Palmitoylated Ras proteins traffic through recycling endosomes to the plasma membrane during exocytosis

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Ras proteins regulate cell growth, death, and differentiation, and it is well established that this functional versatility is accomplished through their different subcellular localizations. Palmitoylated H- and N-Ras are believed to localize at the perinuclear Golgi and plasma membrane (PM). Notably, however, recycling endosomes (REs) also localize to a perinuclear region, which is often indistinguishable from the Golgi. In this study, we show that active palmitoylated Ras proteins mainly localize intracellularly at REs and that REs act as a way station along the post-Golgi exocytic pathway to the PM. H-Ras requires two palmitoyl groups for RE targeting. The lack of either or both palmitoyl groups leads to the mislocalization of the mutant proteins to the endoplasmic reticulum, Golgi apparatus, or the PM. Therefore, we demonstrate that palmitoylation directs Ras proteins to the correct intracellular organelles for trafficking and activity.

Introduction

Ras proteins are small GTPases that regulate cell growth, death, and differentiation. The three ubiquitously expressed Ras isoforms, H-, N-, and K-Ras, are anchored to the inner leaflet of plasma membrane (PM) by two motifs contained in their C-terminal hypervariable domain (for review see Hancock, 2003). The first motif, which is common to all Ras proteins, is a C-terminal CAAX motif that undergoes posttranslational modification by sequential farnesylation, proteolysis, and carboxyl methylation. The second motif varies between Ras isoforms and is comprised of a polybasic domain of six lysine residues for K-Ras and either one or two palmitoylation sites for N-Ras (C181) and H-Ras (C181 and C184). H- and N-Ras acquire these lipid modifications while transiting through the secretory pathway (ER and Golgi; Choy et al., 1999; Apolloni et al., 2000), and recent evidence has revealed that palmitoylated Ras proteins become depalmitoylated at PM, releasing the proteins back to the cytosol, followed by repalmitoylation either on ER or Golgi for another round of exocytic delivery to PM

(Goodwin et al., 2005; Rocks et al., 2005). Another complexity in the trafficking cycle of palmitoylated Ras proteins is that H-Ras associates with endosomes/recycling endosomes (REs; Gomez and Daniotti, 2005; Jura et al., 2006). Gomez and Daniotti (2005) demonstrated that H-Ras mainly localizes at REs in CHO cells, and they proposed that H-Ras localization at REs is maintained by the conventional endocytic pathway, which is governed by Rab5 and Rab11. However, others have found that the retrograde movement of palmitoylated Ras proteins from PM to endomembranes (ER/Golgi) is independent of the conventional endocytic pathway (Goodwin et al., 2005; Rocks et al., 2005), having raised the interesting but counterintuitive question how palmitoylated Ras proteins can reach endosomal compartments.
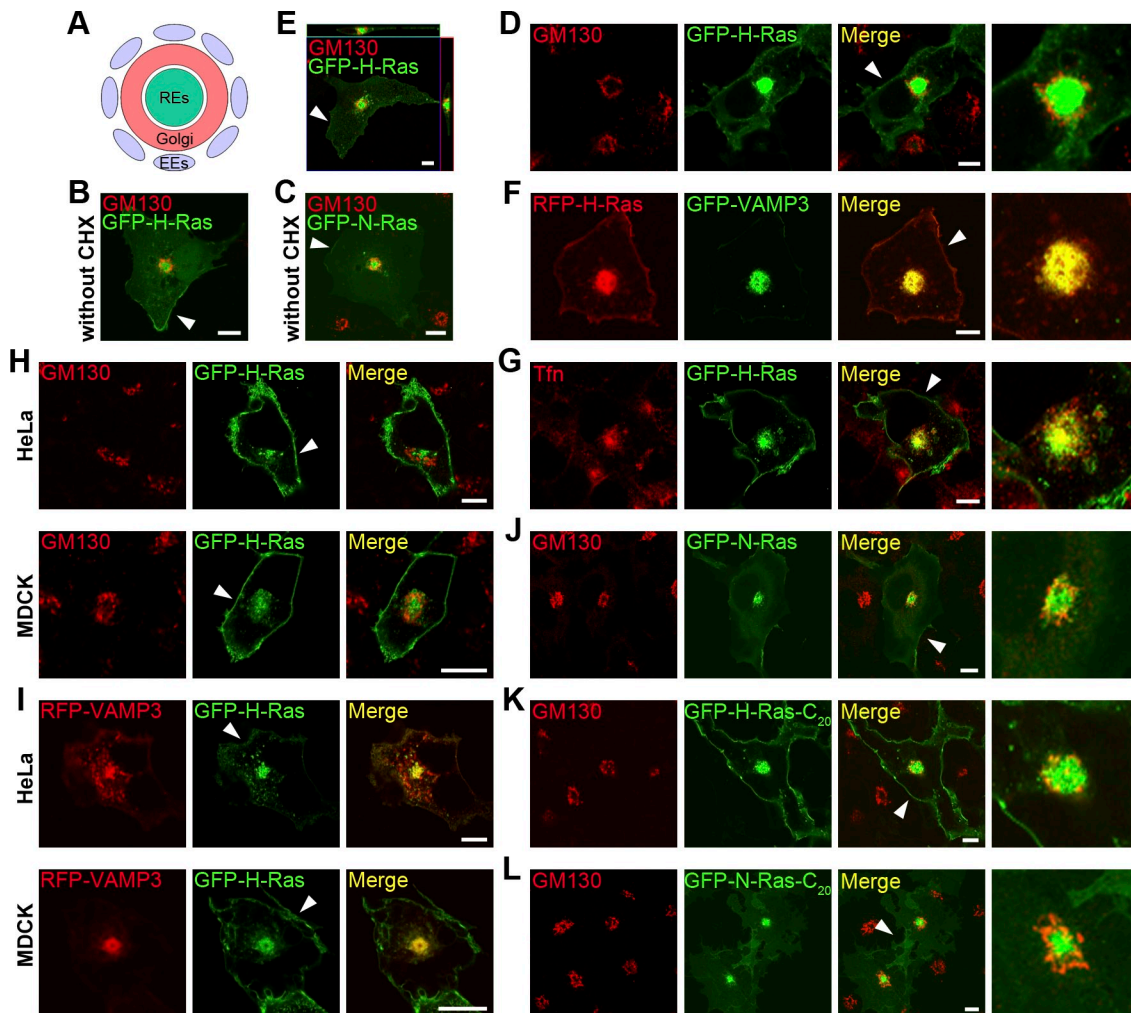
The discrepancy over the palmitoylated Ras proteins, such as their localization (Golgi or endosomes/REs) and their trafficking pathway to reach endomembranes from PM (by the

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Abbreviations used in this paper: 2BP, 2-bromopalmitate; CHX, cycloheximide; PM, plasma membrane; RE, recycling endosome.

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Figure 1. Steady-state distribution of palmitoylated Ras proteins at REs. (A) Schematic illustration of organelle distribution in COS-1 cells. Golgi exhibits a ring-shape appearance (Golgi ring), and REs are confined within Golgi ring. (B–L) The indicated proteins (B, D, E, G, and H, GFP–H-Ras; F, RFP–H-Ras and GFP-VAMP3; I, RFP-VAMP3 and GFP–H-Ras; C and J, GFP–N-Ras; K, GFP–H-Ras–C_{20}; L, GFP–N-Ras–C_{20}) were expressed overnight in COS-1 (B–G and J–L), MDCK, or HeLa cells (H and I). Cells were treated with (D–L) or without (B and C) CHX for 4 h. Cells in G were pulsed for 20 min with transferrin–Alexa Fluor 546 and chased for 10 min to highlight REs. GM130 was immunostained to localize Golgi when necessary. The arrowheads indicate PM. Bars, 10 µm.

conventional endocytic pathway or a pathway involving the cytosol), may arise from the low spatial resolution of organelles at perinuclear regions of many mammalian cells. We recently found that COS-1 cells are well suited for such localization experiments because of their unique spatial organization of organelles: REs are exclusively confined within the ring-shaped structure of Golgi (Golgi ring), and the organelles associated with degradation (early endosomes, late endosomes, and lysosomes) are excluded from inside Golgi ring (Misaki et al., 2007). In this study, we were able to (a) show that REs are the major intracellular compartments for active palmitoylated Ras proteins, (b) solve their entire trafficking pathway leading to PM, and (c) reveal essential roles of palmitoyl groups in RE localization.

Results and discussion

The steady-state distribution of palmitoylated Ras proteins was examined in COS-1 cells. The risk of using a protein synthesis inhibitor, cycloheximide (CHX), was first assessed because CHX is known to inhibit FKBP family of prolyl isomerases (Christner et al., 1999), which might cause any impact on palmitoylated Ras localizations. After overnight expression of Ras proteins followed by 4 h of incubation with 50 µg/ml CHX, we observed extensive endomembrane localizations of GFP–H- and GFP–N-Ras, which were similar to their localizations without CHX (Fig. 1, B and C). Therefore, we used CHX at 50 µg/ml throughout our study. GFP–H-Ras showed very little overlap with a Golgi resident protein, GM130 (Fig. 1, D and E; Pearson coefficient 0.20 ± 0.20). Instead, it was found exclusively within Golgi ring and at PM. The localization within Golgi ring implies its localization at REs, and this was confirmed by colocalization with two RE markers, VAMP3 (Pearson coefficient 0.90 ± 0.06; Fig. 1 F; McMahon et al., 1993; Daro et al., 1996) and transferrin (Pearson coefficient 0.65 ± 0.14; Fig. 1 G; Mellman, 1996; Maxfield and McGraw, 2004). Immunoelectron microscopy showed that H-Ras was mostly associated with tubulovesicular structures adjacent to Golgi, which are characteristic morphological features of REs (Fig. S1; Yamashiro et al., 1984). H-Ras was also found to localize
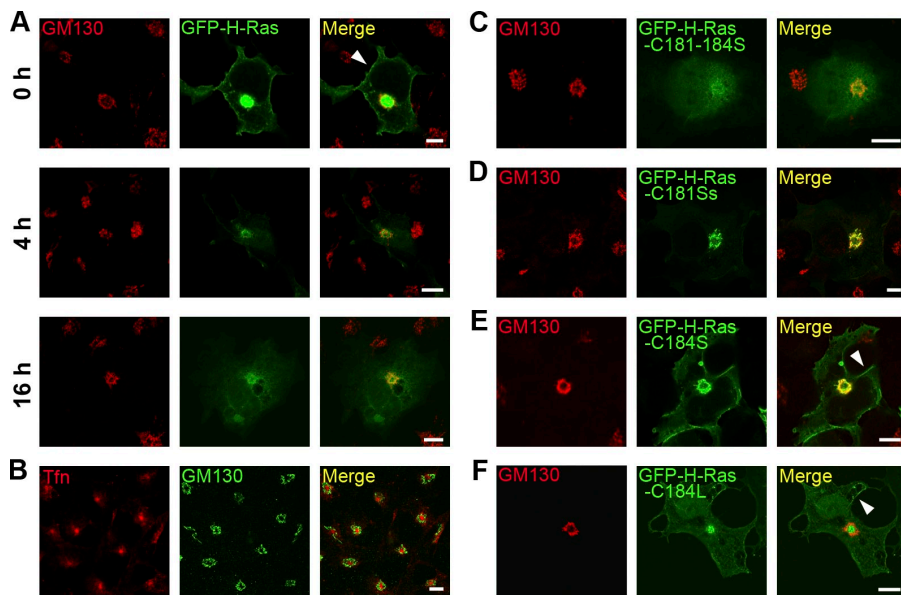
Figure 2. **The essential role of palmitoyl groups of H-Ras in RE targeting.** (A) GFP–H-Ras was expressed overnight. Cells were treated with 25 µM 2BP in the presence of CHX for the indicated times followed by fixation to immunostain GM130. (B) Transferrin–Alexa Fluor 546 was endocytosed to COS-1 cells, which had been treated with 2BP for 16 h. Cells were fixed to immunostain GM130. (C–F) The indicated palmitoyl-deficient mutant proteins (C, GFP–H-Ras–C181-184S; D, GFP–H-Ras–C181S; E, GFP–H-Ras–C184S; F, GFP–H-Ras–C184L) were expressed overnight. Cells were treated with CHX for 4 h followed by fixation to immunostain GM130. The arrowhead indicates PM. Bars, 10 µm.

at REs in CHO cells (Gomez and Daniotti, 2005). MDCK and HeLa cells also showed exclusive localization of GFP–H-Ras at REs/PM (Fig. 1, H and I). The fact that Flag–H-Ras also localized at REs rules out the possibility that the GFP tag interfered with the localization of H-Ras (Fig. S2). The localizations of GFP–N-Ras (Fig. 1 J) and Flag–N-Ras (Fig. S2) were similar to the localization of H-Ras. Furthermore, a 20–amino acid stretch at the C terminus of H-Ras or N-Ras that contains all of the lipid modifications (farnesylation and palmitoylation) was sufficient for their RE targeting (Fig. 1, K and L), demonstrating that H- and N-Ras targeting to REs is primarily lipid based.

To clarify the role of palmitoyl groups in RE localization, we first examined the effect of 2-bromopalmitate (2BP). 2BP inhibits delivery of H- and N-Ras to PM by preventing the repalmitoylation process, leading to Ras accumulation at ER and Golgi (Goodwin et al., 2005). We allowed GFP–H-Ras to be expressed overnight and treated cells with 2BP in the presence of CHX for various times before imaging (Fig. 2 A). During 4–16 h of 2BP treatment, the RE localization of GFP–H-Ras was gradually lost, and GFP–H-Ras started to show strong colocalization with GM130. The Pearson coefficient for colocalization, 0.73 ± 0.07, was significantly higher (P < 0.001) than the overlap seen with GM130 without 2BP. A comparison of the localization of GM130 and transferrin confirmed that cells still maintain a good resolution of Golgi and REs after 16 h of 2BP treatment (Fig. 2 B). Thus, we concluded that H-Ras without palmitoylation accumulates mostly at Golgi and not at REs. Second, to assess how two palmitoyl groups at C181 and C184 of H-Ras are involved in the localization to REs, we generated palmitoyl-deficient mutant proteins by introducing serine point mutations (Roy et al., 2005). The resulting constructs were expressed and localized. In good accord with the result of the 2BP treatment, a null palmitoylation mutant, GFP–H-Ras–C181-184S, did not localize to either REs or PM and accumulated mostly at Golgi with a little staining of ER (Fig. 2 C). A monopalmitoylation mutant, GFP–H-Ras–C181S, was exclusively found at Golgi, revealing that a palmitoyl group on C181 is essential for H-Ras to exit Golgi (Fig. 2 D).

The exclusive Golgi localization of this mutant was further confirmed by immunoelectron microscopy (Fig. S1). The other monopalmitoylation mutant, GFP–H-Ras–C184S, was found at Golgi and PM (Fig. 2 E). No obvious staining at REs was detected in GFP–H-Ras–C184S. The corresponding palmitoyl-deficient mutants tagged with Flag showed the same localization pattern as the GFP-tagged mutants (Fig. S2). The aforementioned localization data raise the counterintuitive question of how N-Ras with the single-palmitoyl group on C181 can access REs. We focused on L184 on N-Ras, which is known to be involved in the N-Ras membrane binding (Huster et al., 2003; Gorfe et al., 2004). A monopalmitoylated mutant, GFP–H-Ras–C184L, was thus generated and localized. This mutant was found at REs and PM (Fig. 2 F) with a great contrast to GFP–H-Ras–C184S localization (Fig. 2 E, Golgi and PM). These results revealed that both palmitoyl groups of H-Ras are required and sufficient for the RE localization and that the palmitoyl group on C184 can be replaced with L184. This finding also explains N-Ras localization to REs.

We next addressed how H-Ras reaches REs. Because a wealth of evidence has established that retrograde movement of palmitoylated Ras proteins from PM to endomembranes (ER/Golgi) is independent of the conventional endocytic pathway (Goodwin et al., 2005; Rocks et al., 2005), we examined the possibility that H-Ras exploits our recently discovered exocytic pathway that directly connects Golgi and REs (Ang et al., 2004). We used a low temperature (20°C) block/release protocol to synchronize post-Golgi trafficking of H-Ras. Treatment of cells with this low temperature is known to retard membrane traffic from Golgi and has been successfully used to examine the post-Golgi membrane traffic of many proteins delivered to PM (Griffiths et al., 1985; Ang et al., 2004). At 20°C with CHX, the RE/PM localization of GFP–H-Ras was gradually lost. GFP–H-Ras localized exclusively at Golgi after 1–2 h, as shown by its colocalization with GM130 (Fig. 3 A, 0 min just before the temperature rise), with little material left at PM. Cells were released from the 20°C block by raising the temperature to 37°C with CHX. After 2 h at 37°C, GFP–H-Ras showed an RE/PM localization (Fig. 3 A, 120 min)

Figure 3. **Post-Golgi trafficking of H-Ras through REs to PM.** (A and E) GFP–H-Ras and GFP–H-Ras–C184S expressed overnight are shown in A and E, respectively. Cells were subjected to 20°C block for 2 h with CHX. 20°C block was released with CHX for the indicated times followed by fixation to immunostain GM130. (right) Magnified images of boxed areas around the Golgi/REs area are shown. Bars, 10 µm. The arrowheads indicate PM. (B) Circles represent the Pearson coefficient between GFP–H-Ras and GM130 from the individual cells shown in A at 0 ($n = 50$) or 120 min ($n = 50$). *, P < 0.001. Error bars indicate mean ± SD. (C) GFP–H-Ras and RFP-Rab6a were coexpressed overnight. Cells were subjected to 20°C block for 2 h with CHX. Immediately upon shifting from 20 to 37°C, the Golgi/REs area was imaged every 10 min for 60 min. Bar, 5 µm. (D) GFP intensity in C was measured every 10 min in two areas: (1) Golgi plus REs (blue) and (2) REs (red). The results of four independent experiments are shown with different symbols. Individual GFP intensities are normalized to the GFP intensity from Golgi plus REs at 0 min.
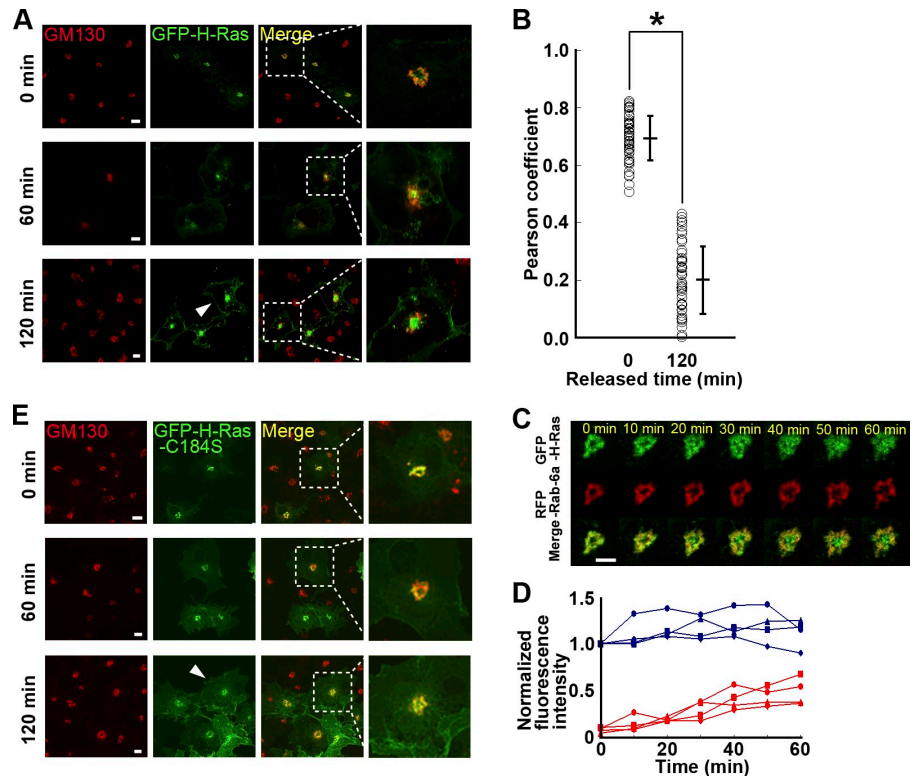
indistinguishable from the localization of GFP–H-Ras before the 20°C treatment, indicating that GFP–H-Ras reached a steady-state distribution. The colocalization between GFP–H-Ras and GM130 significantly decreased by 120 min (Pearson coefficient 0.69 ± 0.08 at 0 min, 0.20 ± 0.11 at 120 min; P < 0.001; Fig. 3 B). We noticed that GFP–H-Ras consistently appeared first at REs 30–60 min after the temperature rise, before prominently appearing at PM after 90–120 min. Flag–H-Ras showed the same localization behavior after the temperature rise (Fig. S2). We then analyzed the post-Golgi trafficking of H-Ras by live cell imaging. Cells were allowed to coexpress GFP–H-Ras and RFP-Rab6a (a Golgi marker) (Goud et al., 1990) and subjected to the 20°C block for 2 h. After the temperature rise, the Golgi/RE region was imaged every 10 min for 60 min to track the initial movement of H-Ras. A portion of GFP–H-Ras started to move centripetally from Golgi, leading to its accumulation at REs, whereas RFP-Rab6a stayed behind at Golgi (Fig. 3 C). GFP intensity was monitored in two areas: (1) Golgi plus REs (the whole area encircled with Golgi ring) and (2) REs, the area within Golgi ring (Fig. 3 D, blue and red curves, respectively). RFP-Rab6a was used as a reference to decide the boundary of Golgi. GFP intensity at REs progressively increased up to 60 min, whereas the total GFP intensity from Golgi plus REs was essentially constant. If H-Ras exploits a pathway involving PM to reach REs, we would expect to see a decrease of the intracellular GFP intensity (the combined fluorescence of Golgi plus REs) at some stage after the temperature rise. However, we did not see such a decrease. These observations indicate that GFP–H-Ras directly moves to REs after its egress from Golgi. We also analyzed the post-Golgi trafficking of GFP–H-Ras–C184S with the low temperature block/release protocol. During the chase up to 2 h at 37°C, we did not find any

noticeable RE localization of GFP–H-Ras–C184S (Fig. 3 E), indicating that its post-Golgi trafficking to PM is direct, not via REs. The exocytic trafficking pathways of palmitoylated Ras proteins delineated by this study are illustrated in Fig. 4.

Recent studies of palmitoylated Ras-mediated signaling reveal that Ras signaling is not limited to PM but also proceeds on endomembranes such as ER and Golgi membranes (Chiu et al., 2002; Bivona et al., 2003; Rocks et al., 2005; Roy et al., 2005; Daniels et al., 2006). Thus, it was of interest to determine whether H-Ras at REs is active. We compared H-Ras activities on different membranes by exploiting the fact that palmitoyl-deficient mutant proteins show distinct subcellular localizations. An RFP fusion protein (RFP fused to the Ras-binding domain of Raf1 that binds only to the active form of Ras, RFP-RBD) was used as a probe for the existence of active Ras proteins (Chiu et al., 2002). GFP–H-Ras recruited RFP-RBD to REs and PM, showing that H-Ras at REs is active (Fig. 5 A). This recruitment is specific to the expressed protein because GFP–H-Ras–C_{20}, which lacks a catalytic domain, did not recruit RFP-RBD either to REs or PM (Fig. 5 G). A palmitoyl-deficient mutant, GFP–H-Ras–C181S, failed to recruit RFP-RBD to Golgi (Fig. 5 B). The other mutant, GFP–H-Ras–C184S, also failed to recruit RFP-RBD to Golgi but not to PM (Fig. 5 C). In contrast, all of the corresponding constitutively active H-Ras proteins harboring the G12V mutation recruited RFP-RBD, irrespective of their intracellular localizations; GFP–H-RasG12V recruited RFP-RBD to REs and PM (Fig. 5 D), GFP–H-RasG12V–C181S recruited RFP-RBD to Golgi (Fig. 5 E), and GFP–H-RasG12V–C184S recruited RFP-RBD to Golgi and PM (Fig. 5 F). This result, together with the observation that a PM pool of GFP–H-Ras–C184S was also active, suggests that the lack of palmitoyl groups does not significantly
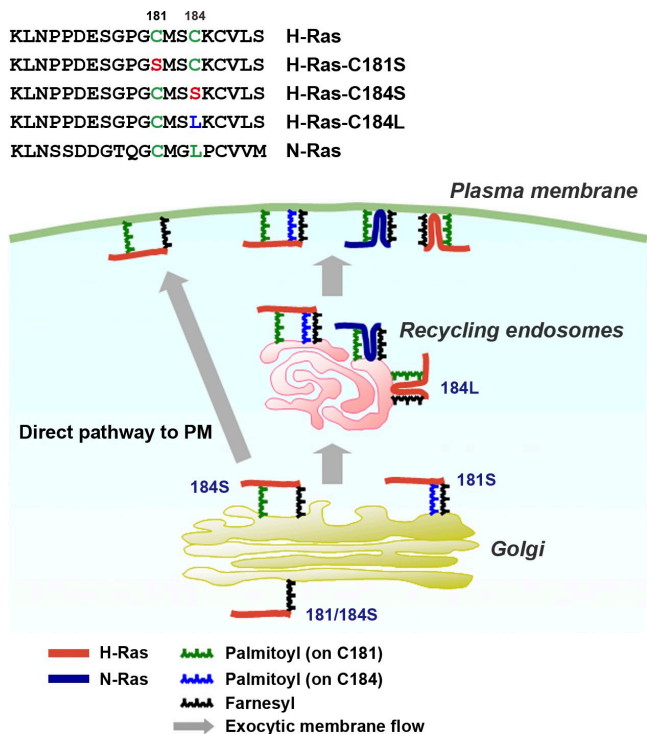
Figure 4. **Exocytic pathway of palmitoylated Ras proteins through REs to PM.** The sequences of C-terminal 20–amino acid stretch of Ras proteins and palmitoylation deficient mutants used in this study are shown. After the exit from Golgi, H-Ras is first transported to REs, then to PM. A palmitoyl group on C181 (green) is required to exit Golgi. Without the palmitoyl group on C184 (blue) upon the exit, mutant H-Ras (H-Ras–C184S) is directly transported to PM. L184 can substitute the palmitoyl group on C184 (H-Ras–C184L can access REs), which explains N-Ras residency on REs.

affect the activity of H-Ras. In summary, we found that H-Ras at REs is active, whereas H-Ras at Golgi is rather dormant.

These results unveil an intimate relationship between palmitoylated Ras proteins and REs (Fig. 4). The palmitoyl group on C181 is essential for H-Ras to exit Golgi; however, without the palmitoyl group on C184 upon the exit, mutant H-Ras is directly transported to PM. Therefore, two palmitoyl groups on C181/C184 appear essential for the correct loading of palmitoylated Ras onto post-Golgi membrane carriers for REs. We found that L184 can substitute the palmitoyl group on C184. In this case, one palmitoyl group on C181 and L184 is sufficient for the protein (H-Ras–C184L and N-Ras) loading to post-Golgi membrane carriers for REs. A recent study (Rocks et al., 2010) shows that palmitoylation is detectable only on Golgi, whereas depalmitoylation occurs everywhere in the cell. Based on our data presented in this study, we demonstrate that palmitoylation reaction on both C181 and C184 has to be completed before H-Ras exit from Golgi for the proper RE targeting of H-Ras. Whether depalmitoylation/repalmitoylation cycle also occurs on REs needs to be elucidated.

The trafficking pathway of palmitoylated Ras proteins leading to PM has been viewed as one involving ER and Golgi (Choy et al., 1999; Apolloni et al., 2000). Our results now demonstrate that the pathway should be revised as ER → Golgi → REs → PM. There is increasing evidence that REs have an important role in the exocytic pathway in different cell types and for different secretory cargos (Ang et al., 2004; Murray et al., 2005). This study suggests

that REs can also serve as an essential way station for cytoplasmically facing palmitoylated proteins on their way to the PM.

The endomembrane pool of palmitoylated Ras proteins has been shown to be separated from the PM pool of palmitoylated Ras proteins with respect to distinct activity profiles, different preferences for downstream signaling pathways, and different roles in cellular proliferation, differentiation, and apoptosis. For example, Ras activation by mitogens is rapid and transient at PM (a few-min onset and 10-min peak) but delayed and sustained (>60 min) at endomembranes (Chiu et al., 2002). Endomembrane (Golgi)-restricted mutant of H-Ras61L, KDELR–H-Ras61L, is potent in its ability to stimulate neurite outgrowth in PC12 cells and is equivalent to natively targeted H-Ras61L (Bivona et al., 2003). During thymic selection of T cells, positive and negative selecting ligands coupled with T cell antigen receptor target early key signal intermediates into distinct subcellular compartments: a positive selector recruits RasGRP1 (Ras-GEF)/Ras/Raf1 to Golgi, whereas negative selectors recruit this signaling complex exclusively to PM (Daniels et al., 2006). Two models have been proposed to account for the activation of palmitoylated Ras proteins on endomembranes (Quatela and Philips, 2006; Rocks et al., 2006). Quatela and Philips (2006) argue that an endomembrane pool of Ras itself becomes activated by Ras-GEF, which is recruited from the cytosol to endomembranes upon mitogen stimulation. Rocks et al. (2006) argue that activated Ras from PM is retrogradely transported to endomembranes via the cytosol. These two models are not necessarily mutually exclusive. In this study, we propose a third model, based on the observation that active palmitoylated Ras proteins mainly localize at REs in COS-1 cells. REs are essential for the recycling of internalized receptors to PM (Mellman, 1996; Maxfield and McGraw, 2004). Activated mitogen receptors at PM were previously believed to be uniformly delivered to lysosomes for degradation, but a recent study reveals that this is not always the case (Sigismund et al., 2008). Activated EGF receptor, if it is endocytosed by the clathrin-mediated pathway, is not targeted for degradation but, instead, is recycled back to PM through REs, which prolongs the duration of signaling (Sigismund et al., 2008). Transferrin and transferrin receptors appear at REs ∼10 min after their uptake from PM, remain at REs for 30 min, and gradually recycle back to PM (Misaki et al., 2007). EGFRs stay >1 h intracellularly and recycle back to PM under conditions in which the clathrin-mediated pathway dominates (Sigismund et al., 2008). These kinetics match well with the profile of H-Ras activation at endomembranes (Chiu et al., 2002). Thus, our model proposes that activated mitogen receptors at PM are delivered to REs and that they can directly activate palmitoylated Ras proteins in situ. We emphasize that this model is not necessarily mutually exclusive with the other two models.

It is noteworthy that RalA, a Ras effector, localizes at REs together with the exocyst complex and that they are involved in membrane traffic through REs to PM (Chen et al., 2006). Another Ras effector, Rho GTPase Cdc42, which is involved in membrane traffic/actin regulation (Kroschewski et al., 1999; Ang et al., 2003), is also found mainly at REs (Fig. S3). Therefore, we propose that Ras-mediated signaling from REs is connected tightly through Ras effectors to a variety of membrane trafficking pathways through REs, such as the recycling, exocytic,

Figure 5. **H-Ras is active at REs.** (A–G) The indicated proteins (A, GFP-H–Ras; B, GFP–H-Ras–C181S; C, GFP–H-Ras–C184S; D, GFP–H-RasG12V; E, GFP–H-RasG12V–C181S; F, GFP–H-RasG12V–C184S; G, GFP–H-Ras–C20) were expressed overnight together with RFP-RBD. Cells were treated with CHX for 4 h. A fluorescence intensity line scan profile was generated along the yellow line, which goes through the Golgi/REs area and shown is in the right column. The arrowheads indicate PM. Bars, 10 µm.
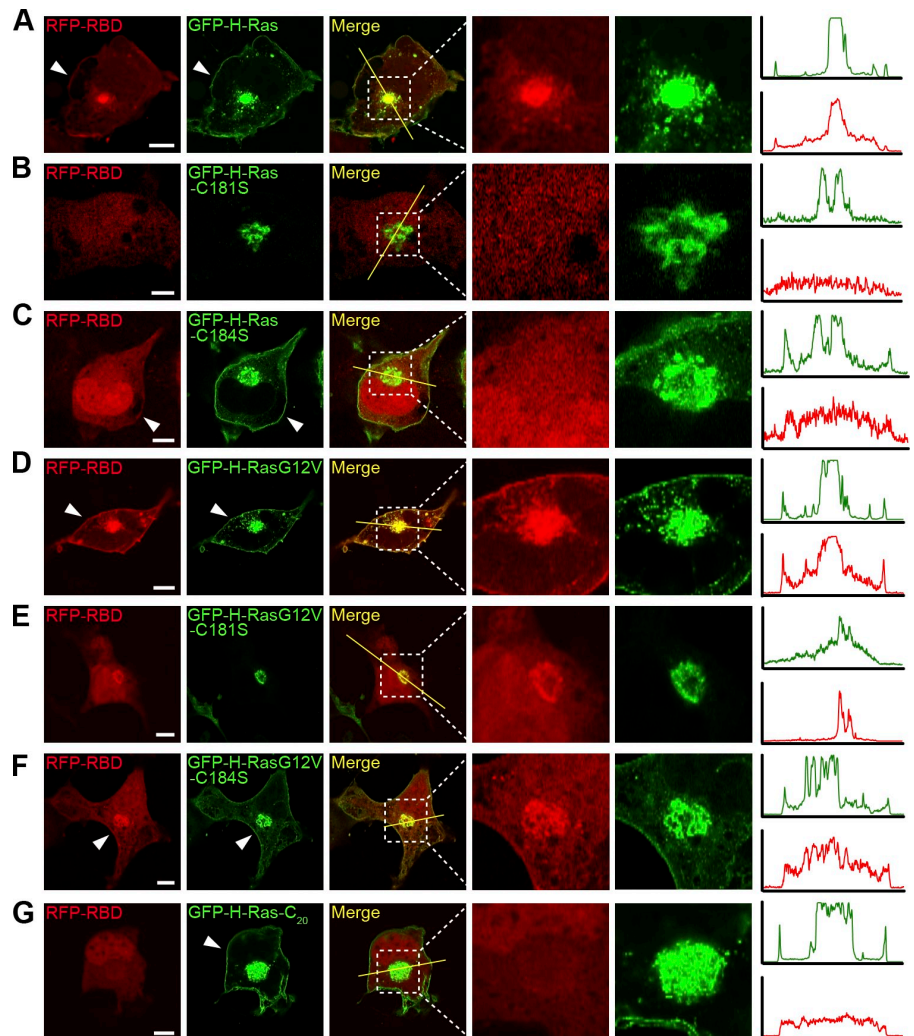
and retrograde pathways. Ras activation at REs, as we proposed in the previous paragraph, may also be regulated by recycling membrane traffic, which delivers activated mitogen receptors from PM to REs.

Materials and methods

Plasmids
Either pCAGGS or pCXN2 vector was used for Ras protein expression. Human VAMP3 cDNA was introduced to pEGFP-C3 vector. Mouse Rab6a cDNA was provided by M. Fukuda (Tohoku University, Sendai, Japan). Constitutively active H-Ras proteins (GFP–H-RasG12V, GFP–H-RasG12V–C181S, and GFP–H-RasG12V–C184S) were provided by J. Hancock (University of Queensland, Brisbane, Queensland, Australia).

Cells and reagents
COS-1, MDCK, and HeLa cells were cultured in 5% CO_2 at 37°C in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal calf serum. Cells grown on µ-dishes (Ibidi) were used for transfection with transfection reagent (FuGENE 6; Roche). The following reagents were purchased from the manufacturers as noted: mouse anti-GM130 monoclonal antibody (BD), sheep anti-TGN46 antibody (AbD Serotec), rabbit anti-Flag epitope antibody (Thermo Fisher Scientific), rabbit anti-GFP antibody (Abcam), goat anti–mouse IgG Alexa Fluor 546, donkey anti–mouse IgG Alexa Fluor 568, donkey anti–rabbit IgG Alexa Fluor 488, donkey anti–sheep IgG Alexa Fluor 555 (Invitrogen), human holo-transferrin, CHX, and 2BP (Sigma-Aldrich). Transferrin–Alexa Fluor 546 was prepared with the aid of Alexa Fluor 546 carboxylic acid succinimidyl ester (Invitrogen).

Transferrin uptake
Cells were starved in serum-free medium at 37°C for 30 min and pulsed for 20 min in serum-free medium with 10 µg/ml transferrin–Alexa Fluor 546. Cells were chased with 20 µg/ml transferrin at 37°C for 10 min.

Immunofluorescence
Cells were fixed with 4% paraformaldehyde in PBS at room temperature for 20 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min, washed twice with PBS, and treated with blocking buffer (5% bovine serum albumin in PBS) at room temperature for 30 min. Cells were incubated with primary antibodies diluted in blocking buffer (1:400 for anti-TGN46 antibody and 1:100 for the others) at 37°C for 30 min. After washing with PBS three times, cells were incubated with Alexa Fluor–conjugated secondary antibodies diluted in blocking buffer (1:400) at 37°C for 30 min. Cells were washed with PBS three times and mounted with aqueous mounting medium (PERMAFLUOR; Beckman Coulter).

Confocal microscopy
Confocal microscopy on fixed cells was performed at 25°C using a laser-scanning microscope in PASCAL (LSM 5; Carl Zeiss, Inc.) with a 63× 1.4 NA Plan Apochromat oil immersion lens. Excitations were performed with a 30-mV argon laser emitting at 488 nm and with a 1.0 mW helium/neon laser emitting at 543 nm. Emissions were collected using a 505–530-nm bandpass filter for Alexa Fluor 488 and GFP and a LP560 filter for Alexa Fluor 546, 555, 568, and RFP.

Live cell imaging
GFP–H-Ras and RFP-Rab6a were coexpressed overnight, and 20°C was applied to cells for 2 h in the presence of CHX. Cells were imaged every 10 min for 60 min immediately upon shifting from 20 to 37°C with an inverted microscope (IX81; Olympus) with a 60× 1.35 NA UPlan SApo

oil immersion lens. Excitation was performed with a 30-mW multi-Argon laser emitting at 488 nm and with a 10 mW helium/neon laser emitting at 543 nm. Emissions were collected using a 500–545-nm bandpass filter for GFP and a 570–670-nm bandpass filter for RFP.

Fluorescent image analysis
Quantitation of images was performed with ImageJ software (National Institutes of Health). The Pearson coefficient was determined with a JACoP plugin and expressed as mean ± SD ($n > 30$).

Statistical significance evaluation
Statistical significance was evaluated with Student's t test (Prism; GraphPad Software, Inc.). $P < 0.001$ was considered significant.

Online supplemental material
Fig. S1 shows localization of GFP–H-Ras and the palmitoylation-deficient mutant GFP–H-Ras–C181S by immunoelectron microscopy. Fig. S2 shows localization of Flag-tagged Ras proteins (Flag–H-Ras, Flag–H-Ras–C181-184S, Flag–H-Ras–C181S, Flag–H-Ras–C184S, and Flag–N-Ras) and the post-Golgi trafficking of Flag–H-Ras after release of low temperature block. Fig. S3 shows RE localization of GFP- and Flag-Cdc42. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200911143/DC1.

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