

RINT-1 was first identified as a Rad50-interacting protein that participates in radiation-induced G₂/M checkpoint control. We have recently reported that RINT-1, together with the dynamin-interacting protein ZW10 and others, is associated with syntaxin 18, an endoplasmic reticulum (ER)-localized SNARE involved in membrane trafficking between the ER and Golgi. To address the role of RINT-1 in membrane trafficking, we examined the effects of overexpression and knockdown of RINT-1 on Golgi morphology and protein transport from the ER. Overexpression of the N-terminal region of RINT-1, which is responsible for the interaction with ZW10, caused redistribution of ZW10. Concomitantly, ER-to-Golgi transport was blocked and the Golgi was dispersed. Knockdown of RINT-1 also disrupted membrane trafficking between the ER and Golgi. Notably, silencing of RINT-1 resulted in a reduction in the amount of ZW10 associated with syntaxin 18, concomitant with ZW10 redistribution. In contrast, no redistribution or release of RINT-1 from the syntaxin 18 complex was observed when ZW10 expression was reduced. These results taken together suggest that RINT-1 coordinates the localization and function of ZW10 by serving as a link between ZW10 and the SNARE complex comprising syntaxin 18.

INTRODUCTION

In eukaryotic cells, communication between membrane-enclosed compartments in the secretory and endocytic pathways is mediated by vesicles or membrane carriers that bud from one compartment and then tether to and fuse with another compartment (Bonifacino and Glick, 2004; Lee *et al.*, 2004). SNAP receptors (SNAREs) play a central role in membrane fusion of transport carriers with the target compartments (Ungar and Hughson, 2003; Burri and Lithgow, 2004; Hong, 2005). SNAREs constitute a superfamily of membrane proteins that are characterized by a homologous coiled-coil domain of 60–70 amino acids (aa) in length, termed the SNARE motif (Weimbs *et al.*, 1997). In mammalian cells, there are at least 36 SNARE members that are uniquely localized in different membrane compartments (Hong, 2005). Depending on their function and localization on two opposing membranes, SNAREs can be classified into v-SNARE (VAMP family proteins) and t-SNARE (syntaxin and SNAP-25 family proteins; Söllner *et al.*, 1993). It has been proposed that the formation of v-SNARE/t-SNARE complex (*trans*-SNARE complex) perturbs the apposing membranes, thereby triggering fusion (Weber *et al.*, 1998; McNew *et al.*, 2000). This complex comprises a bundle

of four α -helices, one supplied from VAMP, one from syntaxin, and two from SNAP-25 (Sutton *et al.*, 1998). In cases where SNAP-25 family proteins are not involved, four SNARE molecules individually supply one helix for the formation of the *trans*-SNARE complex (Fukuda *et al.*, 2000). Depending on the presence of Arg or Gln in the core binding domains of the four-helical bundle, SNAREs are alternatively categorized as R- or Q-SNAREs (Fasshauer *et al.*, 1998).

We have previously identified an endoplasmic reticulum (ER)-associated SNARE, syntaxin 18 (Hatsuzawa *et al.*, 2000), which is most likely the mammalian orthologue of yeast Ufe1p implicated in retrograde transport from the Golgi to the ER (Lewis and Pelham, 1996) and homotypic ER membrane fusion (Patel *et al.*, 1998). More recently, we demonstrated that syntaxin 18 is present in a large complex comprising three SNARE proteins (p31, BNIP1, and Sec22b) and three peripheral membrane proteins (Sly1p, ZW10, and RINT-1; Hirose *et al.*, 2004; Nakajima *et al.*, 2004). p31 is the orthologue of yeast Use1p/Slt1p, which is an unconventional Q-SNARE because of the presence of Asp instead of Gln in the core binding domain (Belgareh-Touze *et al.*, 2003; Burri *et al.*, 2003; Dilcher *et al.*, 2003). BNIP1 likely corresponds to yeast Sec20p, although their sequences as well as molecular weights are markedly different with each other (Sweet and Pelham, 1992; Nakajima *et al.*, 2004). ZW10 and RINT-1, which were originally characterized as checkpoint proteins (Williams *et al.*, 1992; Chan *et al.*, 2000; Xiao *et al.*, 2001), appear to be equivalent to Dsl1p and Tip20p, respectively (VanRheenen *et al.*, 2001; Andag and Schmitt, 2003; Hirose *et al.*, 2004). The equivalents of all proteins identified in the syntaxin 18 complex are present in the Ufe1p complex (Lewis *et al.*, 1997; Andag *et al.*, 2001; Reilly *et al.*, 2001; Hirose *et al.*, 2004; Nakajima *et al.*, 2004), implying that the ER membrane fusion machinery is conserved during evolution (Kraynack *et al.*, 2005).

In the present study we investigated the role of RINT-1 in membrane trafficking between the ER and Golgi. We showed that RINT-1 participates in ER-to-Golgi transport by

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-10-0973>) on March 29, 2006.

^D The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

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Abbreviations used: aa, amino acid; BFA, brefeldin A; ER, endoplasmic reticulum; GFP, green fluorescent protein; GST, glutathione S-transferase; mAb, monoclonal antibody; Man II, mannosidase II; RNAi, RNA interference; siRNA, short interfering RNA; SNARE, SNAP receptor; VSVG, vesicular stomatitis virus-encoded glycoprotein.

regulating the localization and entry of ZW10 into the syntaxin 18 complex.

MATERIALS AND METHODS

Antibodies and Chemicals

A monoclonal antibody (mAb) against human syntaxin 18 and polyclonal antibodies against human syntaxin 18, p31, RINT-1, ZW10, and BNIP1 were produced as described (Hirose *et al.*, 2004; Nakajima *et al.*, 2004). A polyclonal antibody against human Sec31p was prepared in this laboratory. mAbs against human p115 and ERGIC-53 were generous gifts from Dr. M. G. Waters (Princeton University, Princeton, NJ) and Dr. H.-P. Hauri (University of Basel, Switzerland), respectively. mAbs against calnexin, p150^{Gluc}, and Rad50 were obtained from BD Transduction Laboratories (Lexington, KY). A mAb against Hsp47 and a polyclonal antibody against mannosidase II (Man II) were obtained from StressGen Biotechnologies (Victoria, British Columbia, Canada) and Chemicon (Temecula, CA), respectively. A polyclonal antibody against glutathione S-transferase (GST) was from Santa Cruz Biotechnology (Santa Cruz, CA). A mAb against α -tubulin, monoclonal and polyclonal antibodies against FLAG, nocodazole, and brefeldin A (BFA) were purchased from Sigma-Aldrich (St. Louis, MO).

Cell Culture

HeLa cells (Riken Bioresource Center) were cultured in Eagle's minimum essential medium supplemented with 50 IU/ml penicillin, 50 μ g/ml streptomycin, and 10% fetal calf serum. 293T cells were grown in DMEM supplemented with the same materials.

Immunofluorescence Analysis

Immunofluorescence microscopy was performed as described (Tagaya *et al.*, 1996). Cells were fixed with methanol at -20°C for 5 min for staining of endogenous RINT-1 and ZW10, or 4% paraformaldehyde at room temperature for 20 min for Man II, Sec31p, and expressed proteins. Confocal microscopy was performed with an Olympus Fluoview 300 laser scanning microscope (Tokyo, Japan).

Cell Synchronization and Immunoprecipitation

HeLa cells were synchronized by double thymidine block followed by nocodazole treatment, as described by Chan *et al.* (2000). Mitotic cells were collected by gently shaking off flasks. Unsynchronized and synchronized cells were homogenized in lysis buffer and immunoprecipitated with the indicated antibodies (Hatsuzawa *et al.*, 2000).

Plasmid Construction and Transfection

The cDNAs encoding RINT-1 Δ N (aa 220-792), RINT-1 Δ C (aa 1-659), RINT-1N (aa 1-264), RINT-1M (aa 200-585), and RINT-1C (aa 565-792) were amplified by PCR and inserted into pFLAG-CMV 6a (Sigma-Aldrich). The plasmids for Sar1p mutants were constructed in this laboratory (Shimoi *et al.*, 2005). The plasmid (pEBG) encoding GST was a kind gift from Dr. B. Mayer (Harvard Medical School, Boston, MA). For ZW10 binding assays, 293T cells grown on 35-mm dishes were transfected with the plasmid for the RINT-1 constructs (1 μ g each) using LipofectAMINE PLUS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol.

Protein Transport from the ER to the Golgi

The expression plasmid for vesicular stomatitis virus-encoded glycoprotein fused to green fluorescent protein (VSVG-GFP) was kindly donated by Dr. J. Lippincott-Schwartz (National Institutes of Health, Bethesda, MD). The plasmid (1 μ g) was cotransfected with the plasmid for FLAG-RINT-1 full-length or deletion constructs (1 μ g) into HeLa cells grown on 35-mm dishes. The cells were incubated at 40°C for 24 h and then shifted to 32°C to allow transport. The cells were fixed and processed for immunofluorescence analysis.

RNA Interference

Duplex RNAs used for targeting were RINT-1 (268) (5'-gaacaggtacttacaatttc-3'), RINT-1 (1149) (5'-ttagccactgatattctgt-3'), β -COP (276) (5'-gagactttcatgagatgat-3'), and lamin A/C (5'-ctggacttcagaagaacatt-3'). The duplex RNAs were purchased from Japan BioServices (Saitama, Japan). HeLa cells were grown on six-well plates or 100-mm dishes and transfected with duplex RNAs using Oligofectamine (Invitrogen) according to the manufacturer's protocol. Their final concentration was 100 nM. At 72 h after transfection, the cells were processed for immunoblotting, immunofluorescence, and immunoprecipitation. In the case of double transfection, cells were first transfected with duplex RNAs, incubated for 48–54 h, and then transfected with the plasmid for VSVG-GFP or Sar1p mutants. Transport of VSVG-GFP or redistribution of Man II to the ER was monitored after further 18–24-h incubation.

RESULTS

RINT-1 Is Associated with ZW10 and Syntaxin 18 throughout the Cell Cycle

RINT-1 (Rad50-interacting protein 1) was originally discovered as a checkpoint protein that interacts with Rad50 only during late S and G₂/M phases (Xiao *et al.*, 2001). On the other hand, we have previously shown that RINT-1, together with ZW10 and other proteins, is complexed with syntaxin 18 (Hirose *et al.*, 2004), an ER-localized SNARE implicated in membrane fusion (Hatsuzawa *et al.*, 2000). These results suggest that there are, at least, two discrete RINT-1-containing complexes. To gain insight into the relationship between the two complexes, we performed immunoprecipitation experiments using lysates from HeLa cells that were unsynchronized (that is, predominantly interphasic) or synchronized at M phase with nocodazole (Figure 1A). In line with a previous report (Xiao *et al.*, 2001), RINT-1 was coprecipitated with Rad50 from lysates of mitotically arrested cells (lane 6), but not from those of interphase cells (lane 2). In a reciprocal experiment, a small but significant amount of Rad50 was coprecipitated with RINT-1 from mitotic lysates (lane 5). Little, if any, ZW10 was coprecipitated with Rad50 from lysates of interphase cells (lane 2) or mitotic ones (lane 6), and vice versa (lanes 3 and 7). On the other hand, RINT-1 was coprecipitated with ZW10 regardless of whether cells were mitotically arrested or not (lanes 3

Figure 1. RINT-1 is associated with ZW10 and syntaxin 18 throughout the cell cycle. (A) Lysates were prepared from interphasic or mitotic HeLa cells and immunoprecipitated with an antibody against RINT-1 (lanes 1 and 5), Rad50 (lanes 2 and 6), or ZW10 (lanes 3 and 7). The immunoprecipitated proteins were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies (lanes 1–3 and 5–7). Input (6.2% of total) was also analyzed (lanes 4 and 8). LE, long exposure. (B) HeLa cells were treated without (top two rows) or with 30 μ g/ml digitonin (bottom two rows) for permeabilization and double-stained with antibodies against RINT-1 (first column) and calnexin (second column). Merged images are shown on the right. Bar, 5 μ m. (C) Mitotic HeLa cells were fixed and double-stained with RINT-1 (top row) or ZW10 (bottom row) and p150^{Gluc}. Merged images are shown on the right. Bar, 5 μ m.

and 7). These results suggest that, unlike the case of Rad50, RINT-1 is associated with ZW10 throughout the cell cycle.

ZW10, together with Rod and Zwi1ch, has been reported to be localized on kinetochores at prometaphase (Williams *et al.*, 2003). As RINT-1 is associated with ZW10 throughout the cell cycle, we wondered whether or not RINT-1 is associated with kinetochores during mitosis. In interphase cells, RINT-1 gave a reticular staining pattern similar to that of an ER marker, calnexin (Figure 1B). In prometaphase cells, RINT-1 was not localized on kinetochores (Figure 1C), suggesting that it is not a component of a ZW10 complex comprising Rod and Zwi1ch.

The N-terminal Region of RINT-1 Interacts with ZW10

Our previous yeast two-hybrid assay demonstrated that RINT-1 binds directly to ZW10 and BNIP1 (Hirose *et al.*, 2004; Nakajima *et al.*, 2004). To determine which regions of RINT-1 interact with these two proteins, we constructed several truncated mutants (Figure 2A) and performed binding experiments using the yeast two-hybrid system and immunoprecipitation. Yeast two-hybrid analysis showed that deletion of the N-terminal 219 aa (RINT-1ΔN) abolishes the interaction with ZW10, and that the N-terminal 264 aa fragment (RINT-1N) is sufficient for the interaction (Figure 2A). Immunoprecipitation using FLAG-tagged RINT-1 constructs gave similar results (Figure 2B). Because the C-terminally truncated construct (RINT-1ΔC) exhibited a weaker binding to ZW10 than did the N-terminal construct (RINT-1N) in both yeast and mammalian cells, the middle region of RINT-1 might somehow suppress the interaction with ZW10. However, further study is necessary to address this possibility.

Although RINT-1N, as well as full-length RINT-1, was capable of interacting with ZW10, its localization was markedly different from that of the full-length protein. RINT-1N was found to be almost exclusively localized in the cytosol, whereas full-length RINT-1 was distributed in both membranes and cytosol (Supplementary Figure S1A). Consistent with its localization, RINT-1N was not associated with the membrane components, syntaxin 18 and BNIP1 (Supplementary Figure S1B).

We could not define the BNIP1-binding site on RINT-1 because almost all constructs, to a greater or lesser extent, bind to BNIP1 (unpublished data). Perhaps, RINT-1 interacts with BNIP1 through its several distinct regions.

Overexpression of the N-terminal Region of RINT-1 Perturbs Membrane Trafficking between the ER and Golgi

As RINT-1N interacts with ZW10 and localized in the cytosol, it may function as a dominant-negative by competing with endogenous membrane-bound RINT-1 for ZW10. We therefore examined the effect of overexpression of RINT-1N, together with full-length and other truncated constructs, on Golgi morphology (Figure 3A). In cells overexpressing RINT-1N, dispersed patterns for Golgi marker proteins, p115 and Man II, were frequently observed, whereas overexpression of full-length RINT-1 or other truncated constructs had little, if any, effect (top two and middle two panels). Notably, overexpression of RINT-1N, but not the full-length construct, resulted in a significant loss of ZW10 staining at the ER (bottom two panels), suggesting that RINT-1N induced redistribution of ZW10.

Next, we investigated the effect of full-length RINT-1 and RINT-1N on protein transport from the ER to the Golgi. We used a well-known morphological transport assay that utilizes GFP fused to a temperature-sensitive VSVG mutant that is unable to exit the ER at the nonpermissive temperature (40°C; Presley *et al.*, 1997). At 30 min of shifting to the permissive temperature (32°C), VSVG-GFP had exited the ER and accu-

Figure 2. The N-terminal region of RINT-1 interacts with ZW10. (A) Schematic representation of RINT-1 and its deletion constructs. The interaction of RINT-1 or its deletion constructs with ZW10 in yeast cells was evaluated by filter assays for β -galactosidase. Blue color was detected within 1 h (+++), 2 h (++) and 16 h (+). Expression of RINT-1C in yeast cells was confirmed by immunoblotting, whereas this fragment was not expressed in 293T cells (unpublished data). (B) 293T cells were transfected with the plasmids encoding the indicated FLAG-tagged constructs. At 24 h after transfection, cell lysates were prepared, and FLAG-tagged proteins were immunoprecipitated with an anti-FLAG antibody. The precipitated proteins were analyzed by immunoblotting with antibodies against ZW10 and FLAG (lanes 6–10). Lanes 1–5 show the amounts of ZW10 and FLAG-tagged proteins in 4% of the lysate. H.C. and L.C. denote immunoglobulin heavy and light chains, respectively.

mulated at the perinuclear, Golgi region in cells expressing full-length RINT-1, as observed in nontransfected cells (unpublished data) and GST-expressing cells (Figure 3B). In cells expressing RINT-1N, on the other hand, VSVG-GFP had exited the ER but remained in dotlike structures at the cell periphery. Double staining revealed that the distribution of VSVG-GFP fairly overlaps with that of ERGIC-53, a marker for the ER-Golgi intermediate compartment, and β -COP, a COPI component, but less with that of Sec31p, an ER exit site marker (Supplementary Figure S2), suggesting that VSVG-GFP spots observed in RINT-1N-expressing cells represent the ERGIC. Collectively, these results indicate that overexpression of RINT-1N disturbs membrane trafficking between the ER and Golgi.

Depletion of RINT-1 Affects Membrane Trafficking between the ER and Golgi

To assess the requirement of RINT-1 for membrane trafficking, RINT-1 expression was knocked down by RNA inter-

Figure 3. Overexpression of the N-terminal region of RINT-1 perturbs membrane trafficking between the ER and Golgi. (A) HeLa cells were transfected with the plasmids encoding GST or the indicated FLAG-tagged constructs. After 24 h, the cells were fixed and double-stained. Staining for p115 (top two panels), Man II (middle two panels), and ZW10 (bottom two panels). Asterisks indicate cells overexpressing FLAG-RINT-1 full-length (Full) or FLAG-RINT-1N (N). Bar, 5 μ m. The quantitative results are shown on the right. Error bars, SE of the mean for three experiments. (B) The plasmid for VSVG-GFP was cotransfected with the plasmid for GST, as a control, FLAG-RINT-1 full-length, or FLAG-RINT-1N into HeLa cells, and the protein transport assay was performed. To identify cells expressing GST or FLAG-tagged constructs, the fixed cells were stained with an anti-GST or anti-FLAG antibody followed by a Texas Red-conjugated secondary antibody. Only GFP fluorescence is shown. Bar, 5 μ m. The quantitative results are shown at the bottom. "Peripheral dots" denotes the dispersed dotlike distribution pattern of VSVG-GFP. When VSVG-GFP was found to be concentrated in the perinuclear area, this localization pattern was categorized into "Golgi." When VSVG-GFP was detected on the plasma membrane, this pattern was counted as "Plasma membrane." In many of the cells categorized into "Plasma membrane," VSVG-GFP was detected not only on the plasma membrane but also in the perinuclear area.

ference (RNAi). Two short interfering RNAs (siRNAs) named RINT-1 (268) and RINT-1 (1149) markedly blocked RINT-1 expression, although the latter effect was more

prominent (Figure 4A). Concomitant with the reduction in RINT-1 expression, the intensity of RINT-1 staining was decreased, verifying the specificity of the anti-RINT-1 anti-

Figure 4. Silencing of RINT-1 causes dispersion of proteins located in compartments of the early secretory pathway. (A) HeLa cells were transfected without (mock) or with lamin A/C siRNA, RINT-1 (268), or RINT-1 (1149). At 72 h after transfection, the cells were solubilized in phosphate-buffered saline with 0.5% SDS. Equal amounts of total proteins were separated by SDS-PAGE and analyzed by immunoblotting with the indicated antibodies. (B) Immunofluorescence microscopic analysis of mock-treated cells, and cells transfected with lamin A/C siRNA, RINT-1 (268), or RINT-1 (1149). At 72 h after transfection, the cells were fixed and double-stained with antibodies against RINT-1 and p115. Expanded images for p115 are shown on the right. Bar, 5 μ m. (C) Cells were treated as described in B and double-stained with antibodies against Sec31p and ERGIC-53. Bar, 5 μ m.

Figure 5. Delay in VSVG-GFP transport from the ER in cells with reduced expression of RINT-1. HeLa cells were successively transfected with siRNA and the plasmid for VSVG-GFP. Transport of VSVG-GFP from the ER was monitored as described in *Materials and Methods*. Typical images at 0, 30, 60, and 120 min after the temperature shift to 32°C are shown. N indicates the position of the nucleus. Bar, 5 μ m. The quantitative results are shown at the bottom.

body (Figure 4B, bottom two rows). In addition, the distribution of p115 was changed from a compact pattern to a moderately dispersed one, reminiscent of the pattern of p115 staining in cells depleted of ZW10 (Hirose *et al.*, 2004) or syntaxin 18 (unpublished data). The degree of the dispersion of p115 appeared to correlate with the degree of the suppression of RINT-1 expression. Such a change was not observed in mock-treated cells (Figure 4B, top row) or cells transfected with lamin A/C siRNA (second row). In cells where RINT-1 expression was suppressed, another Golgi protein, GM130, also showed a dispersed pattern (unpublished data). The localization of Sec31p and ERGIC-53 was also changed (Figure 4C). We noticed that the overall intensity of ERGIC-53 staining in RINT-1-knockdown cells was

markedly higher than that in control cells. Although the reason for this is unclear at this moment, images obtained by scanning at lower PMT voltage showed dispersion of Sec31p and ERGIC-53 in RINT-1-depleted cells (Supplementary Figure S3). A morphological transport assay revealed that the transport of VSVG-GFP from the ER was substantially delayed in cells transfected with RINT-1 (1149) compared with mock-treated cells or cells transfected with the lamin A/C siRNA (Figure 5).

Depletion of RINT-1 Does Not Substantially Affect BFA- or Sar1p Mutant-induced Redistribution of Man II to the ER

To explore the possibility that RINT-1 is involved in retrograde transport from the Golgi to the ER, we first investi-

Figure 6. Retrograde transport is not significantly impaired in cells with reduced expression of RINT-1. (A) HeLa cells were transfected without (mock) or with RINT-1 (1149). At 72 h after transfection, the cells were incubated with 10 μ M BFA for the indicated times, fixed, and stained with an antibody against Man II. Bar, 5 μ m. (B) At 54 h after transfection, the cells were further transfected with the plasmids for Sar1p mutants and incubated for 18 h. Redistribution of Man II to the ER occurred in almost all RINT-1-depleted and control cells irrespective of the expression levels of Sar1p mutants (unpublished data). Bar, 5 μ m. (C) RINT-1 or β -COP expression was knocked down by RNAi. The expression level of β -COP was decreased by 86% (unpublished data). The cells were incubated with 10 μ M BFA for 30 min, fixed, and double-stained with antibodies against Man II and ERGIC-53. Bar, 5 μ m.

gated the effect of knockdown of RINT-1 on BFA-induced redistribution of Man II. BFA is a well-known reagent that induces the retrograde transport of Golgi enzymes to the ER (Klausner *et al.*, 1992). On BFA treatment, Man II was redistributed to the ER in RINT-1-depleted cells, with kinetics similar to that observed in mock-treated cells, although the localization patterns of Man II in RINT-1-depleted cells and mock-treated cells were substantially different before BFA treatment (Figure 6A). We next examined whether depletion of RINT-1 affects Sar1p mutant-induced redistribution of Man II to the ER. Both the GTP-restricted form, H79G, and the GDP-restricted form, T39N, have been shown to inhibit membrane trafficking from the ER, thereby causing Golgi enzymes to be redistributed to the ER (Kuge *et al.*, 1994; Shima *et al.*, 1998; Ward *et al.*, 2001; Jiang and Storrie, 2005). As shown in Figure 6B, upon expression of Sar1pH79G

Figure 7. Silencing of RINT-1 causes a decrease in the amount of ZW10 associated with syntaxin 18. (A) HeLa cells were transfected without (mock) or with RINT-1 (1149) or lamin A/C siRNA. At 72 h after transfection, lysates were prepared and immunoprecipitated with an anti-syntaxin 18 antibody. The precipitated proteins were detected by immunoblotting with the indicated antibodies (lanes 4–6). Input (4% of total) was also analyzed (lanes 1–3). (B) Cell lysates were prepared as described in A, and immunoprecipitated with an anti-p31 (lanes 3 and 4), anti-BNIP1 (lanes 5 and 6), or control IgG (lanes 7 and 8). Input (2.8% of total) was also analyzed (lanes 1 and 2). (C) HeLa cells were transfected without (mock) or with lamin A/C siRNA or ZW10 (102). At 72 h after transfection, lysates were prepared and immunoprecipitated with an anti-syntaxin 18 antibody. The precipitated proteins were analyzed by immunoblotting with the indicated antibodies (lanes 4–6). Input (4% of total) was also analyzed (lanes 1–3).

or T39N, Man II was redistributed to the ER in RINT-1-depleted cells, as observed in control cells.

Given that RINT-1 depletion had no marked effect on BFA- or Sar1p mutant-induced redistribution of Man II to the ER, we sought to compare the phenotype of cells defective in retrograde transport with that of RINT-1-depleted cells. To this end, β -COP, a component of COP I vesicles involved in retrograde trafficking (Bonifacino and Glick, 2004; Lee *et al.*, 2004), was knocked down by RNAi. On β -COP depletion, Man II became dispersed, whereas ERGIC-53 remained concentrated at the perinuclear region, with loss of its peripheral distribution (Figure 6C, BFA 0 min). Addition of BFA did not induce redistribution of ERGIC-53 from the perinuclear region to the ER (Figure 6C, BFA 30 min). These results suggest that retrograde transport

Figure 8. Silencing of RINT-1 causes redistribution of ZW10. (A and B) HeLa cells were transfected without (mock) or with RINT-1 (1149) or lamin A/C siRNA. At 72 h after transfection, the cells were treated with or without 10 μ g/ml nocodazole for 90 min. The cells were fixed and double-stained with antibodies against ZW10 and α -tubulin. Bar, 5 μ m. (C and D) HeLa cells transfected without (mock) or with ZW10 (102) or lamin A/C siRNA were incubated for 72 h and stained for RINT-1. As observed previously (Hirose *et al.*, 2004), p115 was modestly dispersed in ZW10 (102)-transfected cells (unpublished data), confirming the depletion of ZW10. Alternatively, the cells were incubated with or without 10 μ g/ml nocodazole for 90 min, fixed, and double-stained with antibodies against ZW10 and α -tubulin. Expanded images are shown (bottom row). Bar, 5 μ m.

of the ERGIC is blocked as a consequence of β -COP depletion. Obviously, the phenotype of β -COP-depleted cells is different from that of RINT-1-depleted cells (Figures 4 and 6C).

Loss of RINT-1 Affects the Distribution and Binding of ZW10 to Syntaxin 18

To gain insight into the mechanism underlying the ER-to-Golgi transport defect in RINT-1-depleted cells, we examined the effect of RINT-1 depletion on syntaxin 18 complex assembly. For this purpose, RINT-1 was knocked down, and the syntaxin 18 complex was precipitated with a monoclonal anti-syntaxin 18 antibody (mAb 1E1). As shown in Figure 7A, ZW10, a partner of RINT-1, was not efficiently coprecipitated with syntaxin 18 from lysates of cells transfected with RINT-1 (1149; lane 5) compared with mock-treated cells (lane 4) or lamin A/C siRNA-transfected cells (lane 6). The fact that the relative amount of RINT-1 coprecipitated with syntaxin 18 (lane 5) was larger than that in cell lysates (lane 2) may imply that RINT-1 complexed with ZW10 and other proteins may be more stable than free RINT-1. In contrast to ZW10, the efficiencies of BNIP1 and p31 coprecipitation were indistinguishable between RINT-1-depleted and control cells (lanes 4–6). Similar results were obtained when immunoprecipitation was performed using antibodies against p31 and BNIP1 (Figure 7B, lanes 3–6).

ZW10 in interphase cells has been reported to be distributed in entire ER membranes (Hirose *et al.*, 2004). Given that

overexpression of RINT-1N causes the redistribution of ZW10 (Figure 3A, bottom row), we were interested in the distribution of ZW10 in RINT-1-depleted cells. As shown in Figure 8, A and B, the distribution of ZW10 was significantly changed concomitant with the suppression of RINT-1 expression. ZW10 staining in RINT-1-depleted cells was not observed in the entire ER but rather in dispersed, short filament/dotlike structures. Of note, ZW10 expression was not suppressed in RINT-1-depleted cells (Figure 7). Some of ZW10-positive structures were observed along microtubules. Indeed, nocodazole treatment abolished linear array-like staining for ZW10 (Figure 8B).

Loss of ZW10 does not Affect the Distribution and Binding of RINT-1 to Syntaxin 18

We investigated whether depletion of ZW10 affects the association of RINT-1 with syntaxin 18. Cells were transfected with siRNA named ZW10 (102) (Hirose *et al.*, 2004), lysed, and immunoprecipitated using anti-syntaxin 18 antibody (mAb 1E1). As shown in Figure 7C, RINT-1 was efficiently coprecipitated with syntaxin 18 from lysates of ZW10-depleted cells (lane 6), as well as mock-treated cells (lane 4) and lamin A/C siRNA-transfected cells (lane 5).

We next examined if depletion of ZW10 affects the localization of RINT-1. As shown in Figure 8C, the distribution of RINT-1 was not changed when the expression level of ZW10 was lowered. Interestingly, as seen in RINT-1-depleted cells, residual ZW10 was distributed along microtubules, and this localization was nocodazole-sensitive (Figure 8D).

DISCUSSION

We have previously shown that two cell cycle-related proteins, ZW10 and RINT-1, are tightly associated with syntaxin 18 (Hirose *et al.*, 2004). ZW10 was first characterized as a kinetochore-associated protein involved in spindle checkpoint (Williams *et al.*, 1992; Chan *et al.*, 2000). RINT-1, on the other hand, was shown to interact with Rad50 and participate in G₂/M checkpoint control (Xiao *et al.*, 2001). Our previous results provided evidence that ZW10 during interphase plays a role in membrane trafficking between the ER and Golgi (Hirose *et al.*, 2004). In the present study, we demonstrated that RINT-1 is also implicated in this transport process in interphase cells.

Two-hybrid and immunoprecipitation analyses revealed that the N-terminal region of RINT-1 is responsible for the interaction with ZW10. Overexpression of this region inhibited the ER-to-Golgi transport of VSVG-GFP and caused Golgi disassembly, accompanied by a change in the distribution of ZW10. In contrast, overexpression of full-length RINT-1 neither inhibited protein transport nor induced Golgi disassembly. These results suggest that the N-terminal region of RINT-1 acts as a dominant-negative and raise the possibility that RINT-1 coordinates the localization and function of ZW10. This possibility was supported by the finding that depletion of RINT-1 by RNAi causes the release of ZW10 from the syntaxin 18 complex and affects the distribution of ZW10. In RINT-1-depleted cells, some ZW10 was localized in short filament/dotlike structures along microtubules, whereas it appeared to be distributed in entire ER membranes in normal cells. Perhaps, ZW10 is distributed in both microtubules and ER membranes in normal cells, and its release from the ER, as a consequence of RINT-1 depletion, might accentuate microtubule localization. This explanation may be consistent with the observation that, when ZW10 expression was suppressed by RNAi, residual

ZW10 exhibited a pattern similar to that observed in RINT-1-depleted cells.

The present results suggest that RINT-1 plays a role in anterograde transport from the ER to the Golgi. Given a link between ZW10 and dynamin, a subunit of dynactin that functions as an adaptor for the minus end-directed motor dynein (Starr *et al.*, 1998), it is tempting to speculate that the ZW10/RINT-1 complex acts as an anchor for dynein-dynactin on the ER to facilitate membrane transport to the Golgi. Because a substantial fraction of RINT-1 is associated with the ER membrane (Hirose *et al.*, 2004), RINT-1 may be associated, through its distinct regions, not only with the syntaxin 18 complex but also with other ER proteins that mediate the export of certain secretory and membrane proteins. This possibility is now under investigation.

Tip20p and Dsl1p, the yeast homologues of RINT-1 and ZW10, respectively, form a complex implicated in retrograde transport from the Golgi to the ER (Cosson *et al.*, 1997; Lewis *et al.*, 1997; Reilly *et al.*, 2001; Kraynack *et al.*, 2005). Given that Tip20p directly binds an ER SNARE, Sec20p (Sweet and Pelham, 1993), and that Dsl1p interacts with subunits of coatomer (Andag *et al.*, 2001; Reilly *et al.*, 2001; Andag and Schmitt, 2003), which coats vesicles for retrograde transport (Bonifacino and Glick, 2004; Lee *et al.*, 2004), it has been postulated that the Dsl1p/Tip20p complex functions as a tether in analogy to known tethering complexes (Reilly *et al.*, 2001; Andag and Schmitt, 2003; Lupashin and Sztul, 2005). However, a recent study showed that Tip20p also participates in anterograde transport by prohibiting back-fusion of COPII vesicles that mediate export from the ER (Kamena and Spang, 2004). The finding that the protein responsible for retrograde transport also plays a role in anterograde transport is surprising but understandable, because bidirectional transport is intimately coupled. Hammond and Glick (2000) have proposed that transitional ER sites, where COPII vesicles are formed (Bannykh *et al.*, 1996; Rossanese *et al.*, 1999), are created by retrograde membrane trafficking from the Golgi. Although our results suggest the involvement of RINT-1 in anterograde transport, they could not exclude the possibility that RINT-1 has some role in a retrograde transport process. Our analysis using BFA and Sar1p mutants may not be sensitive enough to assess the role of RINT-1 in retrograde trafficking.

Quite recently, Kraynack *et al.* (2005) reported the results of a detailed analysis of interactions between subunits of the complex comprising Dsl1p and Tip20p. Our present finding that RINT-1 regulates the entry of ZW10 to the syntaxin 18 complex is in accordance with their result that mutations within Tip20p have the most drastic effect on the integrity of the whole complex. However, one marked difference between Tip20p and RINT-1 is that Tip20p is required for the efficient assembly of the Q-SNARE complex consisting of Ufe1p, Sec20p, and Use1p/Slf1p (Kraynack *et al.*, 2005), whereas RINT-1 does not contribute to the formation of the equivalent complex. On the basis of their observation, Kraynack *et al.* (2005) have proposed a model in which the Dsl1p complex stabilizes the Q-SNARE helices and makes them ready for the binding of R-SNARE on retrograde membrane carriers. The different major roles of Tip20p and RINT-1, i.e., Tip20p for retrograde transport and RINT-1 for anterograde transport, may be related to their different modes of interactions with the subunits of the yeast and mammalian complexes.

In support of the original finding by Xiao *et al.* (2001), RINT-1 was found to be associated with Rad50 during mitosis. This was not a consequence of the cell cycle-dependent switch of the interacting partner. RINT-1 was found to

be associated with the syntaxin 18 complex throughout the cell cycle. Recent studies showed the requirement of GRASP-65 and clathrin for mitosis (Sutterlin *et al.*, 2002, 2005; Preisinger *et al.*, 2005; Royle *et al.*, 2005; Yoshimura *et al.*, 2005). At present the reason why proteins involved in membrane trafficking also function in mitosis remains an enigma. Future studies may clarify apparently different, but common traits between membrane trafficking and mitosis.

ACKNOWLEDGMENTS

We thank Drs. M. G. Waters, H.-P. Hauri, J. Lippincott-Schwartz, and B. Mayer for gifts of reagents. This work was supported in part by Grants-in-Aid for Scientific Research (16370089, 16044242, 16048229, and 16657309) from the Ministry of Education, Science, Sports, and Culture of Japan. K.A. is a recipient of a Japan Society for the Promotion of Science research fellowship.

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