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Cis-Golgi Matrix Proteins Move Directly to Endoplasmic Reticulum Exit Sites by Association with Tubules[V]

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The role of *cis*-medial Golgi matrix proteins in retrograde traffic is poorly understood. We have used imaging techniques to understand the relationship between the *cis*-medial Golgi matrix and transmembrane proteins during retrograde traffic in control and brefeldin A (BFA)-treated cells. All five of the *cis*-medial matrix proteins tested were associated with retrograde tubules within 2–3 min of initiation of tubule formation. Then, at later time points (3–10 min), transmembrane proteins are apparent in the same tubules. Strikingly, both the matrix proteins and the transmembrane proteins moved directly to endoplasmic reticulum (ER) exit sites labeled with p58 and Sec13, and there seemed to be a specific interaction between the ER exit sites and the tips or branch points of the tubules enriched for the matrix proteins. After the initial interaction, Golgi matrix proteins accumulated rapidly (5–10 min) at ER exit sites, and Golgi transmembrane proteins accumulated at the same sites ~2 h later. Our data suggest that Golgi *cis*-medial matrix proteins participate in Golgi-to-ER traffic and play a novel role in tubule formation and targeting.

INTRODUCTION

The Golgi complex in mammalian cells is made up of stacked cisternae organized in a ribbonlike structure within a ribosome-free Golgi matrix. The membranes of the cisternae contain many enzymes involved in posttranslational modifications of proteins and lipids as well as molecules involved in sorting of transiting components to their sites of function (Palade, 1975). The matrix is less well defined but has been the focus of a great deal of recent work (reviewed in Shorter and Warren, 2002; Barr and Short, 2003; Gillingham and Munro, 2003). The matrix is composed of an ever-increasing number of coiled-coil proteins, many of which were first identified as autoantigens and termed Golgins (Fritzler and Salazar, 1991; Barr and Short, 2003; Nozawa *et al*., 2005). Matrix proteins interact with the cisternal transmembrane proteins and lipids and provide adhesion of cisternae, tethers for docking of trafficking vesicles and may in some way integrate with microtubules to function in the orientation of the mammalian Golgi at the microtubule organizing center (Barr and Short, 2003; Barr and Egerer, 2005). Golgi matrix proteins are often associated with and activated by small GTPases of the Rab and Arl families (Barr and Short, 2003; Panic *et al*., 2003). In addition, many Golgi matrix proteins are phosphorylated or *N*-myristoylated (Barr *et al*., 1997, 1998; Nakamura *et al*., 1997), but in general the functional significance of their posttranslational modification has not been well established. Originally, Golgi matrix proteins were thought to be stably associated with the matrix once synthesized, but increasing evidence suggests they are dynamic. Fluorescence recovery after photobleaching data show that some *cis*-Golgi matrix proteins rapidly exchange with the cytosol (Ward *et al*., 2001), which suggests they function to continuously proof read or resort (García-Mata *et al*., 2003). Others have shown that *cis*-Golgi matrix proteins are present in tubules and vesicles moving in the anterograde direction from ER to Golgi (Marra *et al*., 2001).

The dynamic nature of Golgi transmembrane proteins has been extensively studied after various perturbations, especially brefeldin A (BFA) (Lippincott-Schwartz *et al*., 1990, 2000; Yang and Storrie, 1998). BFA has been shown to prevent guanine nucleotide exchange factor activation of ADP-ribosylation factor 1 and COPI assembly resulting in Golgi transmembrane proteins moving back to the ER via tubules (Lippincott-Schwartz *et al*., 1990; Orci *et al*., 1991; Donaldson *et al*., 1992a,b; Peyroche *et al*., 1999; Spang, 2002). The effect of BFA on the Golgi matrix is more controversial. Some evidence suggests that matrix proteins distribute to BFA remnants that are distinct from the ER and nucleate the assembly of the Golgi cisternae under various conditions (Seemann *et al*., 2000). In contrast, Lippincott-Schwartz and colleagues have concluded that after BFA treatment, Golgi stackletts reassemble at ER exit sites in the absence of a preassembled *cis*-Golgi matrix (Ward *et al*., 2001). In both of these studies, movement of the *cis*-Golgi matrix molecules from the Golgi to the ER was not clearly resolved. We observed *cis*-Golgi matrix proteins in tubules both in control and BFA-treated cells and decided to analyze these processes in more detail.

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Abbreviations used: BFA, brefeldin A; GalT, galactosyltransferase; Man-II, mannosidase II.

In this study, we followed the movement of *cis*-medial Golgi matrix proteins both in fixed cells and in vivo. Fluorescently tagged matrix and Golgi transmembrane proteins were followed to understand the interactions between these two classes of molecules in recycling between the Golgi and ER. We found that in the presence of BFA, five *cis*- and medial-Golgi matrix proteins move to the ER associated with the same tubules as transmembrane proteins. The tips of these retrograde tubules are enriched in matrix proteins at early time points followed at later times by enrichment of the transmembrane proteins. At later time points, the transmembrane and matrix proteins do not colocalize and seem to sort into alternative domains within the same tubule. Surprisingly, the tubules move directly to ER exit sites, and once attached, the tubules collapse into the ER exit sites. This process delivers a wide range of Golgi matrix proteins followed by transmembrane proteins to the ER. We encourage the reader to view the videos, because the data are best seen in this format.

MATERIALS AND METHODS

DNA Constructs and Cell Transfection

The following plasmids were used: GalT-CFP (Zaal *et al*., 1999) and Sec13-YFP and p58-YFP (Ward *et al*., 2001; J. Lippincott-Schwartz, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD); GRASP55-GFP and GRASP65-GFP (Barr *et al*., 1998; Shorter *et al*., 1999; F. A. Barr, Max-Planck-Institute of Biochemistry, Martinsried, Germany); p115-YFP (E. Sztul, University of Alabama Medical School, Birmingham, AL); and GM130-CFP and GM130-YFP (Marra *et al*., 2001; A. De Matteis, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy).

Normal rat Kidney (NRK) cells were grown in DMEM supplemented with 10% fetal calf serum, glutamine, penicillin, and streptomycin (Invitrogen, Carlsbad, CA). Transient transfections were performed using Lipofectamine 2000 transfection reagent (Invitrogen) according to the manufacturer's instructions. Stable cell lines were produced by selection for G418 (Geneticin) resistance (at 500 μg/ml; Invitrogen) expressed by the appropriate plasmid.

Cell Reagents and Immunofluorescence

BFA (Sigma-Aldrich, St. Louis, MO) was used at 5 μg/ml for different incubation times before either fixation or in vivo visualization. Monoclonal antibodies against the following antigens were used: GM130 and GRASP55 (BD Transduction Laboratories, San Diego, CA), rat mannosidase II (Man-II) (Covance, Berkeley, CA), Giantin (Linstedt and Hauri, 1993), β-COP, clone M3A5 (Duden *et al*., 1991), β-tubulin (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom), and GalNAc-transferase 1 and GalNAc-transferase 2 (Mandel *et al*., 1999) (from U. Mandel, University of Copenhagen, Nørre Alle, Denmark). Polyclonal antibodies against the following antigens were used: human mannosidase II (Chemicon International, Temecula, CA), rat mannosidase II (Carbohydrate Research Center, University of Georgia, Athens, GA), p115 (Barroso *et al*., 1995), GM130 (Nelson *et al*., 1998), GRASP65 (from G. Warren, Yale University School of Medicine, New Haven, CT), p58 (Hendricks *et al*., 1991), and Sec23 (Affinity Bioreagents, Golden, CO). Fluorescently labeled secondary antibodies, anti-mouse Alexa Fluor-488 and -594, and anti-rabbit Alexa Fluor-488 and -594 were from Invitrogen. Samples were prepared for immunofluorescence analysis as described previously (Wu *et al*., 2004). Fixation of cells with methanol or 4% paraformaldehyde gave virtually the same results; however, tubular profiles were best preserved in cells fixed with paraformaldehyde. Images were obtained with an inverted Zeiss Axiovert 200M deconvolution microscope (Carl Zeiss, Thornwood, NY) used with a 63× 1.4 numerical aperture (NA) Zeiss Plan apochromat oil immersion objective and processed using SlideBook acquisition software without deconvolution of the images (Intelligent Imaging Innovations, Denver, CO). Three hundred cells were counted for each experiment.

Time-Lapse Imaging and Microscopy

Live cells were held at 37°C on a microscope heater stage (Warner Instruments, Hamden, CT), and imaging was performed using an inverted Olympus IX81 microscope (Olympus America, Melville, NY) with an attached Hamamatsu ORCA IIER monochromatic charge-coupled device camera (Hamamatsu, Bridgewater, NJ), and images were captured with a 60× 1.4 NA Olympus oil immersion objective using SlideBook acquisition software (Intelligent Imaging Innovations). Filter sets were from Chroma Technology (Brattleboro, VT). Images were processed and converted to QuickTime movies with MetaMorph software (Molecular Devices, Sunnyvale, CA). To prepare figures, single frames were processed with Adobe Photoshop 7 (Adobe Systems, Mountain View, CA).

RESULTS

Golgi Matrix Proteins Are Redistributed to ER Exit Sites Associated with Tubules in BFA-treated Cells

During BFA treatment, the COPI coat complex dissociates from Golgi membranes, and *cis*- and medial-Golgi-resident transmembrane proteins are redistributed to the ER via long tubules (Klausner *et al*., 1992). In contrast, *cis*- and medial-matrix proteins are reported to occur in peripheral structures after 30 min (Seemann *et al*., 2000; Ward *et al*., 2001; Kasap *et al*., 2004). Our initial findings showed that Golgi matrix proteins redistributed in tubules within 2 min of BFA treatment (5 μg/ml) as shown by immunolocalization of an endogenous transmembrane protein, mannosidase II, and the matrix protein GRASP65 in time-course experiments (Figure 1). Within the first 2 min, tubules positive for GRASP65 emerge from the Golgi. These initial tubules do not contain Man-II (Figure 1, BFA, 2 min). After 2 min, approximately half of the cells contain a single GRASP65-positive tubule with lengths ranging from 2 to 10 μm. Between 5 and 10 min after BFA treatment, Man-II starts to emerge in the same tubules, and these tubules extend up to 35 μm from the Golgi. By 15 min, all cells contain branched tubules emanating from the Golgi that are positive for both transmembrane and matrix proteins. By 45 min, GRASP65 is accumulated at puncta similar to ER exit sites, whereas Man-II is more widely distributed. Surprisingly, at late time points (150 min), both matrix and transmembrane proteins colocalize at the peripheral puncta. These puncta are close to and overlapping with ER exit sites at 150 min, as demonstrated by colocalization of another *cis*-Golgi matrix protein, GM130, and Sec23, a COPII subunit (Figure 2). As a control, after 45 min of BFA treatment, additional BFA (at the same concentration) was added at 45-min intervals up to 5 h without any change in the distribution of the markers. Similar data were obtained with medial-matrix (GRASP55) and transmembrane (Man-II) proteins (Figure 3).

Other *cis*-Golgi matrix proteins show similar dynamics as GRASP65 and GRASP55 after BFA treatment. Figure 4 shows the dynamics of GM130 (Figure 4A); Giantin, a transmembrane matrix protein (Figure 4B); and p115 (Figure 4C); and a comparison between the *cis*-Golgi matrix protein GRASP65 and the medial-Golgi matrix protein GRASP55 (Figure 4D). Tubules positive for the matrix proteins extend from the Golgi between 2 and 7.5 min after BFA treatment and by 45 and 150 min they are distributed at ER exit sites. The best colocalization was observed with GM130-GRASP65 (Figure 4A), as might be predicted because GRASP65 has been shown to form a very stable complex with GM130 in vitro, although it may not be responsible for the in vivo association of GM130 with the *cis*-Golgi membranes (Barr *et al*., 1998; Sütterlin *et al*., 2005). The staining of p115 is slightly different. It is discontinuous along the length of the tubule (Figure 2C, 7.5 min); in almost all of the cells, the p115 is in small (2–7 μm) tubulovesicular profiles radiating from the juxtanuclear region to the periphery of the cells. Often these small tubules were connecting ER exit sites identified by Sec23 localization (our unpublished data). For p115, this staining pattern was stable for at least 45 min, suggesting that it remains in tubular form longer than the other matrix proteins. Together, these data suggest both Golgi matrix proteins and Golgi transmembrane proteins move in association with tubules after BFA treatment, with the *cis*-medial matrix proteins localized to the tip of the tubule. Addition-

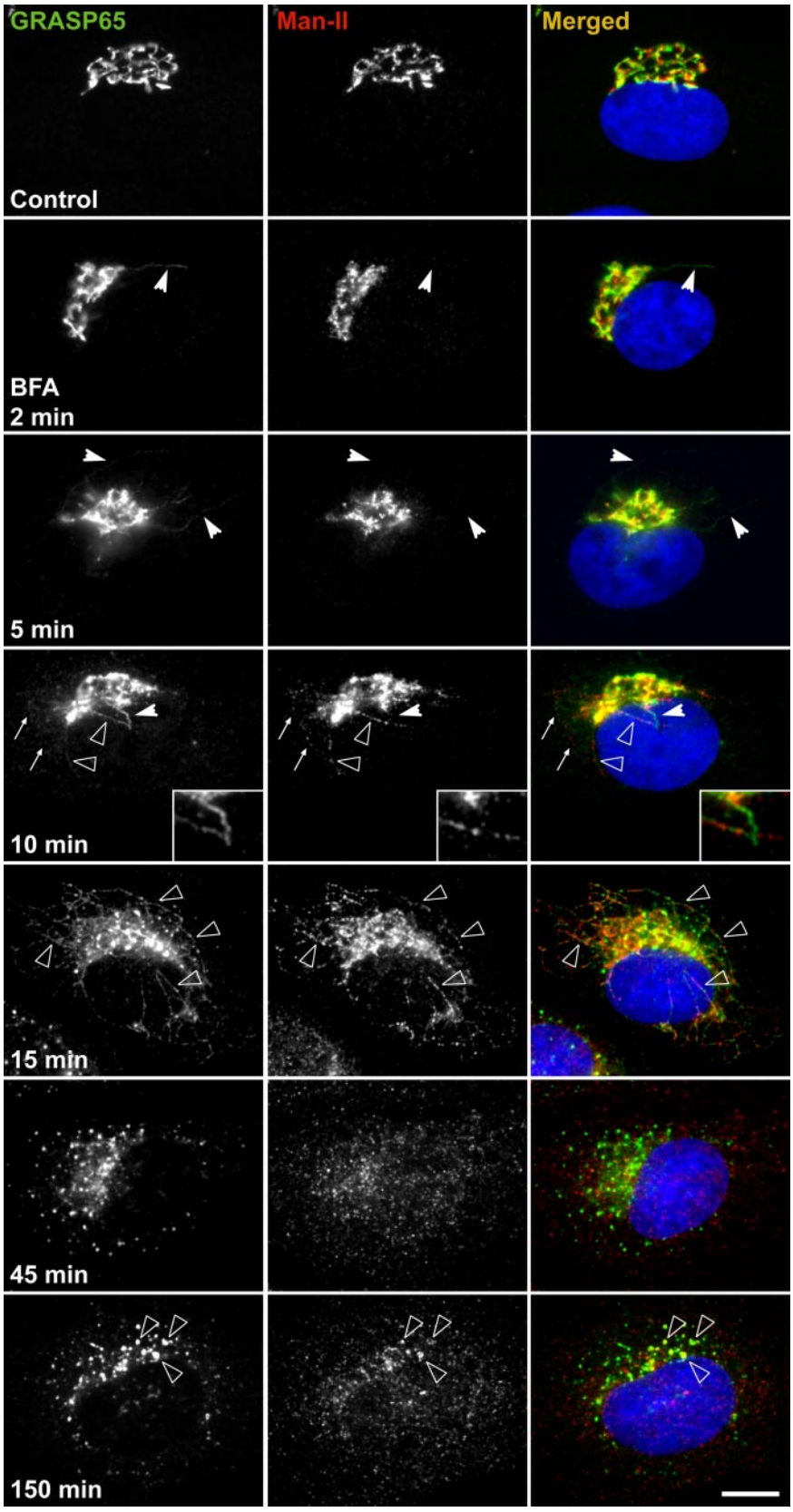

Figure 1. Time course of the *cis*-Golgi matrix protein GRASP65 and mannosidase II tubulation in BFA-treated cells. NRK cells were untreated (control) or treated with 5 μg/ml BFA for the times shown in the left panel (from 2 to 150 min). Cells were fixed and processed for immunolabeling with antibodies against GRASP65 (left) and Man-II (middle) and merged (right). Alexa Fluor-488 and Alexa Fluor-594 were used as fluorescent secondary probes. At 2 min of BFA treatment, 70% of the cells extend a single tubule containing exclusively GRASP65 emerging from the Golgi (white arrowheads). By 5 min, multiple tubules extended up to 35 μm from the Golgi and contained both GRASP65 and Man-II, whereas 10% remained only GRASP65 positive. By 10 min 50% of the cells contained three or more tubules with both GRASP65 and Man-II (outlined arrowheads), whereas other tubules contained only GRASP65 (white arrowheads and enlargement) or only Man-II (arrows). The boxed regions are shown at higher magnification in the lower right corner. By 15 min, only 15% of the cells retained tubules, and these contained both GRASP65 and Man-II (outlined arrowheads). By 45 min, all cells showed complete redistribution; GRASP65 at or in proximity to puncta, which resembled ER exit sites, whereas Man-II was redistributed throughout the ER. By 150 min, in 30% of the cells Man-II started to accumulate at the same regions as GRASP65 (outlined arrowheads). Bar, 10 μm.

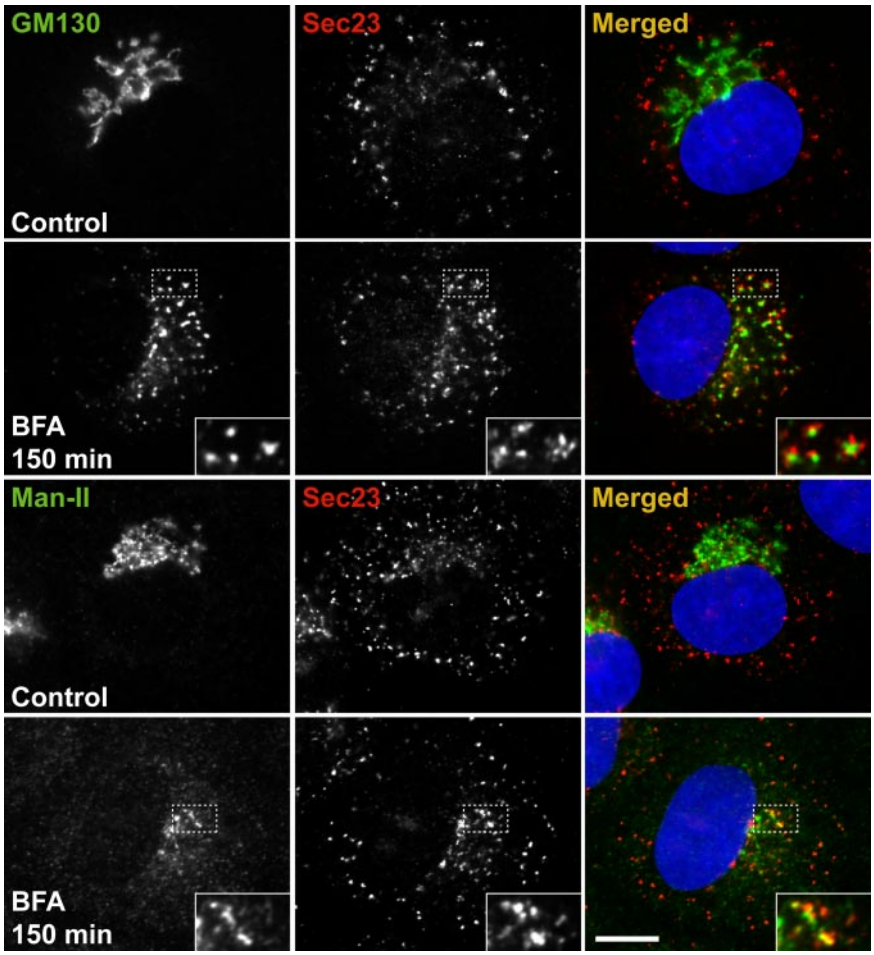

Figure 2. Mannosidase II accumulates at ER exit sites at prolonged incubation with BFA. NRK cells were untreated (control) or treated with 5 μg/ml BFA for 150 min. Cells were fixed and processed for immunolabeling with antibodies against GM130 and Man-II (left), Sec23 (middle), and merged (right). Alexa Fluor-488 and Alexa Fluor-594 were used as fluorescent secondary probes. A hundred percent of the cells positive for GM130 (top two panels) and 30% of the cells positive for Man-II (bottom two panels) showed accumulation of these proteins in puncta after 150 min of BFA treatment. All GM130- or Man-II-positive puncta are near and/or at ER exit sites, defined by colocalization with Sec23, a component of the COPII coat (middle and merged images on right). Boxed regions are shown at higher magnification in the lower right corner. Bar, 10 μm.

ally, both transmembrane and matrix proteins accumulate at ER exit sites by 150 min.

Finally, as expected, Golgi matrix-positive, BFA-induced tubules were sensitive to microtubule-disrupting agents (33 μM nocodazole), energy poisons (50 mM 2-deoxy-D-glucose, and 0.02% sodium azide for 3 min before BFA addition), and reduced temperatures (16°C) (our unpublished data), similar to the sensitivity previously characterized for tubules containing Golgi-resident enzymes (Lippincott-Schwartz *et al*., 1990; Klausner *et al*., 1992).

These experiments using endogenous proteins demonstrate that *cis*-medial matrix proteins associate with tubules and ultimately localize at ER exit sites with Golgi transmembrane proteins. In vivo imaging of expressed forms of *cis*-Golgi matrix and transmembrane proteins tagged with different variants of green fluorescent protein (GFP) were used to understand the dynamics of this process.

In Vivo Dynamics of Golgi Matrix Tubules

To evaluate the dynamics of Golgi matrix and Golgi transmembrane proteins, differentially fluorescently tagged molecules were followed in vivo. After transfection, these molecules were studied by live cell imaging in untreated NRK cells or after treatment with 5 μg/ml BFA. Previous studies using similar techniques failed to observe Golgi matrix proteins moving in tubules, instead proposing that they "relocate abruptly to ER exit sites" (Ward et al., 2001). Functional assays have shown that the GFP-tag has no effect on the localization or activity of these proteins (Barr *et al*., 1998; Marra *et al*., 2001; Ward *et al*., 2001; Bevis *et al*., 2002; Niu *et al*., 2005).

Time-lapse images in untreated cells expressing GRASP55-GFP show, in addition to the Golgi localization, highly mobile peripheral puncta. Retrograde tubules extending from the Golgi containing GRASP55-GFP are observed concentrated around the Golgi and distributed throughout the cytoplasm in control cells (Figure 5A and Video 1). Similar data have been reported for anterograde GM130-GFP-positive tubules (Marra *et al*., 2001). In BFA-treated cells, tubules extend from the Golgi within 2–4 min (Figure 5B). These GRASP55-GFP-positive tubules extend toward the periphery of the cell and are visible for 6–14 min before "blinking out" (9:46 min in Figure 5B and Video 2). The blink out is assumed to be the point when the tubules fuse with a larger compartment such as the ER or when a dynamic protein becomes cytoplasmic (Sciaky *et al*., 1997). After the blink out, the peripheral structures increase in fluorescence (11:19 min in Figure 5B), whereas the Golgi-localized fluorescence is diminished. The peripheral GRASP55-GFP structures seem small and are very dynamic, transiently emerging from and rapidly retracting back into these peripheral structures (~30 s for the extension-retraction; Video 2). Because BFA is reported to not directly perturb COPII-dependent ER export (Orci *et al*., 1993; Lippincott-Schwartz *et al*., 2000), this behavior may reflect a novel dynamic function within ER exit sites. Similar tubulation was observed in BFA-treated cells expressing GRASP65-GFP

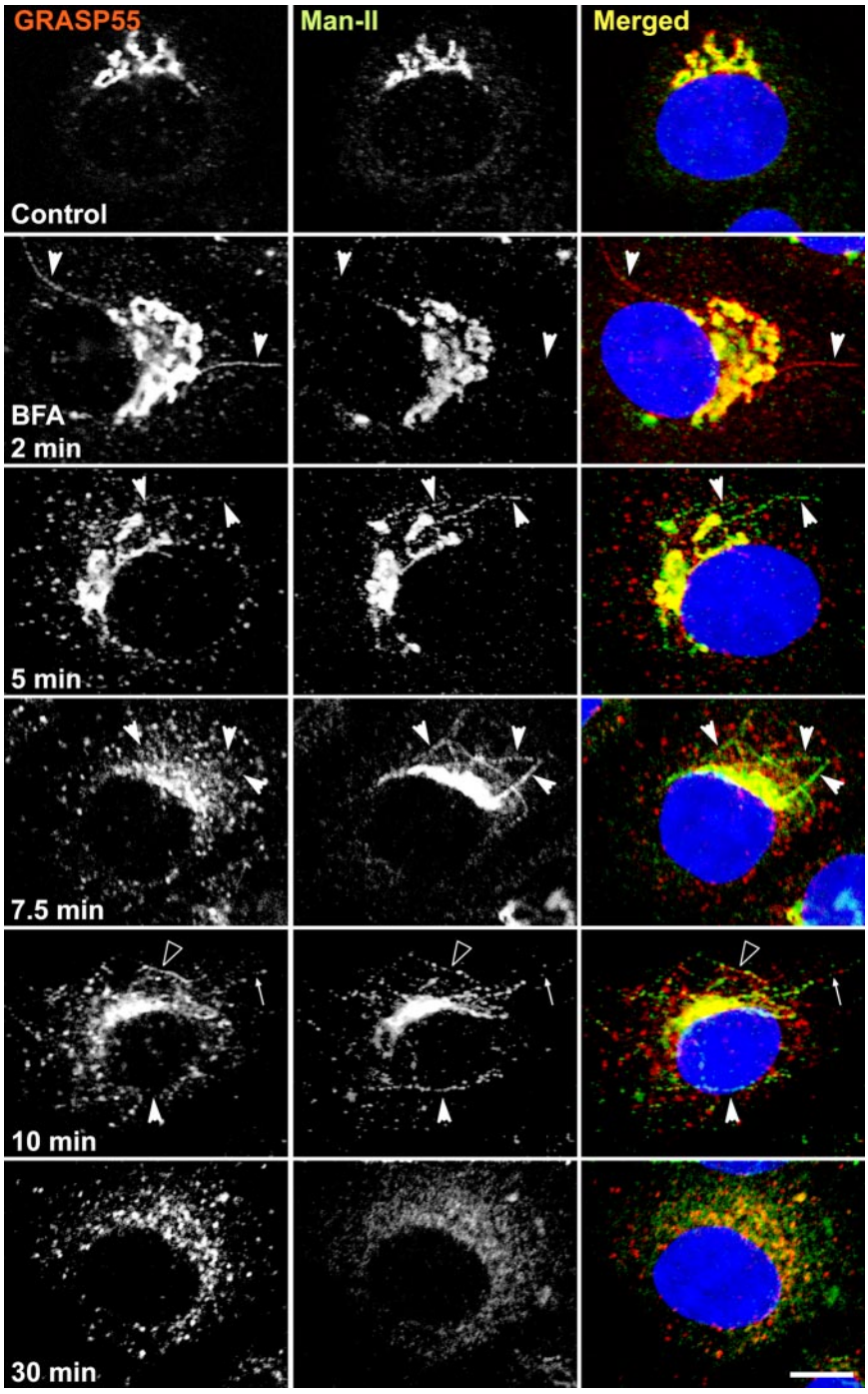

Figure 3. Time course of the medial-Golgi matrix protein GRASP55 and mannosidase II tubulation in BFA-treated cells. NRK cells were untreated (control) or treated with 5 μg/ml BFA for the times shown on the left (from 2 to 30 min). Cells were fixed and processed for immunolabeling with antibodies against GRASP55 (left) and Man-II (middle) and merged (right). Alexa Fluor-488 and Alexa Fluor-594 were used as fluorescent secondary probes. At 2 min of BFA treatment 70% of the cells extended from the Golgi one or two tubules containing only GRASP55 (white arrowheads). By 5 min, multiple tubules extended from the Golgi and were positive for both GRASP55 and Man-II (white arrowheads), whereas 10% remained only GRASP55 positive (not shown); 85% of the cells showed partial redistribution of GRASP55 to peripheral puncta, whereas Man-II was still mainly Golgi associated. By 7.5 min, 100% of the cells presented three or more tubules containing Man-II (white arrowheads), but only 50% showed GRASP55 associated to these tubules; 100% of the cells showed also GRASP55 redistributed in peripheral puncta. By 10 min, only 50% of the cells contained three or more tubules with either, both GRASP55 and Man-II (outlined arrowheads), or only Man-II (white arrowheads); the tip of some tubules were enriched in GRASP55 (arrow). By 30 min, 90% of cells showed complete redistribution; GRASP55 at or in proximity to puncta, which resembled ER exit sites, whereas Man-II was redistributed throughout the ER. Bar, 10 μm.

(n = 2), GM130-YFP (n = 9), and p115-YFP (n = 7) (our unpublished data).

As a control, NRK cells transiently transfected with GFP-tagged Golgi matrix proteins as well as a cell line stably expressing GRASP55-GFP were treated with BFA, fixed after various times, and immunolabeled with antibodies against the respective endogenous protein. The tubules containing endogenous Golgi matrix proteins were indistinguishable from those containing the fluorescently tagged protein (our unpublished data). We conclude that *cis*-Golgi matrix proteins move to ER exit sites and are dynamic within the exit sites. These data suggest that *cis*-Golgi matrix proteins have a wider function than previously described.

Temporal and Spatial Relationship of Golgi Transmembrane and Matrix Proteins after Tubule Formation

To study the temporal and spatial relationship between the two classes of Golgi proteins in vivo, GM130-YFP (Figure 6A) or p115-YFP (Figure 6B) was coexpressed with a GalT-CFP construct coding for the transmembrane domain of galactosyltransferase (GalT) fused to cyan fluorescent protein (CFP) at the luminal domain (Cole *et al*., 1996). Approximately 10 min after BFA treatment, tubules emerging from the Golgi are positive for both GalT-CFP and GM130-YFP (Figure 6A, 10:07–11:31, and Video 3). At this intermediate

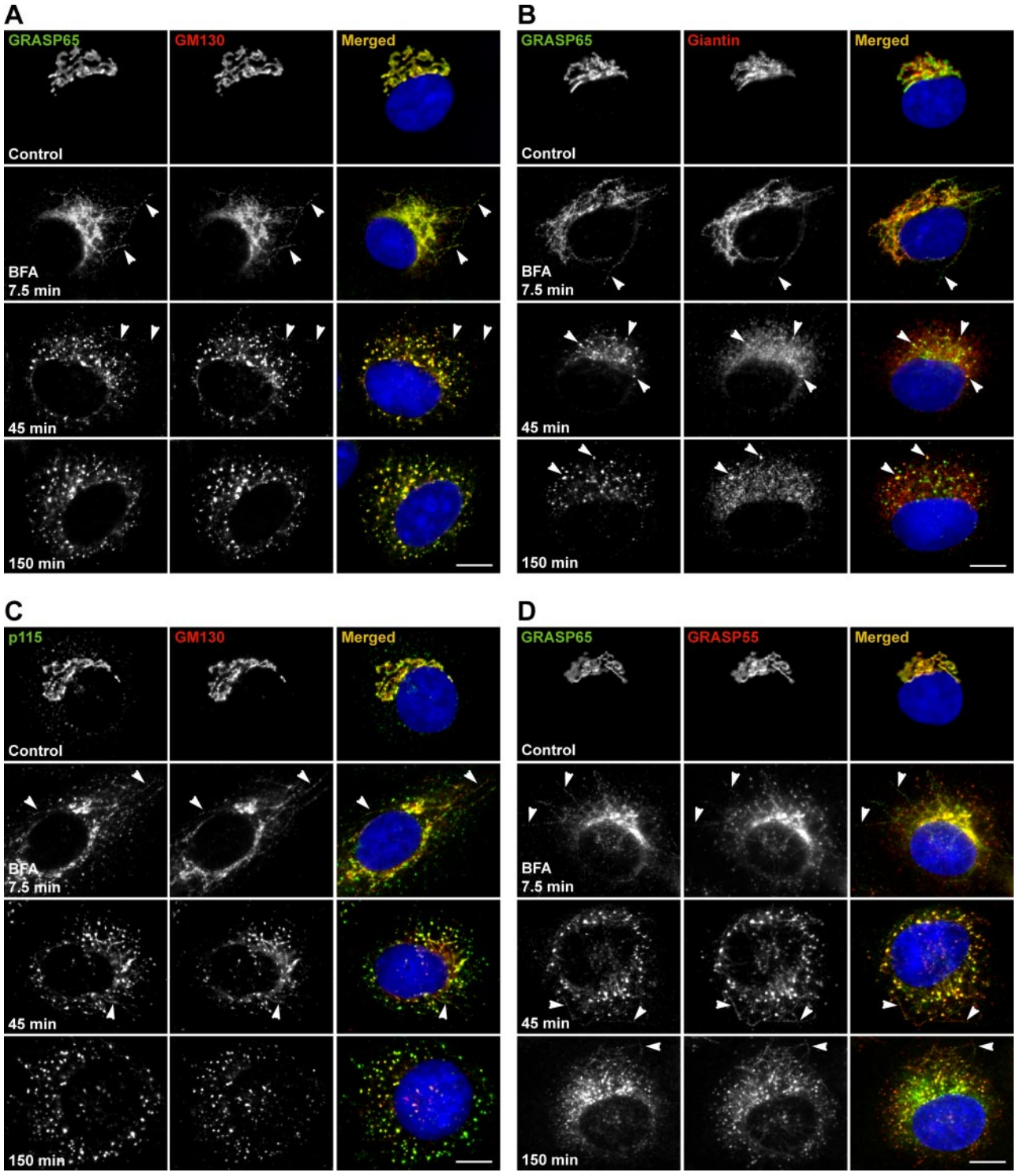

Figure 4. *cis*-medial Golgi matrix proteins redistribute to peripheral structures via tubules in BFA-treated cells. NRK cells were treated with 5 μg/ml BFA for various times, and after fixation they were processed for immunolabeling with antibodies against five different *cis*-medial Golgi matrix proteins. Tubulation was observed in 95% of the cells for all the *cis*-medial Golgi matrix proteins between 2 and 7.5 min after BFA treatment, with extensive colocalization between the *cis*-matrix proteins (7.5 min is shown, white arrowheads). (A) GRASP65 (left), GM130 (middle), and merged images (right). (B) GRASP65 (left), Giantin (middle), and merged images (right). (C) p115 (left), GM130 (middle), and merged images (right). (D) GRASP65 (left), GRASP55 (middle), and merged images (right). The best colocalization was with GRASP65 and GM130 (A). An intermediate degree of colocalization was observed for the other *cis*-matrix proteins. After 45 min, 100% of the cells stained for Golgi matrix proteins were relocated to peripheral puncta, and all colocalized. However, only 40% of the transmembrane matrix protein Giantin localized to ER exit sites; the remaining 60% was distributed throughout the ER membrane. In addition with GRASP65, GM130, p115, and GRASP55, but not for the transmembrane protein Giantin, small tubulovesicular profiles were directed toward the periphery of the cell (arrowheads in A, C, and D), which persisted after 150 min (arrowheads in D). After 150 min, in all cells the proteins followed, including Giantin, were localized to ER exit sites. Bars, 10 μm.

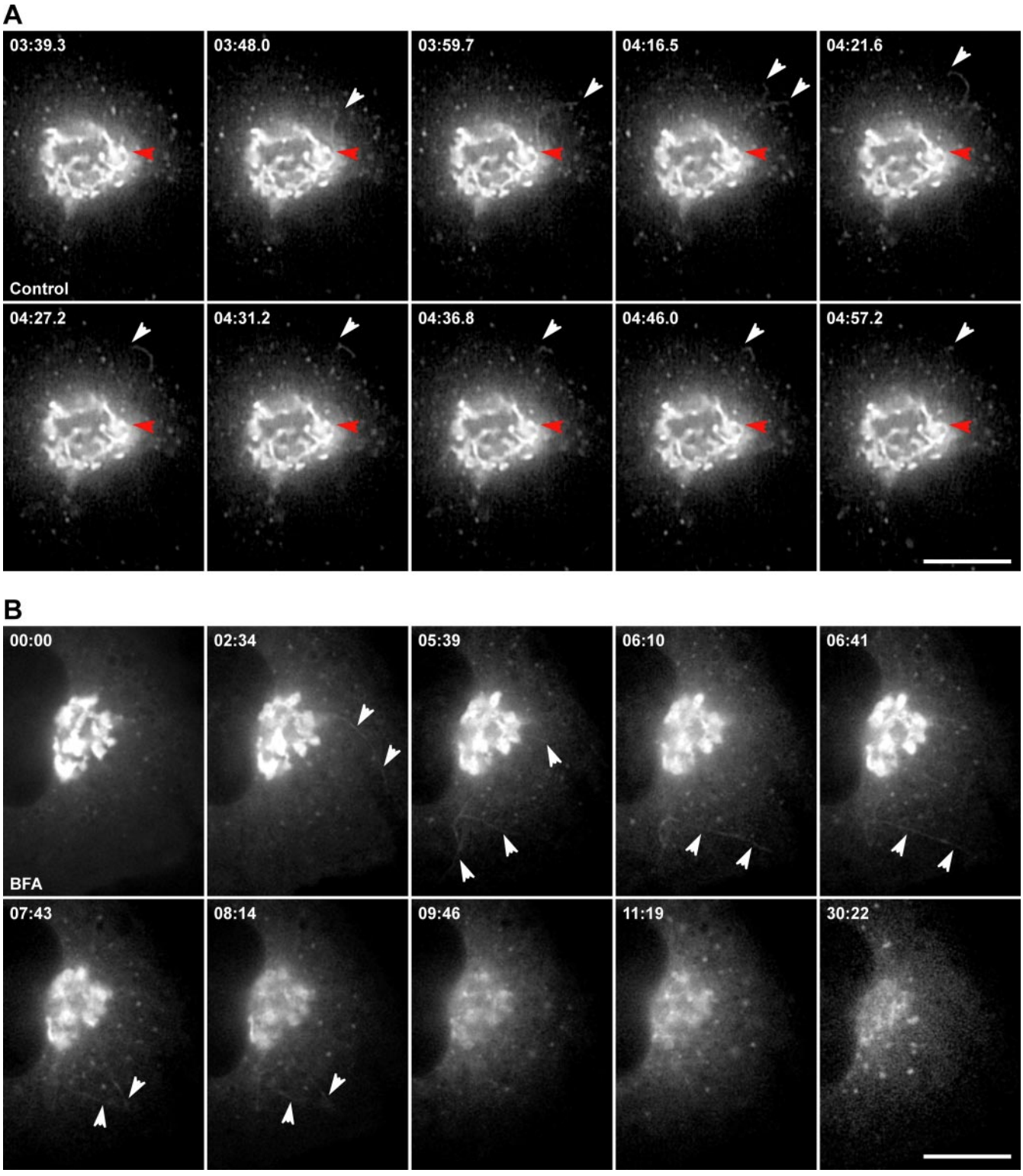

Figure 5. In vivo redistribution of GRASP55-GFP on treatment with BFA. NRK cells transiently expressing GRASP55-GFP were held in a microscope stage at 37°C, and images were recorded for 30 min in control (n = 7; A) and cells treated with 5 μg/ml BFA (n = 14; B). All cells showed the same tubulation and movement to peripheral sites. Time after initiation of imaging is shown in upper left of each panel in minutes:seconds. Video 1, 3:39.3 to 4:57.2 (A) and Video 2, 0:00 to 30:22 (B). (A) A tubule emerges from the perinuclear Golgi region, branches, retracts (04:16.5), and coalesces in a peripheral puncta (red arrowheads point to the origin, white arrowheads follow the movement). (B) In BFA-treated cells, longer tubules containing GRASP55-GFP emerge from the Golgi (02:34) and continue for ~6 min (08:14). During this time (~6 min), increasing concentrations of GRASP55-GFP accumulate in peripheral puncta with a corresponding decrease in fluorescence at the Golgi region. At 9:46, blink out of fluorescence at the Golgi is followed by a continuous increase at peripheral sites that remain until the end of the imaging (30:22) (white arrowheads point to tubules). Bars, 10 μm.

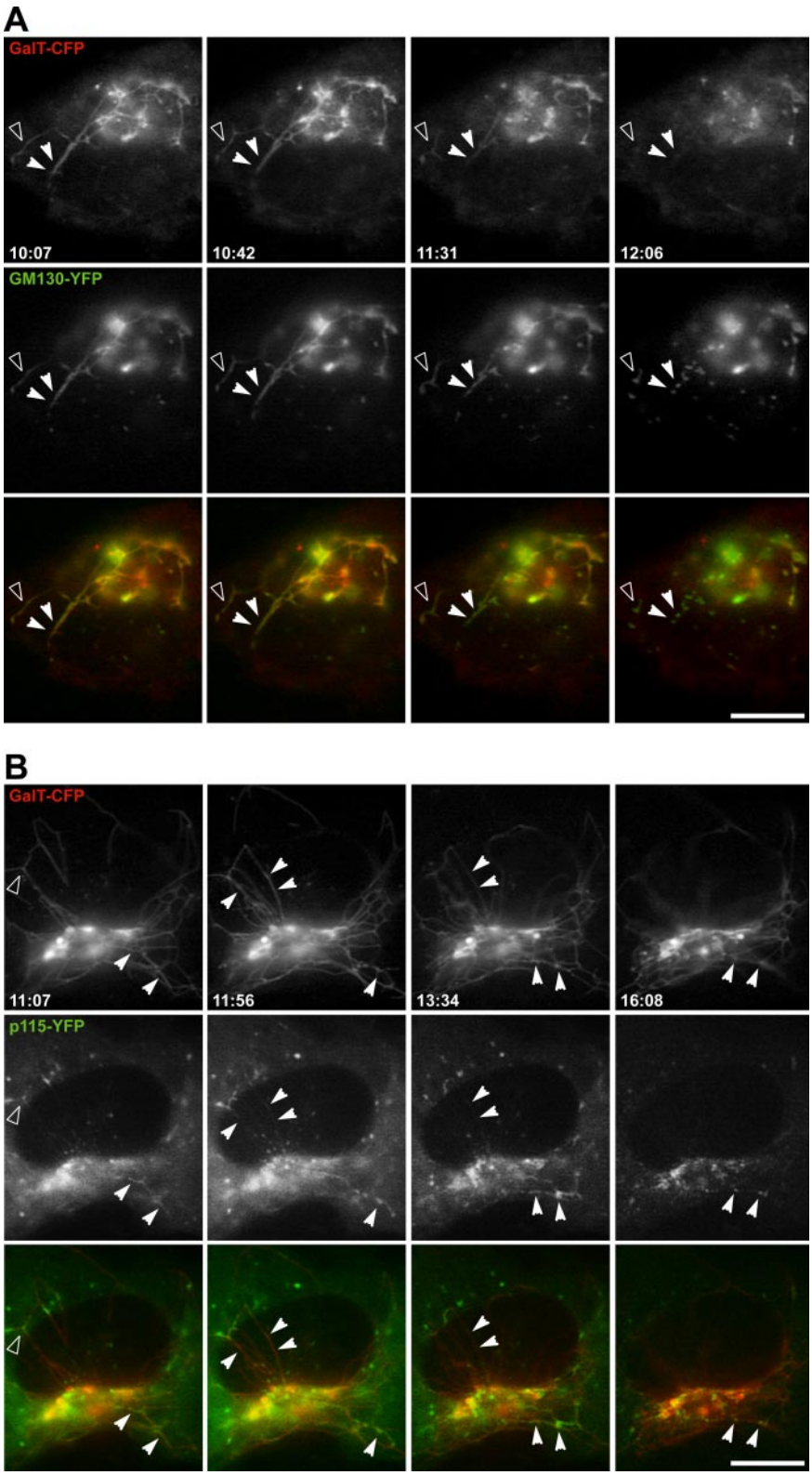

Figure 6. In vivo relationship between a Golgi transmembrane protein and Golgi matrix proteins during BFA treatment. Images were captured at 37°C over 30 min after treatment with 5 μg/ml BFA. NRK cells transiently coexpressing GalT-CFP and GM130-YFP (starting at 10:07 for A) or GalT-CFP and p115-YFP (starting at 11:07 for B). These series show tubules that contain both GalT-CFP and GM130-YFP (white arrowheads, A; and Video 3) or GalT-CFP and p115-YFP (white arrowheads, B; and Video 4, A and B). The GalT-CFP tubular pattern is more continuous than GM130-YFP or the p115-YFP. The *cis*-Golgi matrix proteins occur in domains located at branching points of GalT-CFP-containing tubules (11:07 outlined arrowheads, B). At later time points the fluorescence pattern of GM130-YFP and p115-YFP becomes fragmented and concentrated in regions of the GalT-CFP tubules (white arrowheads, 11:31 in A and 11:56 to 16:08 in B). Matrix fragments concentrate in puncta before and after (white arrowheads, 12:06 in A and 16:08 in B) GalT-CFP redistribution to ER. For each experiment, n = 6. Bars, 10 μm.

time, both matrix and transmembrane proteins are associated with the same tubules (Figure 1). These tubules are either single or branched. In addition, there are peripheral structures containing GM130-YFP that are devoid of GalT-CFP (Figure 6A, left). These data demonstrate that Golgi matrix proteins relocate early to peripheral ER exit sites, whereas Golgi transmembrane proteins redistribute throughout the ER. Similar observations were made in cells expressing p115-YFP and GalT-CFP (Figure 6B, 11:07–13:34, and Video 4, A and B).

In these studies, the two matrix proteins are continuous along the length of the tubule at intermediate time points (~10 min) and then concentrate in regions along the tubule, whereas the transmembrane protein seems to transiently concentrate at specific regions throughout the length of the tubules (Figure 6B, 11:56–16:08). Even greater differences were observed just before the blink out of the GalT-CFP. The GM130-YFP (12:06 panel) and p115-YFP (16:08 panel) fluorescence concentrated in spots aligned in a footprint of the original tubule and persisted after the GalT-CFP blink out (Figure 6, A and B, right). These data further support an intimate relationship between the Golgi matrix and transmembrane proteins in retrograde pathway from the Golgi to the ER.

Tubules Containing Golgi Matrix Proteins Fuse at ER Exit Sites

To better define where the tubules containing the Golgi matrix proteins are docking and fusing, the ER exit sites were labeled with two different markers, p58-YFP (also known as ERGIC53) and Sec13-YFP. The p58 construct codes for the full-length transmembrane protein fused with yellow fluorescent protein (YFP) at the luminal domain (Ward *et al*., 2001). This protein is a transmembrane recycling protein involved in ER-to-Golgi trafficking of specific cargo (Appenzeller *et al*., 1999), and after BFA treatment, it occurs in peripheral structures associated with ER exit sites (Tang *et al*., 1995; Ward *et al*., 2001). The focus of these experiments is to follow the tubules at the moment they collapse (blink out) at the ER. The p58-YFP fluorescence is most likely in vesicular tubular clusters (VTCs) and seems fragmented even before BFA treatment (Ward *et al*., 2001; Ben-Tekaya *et al*., 2005). After BFA treatment, the GalT-CFP colocalizes with p58-YFP (Figure 7A and Video 5, A and B) and with Sec13-YFP (Figure 7B). Unexpectedly, early in tubulation p58-positive puncta line up with GalT-CFP tubules and remain aligned throughout tubulation (6:45–17:30, Figure 7A, and Video 5, A and B). Even after tubule blink out, the p58-positive puncta are stable. When coexpressed with Sec13-YFP, it was obvious that both the tips and branch points of the GalT-CFP tubules colocalize with the ER exit sites (Figure 7B and Video 6, A and B).

This colocalization between the tip of a tubule labeled with GalT-CFP led us to ask whether tubules also carried Golgi matrix proteins directly to ER exit sites. In cotransfection experiments with GM130-CFP and Sec13-YFP, the GM130 fluorescence at the tip of a tubule colabeled with Sec13-YFP and the association seemed to be stable even though the tubule continues to move extensively (Figure 8 and Video 7A). For additional examples of the interaction between GM130-CFP tubules and Sec13-YFP-labeled exit sites, see Videos 7B and 7C. This interaction at ER exit sites is specific because tubules from the *trans*-Golgi network never colocalize with ER exit sites (our unpublished data). A dramatic example of the interaction of tubules with ER exit sites was observed with the *cis*-Golgi matrix protein p115-YFP and GalT-CFP at a time when a major portion of p115-YFP is present at the ER exit site (Figure 9A, low magnification; and B, higher magnification of area within the dotted box). The tip of the tubule containing GalT-CFP expands as it approaches the ER exit site labeled with p115-YFP (25:55). Then, the tubule detaches from the Golgi region and shortens from that end (26:10–26:35). Finally, the expanded tip remains for a few seconds and then completely disappears, whereas the p115-labeled exit site remains stable (28:20). Other points of tubule contact are observed in the same cell (arrowheads in Figure 9A and Video 8). Our data allow us to conclude that retrograde tubules from the Golgi associated with both *cis*-Golgi matrix proteins (GM130-CFP and p115-YFP) and transmembrane protein (GalT-CFP) travel directly to ER exit sites and enter the ER at or near these sites.

DISCUSSION

Our goal has been to define the differences in the behavior of *cis*-medial Golgi matrix proteins and Golgi transmembrane proteins in the retrograde pathway between the Golgi and ER. To amplify this pathway, the fungal metabolite BFA is regularly used (Klausner *et al*., 1992; Lippincott-Schwartz *et al*., 2000; Storrie *et al*., 2000). Our findings are unexpected in that both in control and BFA-treated NRK cells redistribution of *cis*-Golgi matrix proteins to the ER occurs associated with the same tubules as Golgi transmembrane proteins. The matrix proteins are enriched at the tip of the tubules and the transmembrane proteins follow within the same tubular structures. Strikingly, the matrix-enriched tip of the tubule moves directly to ER exit sites. The matrix proteins remain at and around the ER exit sites, whereas the trailing transmembrane proteins enter the ER at these sites and then redistribute throughout the ER.

Cis-*Medial Golgi Matrix Proteins Are Redistributed Associated with Retrograde Tubules*

Movement of *cis*-medial Golgi matrix proteins on retrograde tubules has not been the focus of other studies, although GM130 has been found in retrograde tubules in cells subjected to osmotic stress (Lee and Linstedt, 1999; Jiang and Storrie, 2005), and GM130-GFP and other endogenous matrix proteins were shown to be associated to short tubules described as anterograde and retrograde in control cells (Marra *et al*., 2001). Because our observation that *cis*-Golgi matrix proteins are enriched at the tips of retrograde tubules at the initiation of tubule formation from the Golgi is novel, we felt it was important to carry out these experiments by both immunolabeling of fixed cells and by in vivo imaging of expressed forms of *cis*-Golgi matrix and transmembrane proteins tagged with different variants of GFP. These experiments provided evidence that the tubules are present at endogenous protein levels, and the in vivo imaging provided an understanding of the dynamics of tubule formation and consumption. We studied five *cis*-medial Golgi matrix proteins—GM130, p115, Giantin, GRASP65, and GRASP55—and all, both cytoplasmic and transmembrane matrix proteins, are associated with tubule tips at the initiation of tubule formation and also distribute along the tubule as it elongates and reaches its site of fusion (Figure 4).

Cis-Golgi matrix proteins GM130, p115, Giantin, and GRASP65 and the medial matrix protein GRASP55 function in both anterograde and retrograde trafficking between the ER and Golgi and provide tethering for vesicles, tubules, and VTCs (Shorter and Warren, 2002; Barr and Short, 2003; Gillingham and Munro, 2003). The matrix proteins also are thought to provide structural support for Golgi cisternae (Shorter and Warren, 2002). This property seems to be redundant because microinjection of antibodies against Giantin, a conditional mutant with undetectable expression of GM130, or RNA interference depletion of GRASP65 showed no significant changes in overall Golgi organization (Puthenveedu and Linstedt, 2001; Vasile *et al*., 2003; Sütterlin *et al*., 2005). Coiled-coil domains forming an extended rod-like conformation are the dominant structural characteristic of

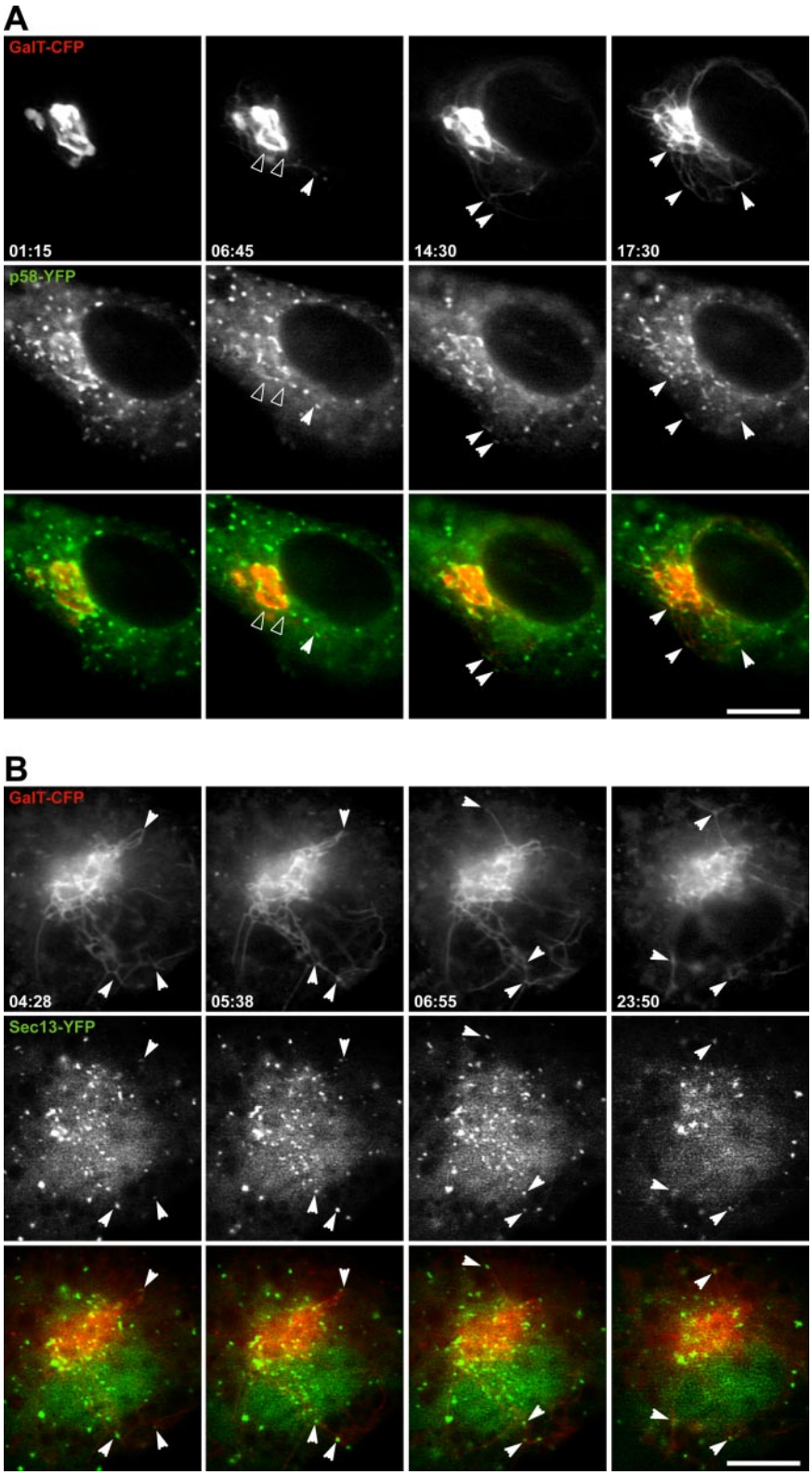

Figure 7. Association between Golgi-derived tubules and ER exit sites. Images were captured at 37°C at various times after treatment with 5 μg/ml BFA. The cells transiently coexpress GalT-CFP and p58-YFP (A; n = 7, imaging initiated at 01:15) or GalT-CFP and Sec13-YFP (B; n = 7, imaging initiated at 04:28). Tubules that contain GalT-CFP are associated with p58-YFP-positive puncta very rapidly after the tubulation event (~6 min) with p58-YFP-containing puncta remaining attached throughout the period of tubulation (~10 min, white arrowheads in A; and Video 5, A and B). There are also p58-YFP puncta that seem not to connect with GalT-CFP tubules (A, 06:45 outlined arrowheads). The tips or tubule branching points of all GalT-CFP-positive tubules associate with the Sec13-YFP-positive ER exit sites (white arrowheads in B; and Video 6, A and B). Bars, 10 μm.

these proteins, and many functions have been attributed to these structural features (Barr and Short, 2003; Gillingham and Munro, 2003). Because our data show that all of the matrix proteins act similarly, we do not discuss the individual properties of these proteins. However, a number of insightful reviews on these molecules provide this information (Shorter and Warren, 2002; Barr and Short, 2003; Gillingham and Munro, 2003). More recently, an essential role of GRASP65 in the dynamics of the mitotic spindle was demonstrated (Sütterlin *et al*., 2005).

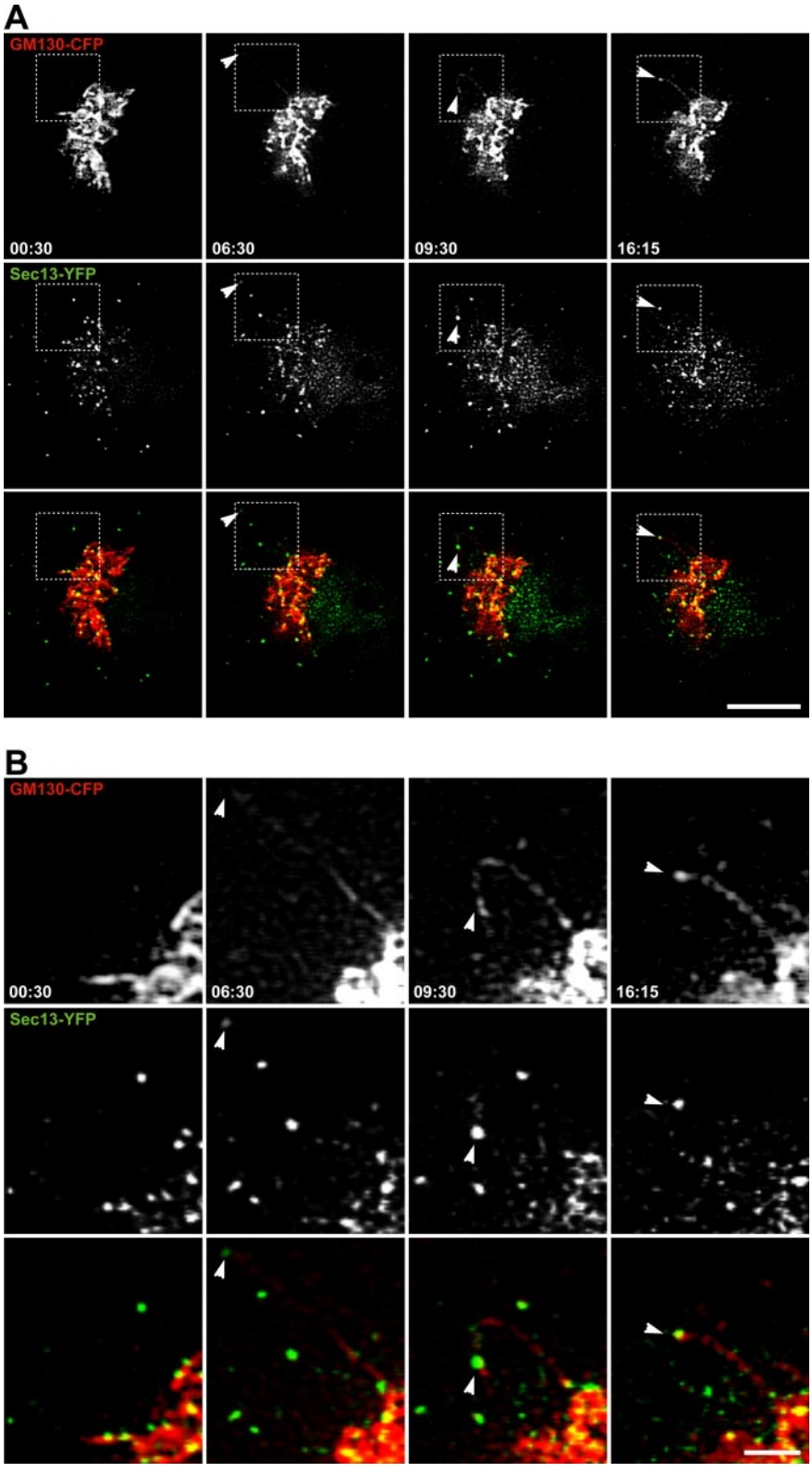

Figure 8. Tubules containing GM130-CFP are associated to ER exit sites in BFA-treated cells. Images of cells transiently coexpressing GM130-CFP and Sec13-YFP were captured at 37°C at various times after treatment with 5 μg/ml BFA(n = 5) (A, low magnification; B, higher magnification of boxed regions). Images shown begin 00:30 after the addition of BFA. A tubule containing GM130-CFP extends from the Golgi and by 06:30 its tip reaches the Sec13-YFP-containing ER exit sites (white arrowheads). After almost three additional minutes (09:30), the tubule tip turns and associates with a second ER exit site. The tip of the GM130-CFP tubule remains associated with ER exit sites for ~10 min (arrowheads and Video 7A). Bars, 10 μm (A) and 2 μm (B).

Regulation of Golgi Matrix Protein Association with Membranes

It is becoming increasingly clear that Golgi matrix proteins are highly dynamic in their association with membranes. Photobleaching experiments of ER exit sites and control Golgi revealed rapid exchange between the membrane and cytosolic pools of these proteins (Ward *et al.*, 2001). Golgi matrix proteins interact with or are recruited to membranes by small GTPases of the Rab and Arl families (Shorter and Warren, 2002; Barr and Short, 2003).

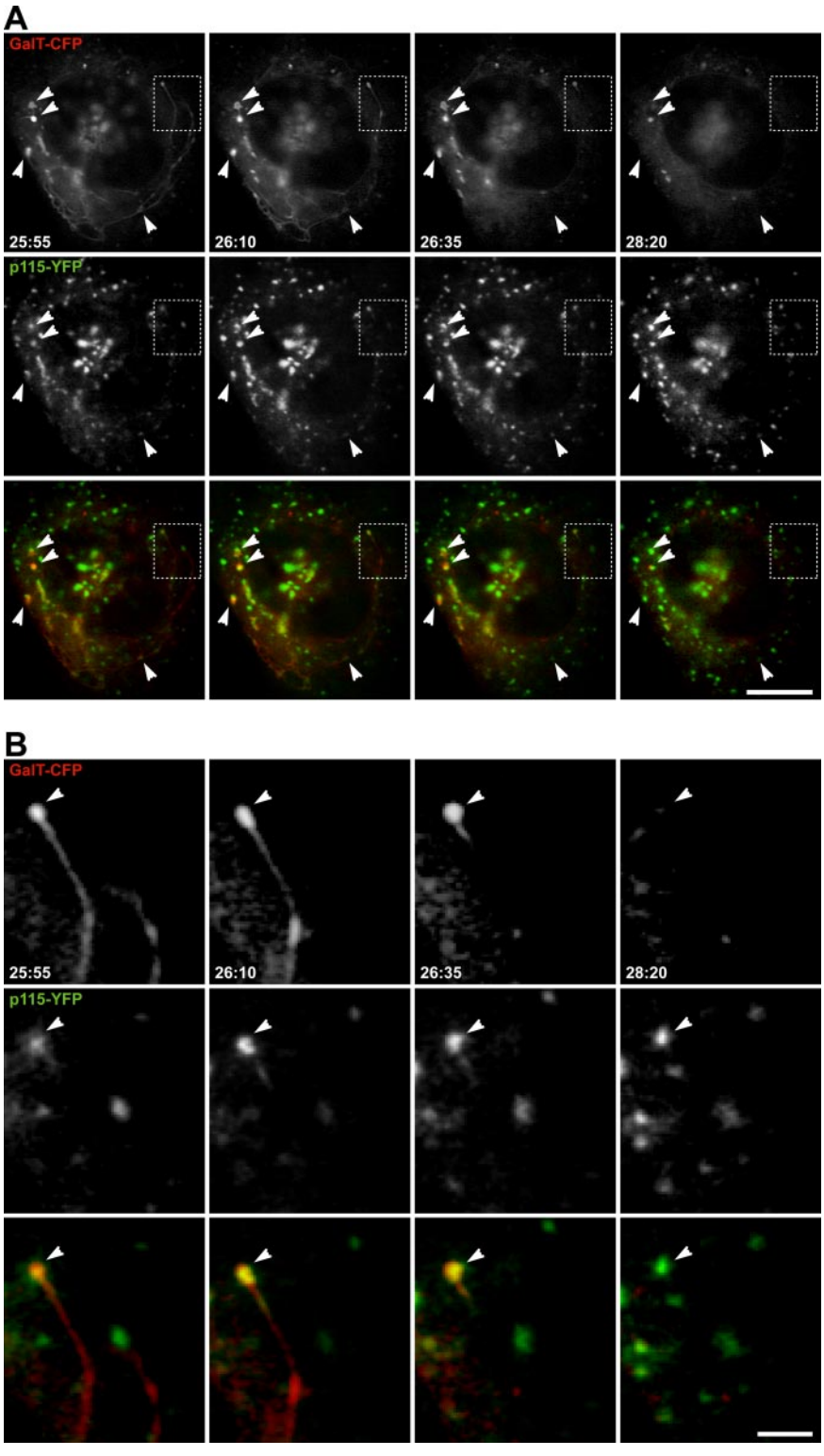

Figure 9. Tubules containing GalT-CFP fuse at ER exit sites in BFA-treated cells. Images of cells transiently coexpressing GalT-CFP and p115-YFP were captured at 37°C at various times after treatment with 5 μg/ml BFA (n = 6) (A, low magnification; B, higher magnification of boxed regions). Recording was initiated at 25:55 after tubulation of GalT-CFP was observed. A tubule that contains GalT-CFP is associated at its tip with p115-YFP, which is already redistributed to the ER exit site by this time (25:55, boxed in A; and white arrowhead in B). In this region, there are several nontubular puncta that contain GalT-CFP as well as p115-YFP at ER exit sites (25:55, arrowheads in A). Rapidly, GalT-CFP containing tubules and peripheral elements collapse into those regions defined by p115-YFP (arrowheads in A and B), whereas the p115-YFP fluorescence persists until the end of the recording (Video 8). Bars, 10 μm (A) and 2 μm (B).

Matrix proteins also are regulated by different posttranslational modifications. GM130 and p115 are phosphorylated by mitotic kinases (Lowe *et al*., 1998; Dirac-Svejstrup *et al*., 2000). GRASPs are N-terminally myristoylated and in a recent proteomics study of the Golgi, we found two Golgi matrix proteins, GRASP55 and Golgin 84, that were dimethylated on arginines (Wu *et al*., 2004). We know little about the function and dynamics of these modifications or whether they play a role in tubulation.

Cis-*Golgi Matrix Tubules Move Directly to ER Exit Sites*

The ability to follow in vivo imaging of expressed forms of *cis*-Golgi matrix and transmembrane proteins tagged with different variants of GFP has allowed us to make three

additional unique observations. First, the same tubules positive for the *cis*-Golgi matrix proteins also contain transmembrane proteins, with matrix proteins enriched at the tip of the tubule. Second, the matrix–tubule tips recognize ER exit sites and deliver both the transmembrane and matrix proteins to the ER at these well-defined sites, which labeled with both p58 and Sec13 (Figures 7–9). These data suggest a distinct recognition between molecules in the retrograde tubule tip and the ER exit sites and further imply that all retrograde transmembrane proteins enter the ER at ER exit sites. Finally, the transmembrane and matrix proteins seem to sort into alternative domains within the same tubule, suggesting the matrix proteins function in proofreading or resorting within the tubule.

Retrograde tubules containing *cis*-Golgi matrix from the Golgi have not been seen by others performing very similar experiments (Seemann *et al*., 2000; Ward *et al*., 2001). A possible explanation for this could be that the *cis*-Golgi matrix proteins were observed too late after the tubules interacted or fused at ER exit sites, forming the BFA remnants of Seemann *et al*. (2000). Alternatively, the live cell imaging was not conducted at 37°C, a temperature providing optimal formation of the tubules (Ward *et al*., 2001). We believe the more likely explanation is that the sensitivity and speed of cameras for live cell imaging has improved significantly in the past few years, allowing small tubules to be visualized.

Using a variety of *cis*-Golgi matrix proteins, others have shown that after 30 min of BFA treatment, matrix proteins accumulate as BFA remnants, distinct from the ER (Seemann *et al*., 2000). These data has been used to put forward the notion that the Golgi has the capacity to self-assemble and matrix proteins are required for this self-assembly process (Shorter and Warren, 2002). Similar results demonstrated that the BFA remnants were actually ER exit sites that colocalized with Sec13-YFP (Ward *et al*., 2001). These data were interpreted to show that the Golgi has the capacity to undergo de novo biogenesis from the ER (Ward *et al*., 2001). Our data offer integration of the two models. First, Golgi matrix proteins move to ER exits sites and redistribute within the ER network as evidenced by the blink out of GRASP55 (Figure 5B) and then very rapidly collect at ER exit sites. Seconds later, transmembrane proteins move to the ER via the same tubules, and after ~2 h, move to the ER exit sites. Reassembly of the Golgi could then start at ER exit sites where *cis*-Golgi matrix proteins are present to provide the necessary structural components (Puri and Linstedt, 2003; Kasap *et al*., 2004). The temporal difference between the delivery of matrix and transmembrane proteins to the ER and the fact that the matrix proteins reassemble extremely rapidly at exit sites, whereas the transmembrane proteins accumulated after a significant delay, may explain the lack of colocalization observed by others (Seemann *et al*., 2000).

Our observation that tubules intersected with several ER exits sites both along the tubule length and at the tubule tip raises a number of questions (Figure 7). It is possible that ER exits sites are aligned with microtubule tracks and hence tubules following these tracks will intersect with the exit sites in either a functional or nonfunctional way. This interaction might be purely structural in nature, for example, to extend the length of the tubule or provide support for a growing tubule. Alternatively, there could be delivery of Golgi molecules to the ER at each site. Our data suggest that the tubules are contiguous with the ER at each site because the blink out often occurs simultaneously at several sites along the length of the tubule.

Regulation of Tubule Formation

It is unclear what function the *cis*-Golgi matrix plays in tubule formation. In other words, is the matrix an essential component for tubule formation? To produce a tubule or vesicle from a lipid bilayer, an external force is required (Upadhyaya and Sheetz, 2004). Additionally, the coiled-coil domains of the matrix proteins may interact directly or via another protein with components of the cytoskeleton as shown for matrix proteins of the *trans*-Golgi, bicaudal 1 and bicaudal 2, which link vesicles to microtubules via dynactin (Hoogenraad *et al*., 2001; Matanis *et al*., 2002).

Future work will be directed at understanding the regulation of the *cis*-medial Golgi matrix in the formation, fission, and fusion of retrograde tubules from the Golgi to the ER and their role in mitosis. Our finding that the tubules go directly to ER exit sites and deliver both *cis*-Golgi matrix and transmembrane proteins implies that this, too, is a regulated process and that there are distinct recognition molecules within the ER exit sites. These new data extend the functions of the *cis*-Golgi matrix molecules in retrograde transport from the Golgi.

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