

## Intracellular localization and in vivo trafficking of p24A and p23

Robert Blum<sup>1</sup>, Fatima Pfeiffer<sup>1</sup>, Peter Feick<sup>1</sup>, Wolfgang Nastainczyk<sup>2</sup>, Bärbel Kohler<sup>1</sup>, Karl-Herbert Schäfer<sup>3</sup> and Irene Schulz<sup>1,\*</sup>

<sup>1</sup>Institute of Physiology II, <sup>2</sup>Medical Biochemistry and <sup>3</sup>Department of Anatomy, University of the Saarland, D-66421 Homburg Saar, Germany

\*Author for correspondence (e-mail: schulz@med-ph.uni-sb.de)

Accepted 9 December 1998; published on WWW 25 January 1999

### SUMMARY

Recently, p24A and p23 (also termed Tmp21), two members of the p24 protein family, have been proposed to function as integral receptors for the COPI-vesicle coat. This study describes the intracellular localization and trafficking of p24A in comparison to p23. For immunolocalization of p24A and p23, strong reduction and denaturation conditions were necessary to allow antibody interaction. Both p24A and p23 cycle continuously between intermediate compartment (IC) elements and the cis-Golgi network. In vivo trafficking of p24A and p23 tagged to green fluorescent protein (GFP) revealed that both proteins travel by large (up to 1  $\mu\text{m}$  in length) microtubule-dependent pre-Golgi carriers with a maximum speed of up to 1.6  $\mu\text{m s}^{-1}$  from the IC to the Golgi cisternae. Aluminum

fluoride, a general activator of heterotrimeric G-proteins, blocked peripheral pre-Golgi movements of GFP-p24A/p23 and inhibited fluorescence recovery after photobleaching in the perinuclear Golgi area. p24A and p23 are predominantly colocalized. Overexpression of GFP-p24A, to an extent which did not destroy the Golgi complex, induced delocalization of part of the proteins into ER elements. This study therefore gives new insights into the localization and trafficking behavior of the two COPI-binding proteins p24A and p23.

Key words: Membrane protein, Golgi apparatus physiology, Microtubule-drug effect, Green fluorescent protein.

### INTRODUCTION

Transport of proteins along the secretory pathway of eukaryotic cells is mediated by vesicular carriers, which bud off from a donor compartment and fuse with an acceptor compartment (Palade, 1975). Formation of these transport vesicles is dependent on recruitment of cytosolic coat proteins on the surface of the donor compartment membrane. Two types of coat structures, COPI and COPII, have been shown to mediate the transport between the endoplasmic reticulum and the Golgi apparatus (for a review see Rothman and Wieland, 1996). Whereas the COPI coat is involved in anterograde ER to Golgi (Pepperkok et al., 1993; Peter et al., 1993; Bednarek et al., 1995), intra-Golgi (Orci et al., 1986), and retrograde Golgi to ER transport (Cosson and Letourneur, 1994; Letourneur et al., 1994), COPII seems to mediate exclusively anterograde transport from the ER to pre-Golgi structures (Barlowe et al., 1994; Aridor et al., 1995). Vesicles are not the only vehicle for ER to Golgi transport. Recently it has been shown that anterograde intermediate compartment (IC) to Golgi transport can occur by pre-Golgi carriers (Presley et al., 1997; Scales et al., 1997). These are non-vesicular membrane structures, sometimes larger than 1.5  $\mu\text{m}$  in diameter, which move en masse on microtubule tracks in a 'stop-and-go' fashion towards the Golgi complex (Presley et al., 1997).

Recently we have cloned and characterized p23 (Tmp21) and p24A from rat pancreatic microsomal membranes (Blum

et al., 1996). These proteins exhibit weak homology (23% identity). They are members of the p24 protein family, which were initially described by Stannes et al. (1995). All p24 members share the same type I topology, with a large luminal domain, followed by a C-terminal located membrane anchor and a short highly conserved cytoplasmic tail. The function of p24 proteins is not yet known, but experiments with the yeast p24 proteins Emp24p and Erv25p indicate that they are involved in the sorting and/or concentration of specific cargo proteins in ER-derived COPII-transport vesicles (Schimmöller et al., 1995; Belden and Barlowe, 1996). p24 proteins exhibit all the features of putative cargo receptors, as suggested by Balch et al. (1994). So far, no direct or indirect interaction of p24 proteins and cargo proteins has been shown.

The only mammalian p24 member for which steady-state localization has been described is p23. It is localized to Golgi cisternae (Sohn et al., 1996), is concentrated into COPI vesicles, and is able to bind COPI proteins by its cytosolic tail peptide in vitro. It therefore had been assumed to be an integral Golgi-specific receptor for the COPI coat. However, Rojo et al. (1997) showed that p23 is a major component (30% of the total protein) of intermediate compartment/cis-Golgi network membranes, is de-enriched in COPI-coated vesicles, and is not required for COPI recruitment onto donor membranes.

Here we describe the intracellular localization of p24A and compare it with p23. We have localized p24A to membranes

of the cis-Golgi network and the intermediate compartment. In vivo trafficking of p24A and p23, tagged to 'green fluorescent protein' (GFP), showed that both proteins are members of pre-Golgi-carriers, whose movement from IC elements to the Golgi complex is microtubule-dependent. Peripheral movements of GFP-p24A are sensitive to aluminum fluoride ( $\text{AlF}_4^-$ ), a general activator of trimeric-G-proteins. p24A and p23 exhibit the same trafficking behavior and are predominantly colocalized.

## MATERIALS AND METHODS

### Antibodies

The polyclonal p23 antibody (anti-MAL-p23) was produced by immunization of rabbits with recombinant p23 obtained from expression in *E. coli* with the pMal-C2 vector system (New England Biolabs). The protein p24A was expressed in the same manner. Anti-MAL-p24A was produced in mice. Other rabbit antibodies raised against the N terminus of p23 (anti-NT-p23) and p24A (anti-NT-p24A) have been described previously (Blum et al., 1996). The following antibodies were obtained from the indicated distributors: anti-rat 135 kDa mannosidase II (Hiss diagnostics, Freiburg, Germany),  $\gamma$ -adaptin (Dianova, Hamburg, Germany), 58K (Sigma). The mouse antibody against hum-ERGIC-53 was a generous gift of H. P. Hauri (Biocenter Basel, Switzerland). Cy3-conjugated anti-mouse and anti-rabbit IgG from goat, fluorescein (DTAF)-conjugated goat anti-rabbit IgG (Dianova), and Texas-Red-X goat anti-rabbit and anti-mouse IgG (Molecular Probes), were used as secondary antibodies in indirect immunofluorescence experiments.

### Cells

COS-1 cells were grown in Minimum Essential Medium (MEM $\alpha$ ) containing heat-inactivated 10% fetal calf serum (FCS). NRK-52E cells were grown in Dulbecco's modified Eagle medium and 10% FCS. Long-term cultures of neonatal rat pancreatic acinar cells were isolated and cultivated as described by Anderson and McNiven (1995). For indirect immunofluorescence experiments, cells were grown on coverslips. Brefeldin A (BFA; 5  $\mu\text{g}/\text{ml}$ , Sigma), cycloheximide (10  $\mu\text{g}/\text{ml}$ , Sigma), nocodazole (NZ; 5  $\mu\text{g}/\text{ml}$ , Calbiochem) and aluminum fluoride (30 mM NaF, 50  $\mu\text{M}$   $\text{AlCl}_3$ ) were added to the medium at indicated concentrations.

### Indirect immunofluorescence

Cells were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 20 minutes at 37°C, and then washed twice in PBS buffer. For detection of p24A, p23 and ERGIC-53, fixed cells were permeabilized, reduced and denatured for 30 minutes at 37°C in PBS buffer containing 0.5% SDS, 5%  $\beta$ -mercaptoethanol and 10% FCS. Then, cells were washed five times with PBS containing 4% FCS and 0.1% Triton X-100 (PFT buffer). For detection of mannosidase II,  $\gamma$ -adaptin and ERGIC-53, fixed cells were permeabilized in PFT buffer for 30 minutes at 37°C. The following incubation and washing steps were performed in PFT buffer. Cells were incubated with primary antibodies for 1 hour at 37°C. Antibodies were removed and the cells washed three times. Cells were then incubated for 1 hour at 37°C with secondary antibodies and subsequently washed five times in PFT buffer and finally in  $\text{H}_2\text{O}$ . For double immunofluorescence, DTAF-conjugated anti-rabbit IgG and Texas-Red-X anti-mouse IgG from goat were applied simultaneously. Indirect detection of one protein was performed using Cy3-coupled secondary antibodies. Labeled cells were embedded in Mowiol 4-88 (Hoechst, Frankfurt). Images were recorded with a color video camera (Sony DXC-950), using an Olympus BX50F microscope, equipped with a 40 $\times$  and a 100 $\times$  Olympus UPlanFI objective and filters for fluorescein isothiocyanate- and rhodamine-derived fluorescence. The colocalization was

visualized by combining RGB pictures of the DTAF- and the Texas-Red-X label with Adobe Photoshop D1-3.0.

### Construction and transfection of p23/p24A-GFP fusion proteins

All cloning procedures were performed according to standard procedures. The signal peptide of p23, which had been characterized in our previous study (Blum et al., 1996), was amplified by PCR with the following primers: 5'-TACCGGTCGCCACCATGTCTGG-TTTGTCTGGCCC-3'; 5'-GACCGGTAGGGCAAGGACCAATCTGGG-3'. The resulting PCR product was subcloned in the vector pTOPO2.1 (Invitrogen) and then cloned in the *Age*I-restriction site of the eukaryotic expression vector pEGFP-C1 (Clontech), resulting in the construct 'luminal-GFP'. The DNA sequences encoding the mature p23 (accession no. X97443) and p24A (accession no. X92098) were then PCR-amplified using the following primers: p23for: 5'-TCGAATTCTATCTCCTTCCATCTACCC-3'; p23rev: 5'-GGTG-GATCCATTACTCTATCAACTTCTTG-3', p24for: 5'-TCGAATTC-TTATTTCGTTAGCATCGACG-3', p24rev: 5'-GGTGGATCCTT-AAACAACCTCTCCGGAC-3'. The resulting PCR products were cleaved with *Eco*RI/*Bam*HI and ligated to the 3' terminus of 'green fluorescent protein' (GFP), resulting in GFP-p24A and GFP-p23. The GFP-KDEL construct was produced by cloning annealed oligonucleotides encoding a 'KDEL' motif to the 3' terminus of the signal-peptide-GFP construct (see above). The sequences of the KDEL-encoding oligonucleotides were: KDEL-sense: 5'-GCGAATTCTAAGGACGAGCTCTAAGGATCCGCG-3', KDEL-antisense: 5'-CGCGGATCCTTAGAGCTCGTCCCTAGAAATTCGC-3'. The nucleotide sequence of all constructs was confirmed by DNA sequencing using the dideoxynucleotide method. COS-1 and NRK-52E cells were grown on glass coverslips and transfected with the GFP-vector constructs using Lipofect-AMINE reagent (Gibco BRL). In vivo studies were started 16-20 hours after transient transfection. In double immunostaining experiments against GFP-fusion proteins, antigens of interest were detected as described above, but Texas Red-conjugated secondary antibodies were used. The integrity of the constructs was tested by western blot analysis of the GFP-fusion proteins with a polyclonal antibody against GFP (Clontech) and the antibodies anti-Mal-p23 and anti-NT-p24A.

### Fluorescence measurements

Transfected COS-1 cells were grown on glass coverslips, which were attached to the bottom of a heatable perfusion chamber. Hepes-buffered cell culture medium containing 10  $\mu\text{g}/\text{ml}$  cycloheximide was added. The GFP fluorescence was recorded by means of an inverted microscope (Zeiss, Axiovert 35, Plan-Neofluar 63 $\times$ /oil) equipped with a confocal laser-scanning and image processing system (Bio-Rad, MRC 600). The cells were excited at 488 nm with an argon ion laser and fluorescence was measured at  $\geq 515$  nm. To reduce bleaching of GFP fluorescence, the laser emission was attenuated by means of a neutral density filter. Fluorescence images of 256 $\times$ 256 pixels with a resolution of 0.097  $\mu\text{m}/\text{pixel}$  were recorded every 2 seconds. The recorded images were stored on a hard disc. Movements of intracellular structures were analyzed with the Bio-Rad software. For photobleaching of GFP, a small area within the observed cell was scanned with high laser energy (10 mW, 488 nm band of the argon ion laser). Fluorescence recovery after photobleaching (FRAP) was monitored by images of 512 $\times$ 512 pixels with a resolution of 0.073  $\mu\text{m}/\text{pixel}$  every 60 seconds. All experiments were carried out at 32°C. Final figures were arranged with Adobe Photoshop D1-3.0.

## RESULTS

### Properties of the antibodies

The antibodies anti-NT-p23 and anti-NT-p24A raised against

the N-terminal end of p23 and p24A, respectively, recognize their corresponding natural counterparts in western blot analysis (Blum et al., 1996). For the present study we have produced antibodies against recombinant rat-p23 and hum-p24A (anti-MAL-p23 and anti-MAL-p24A). The specificity of the antibodies was tested against natural p23/p24A and recombinant MAL-p23/p24A, cleaved from the fusion protein by treatment with factor Xa. Natural and recombinant p23 and p24A were recognized at their close N-terminal epitope with anti-NT-p23/p24A. Anti-MAL-p23/p24A recognized luminal epitopes of p24A and p23 (not shown).

### Strong denaturation and reducing conditions allow immunolocalization of p23 and p24A

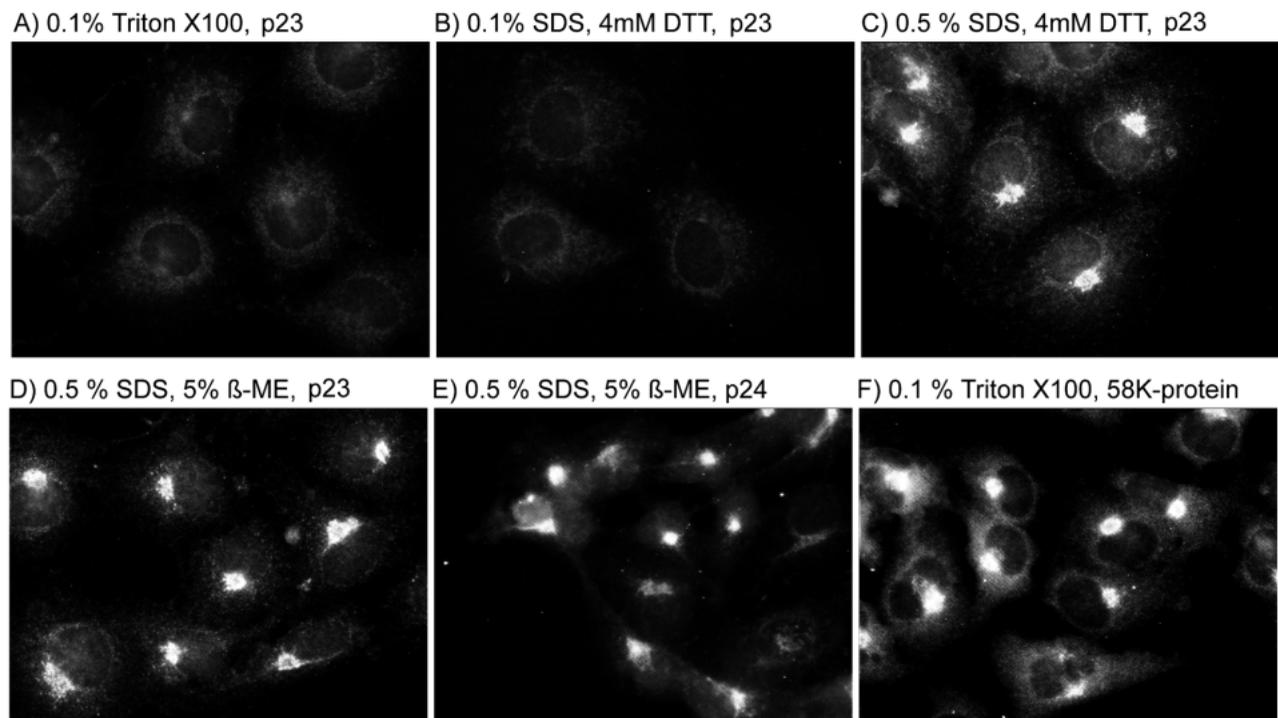
Natural p24A and p23 could not be immunodetected in cells fixed with paraformaldehyde and permeabilized with Triton X-100 (Fig. 1A). Permeabilization of cells with 0.1% SDS plus 4 mM dithiothreitol (DTT) (Fig. 1B) was also not sufficient to allow detection of p23 and p24A. However, permeabilization with 0.5% SDS plus 4 mM DTT allowed staining of Golgi-like structures in COS-1 cells (Fig. 1C). Better results for all described p23/p24A-specific antibodies were obtained in paraformaldehyde-fixed and permeabilized cells using denaturing and reducing conditions with 0.5% SDS and 5%  $\beta$ -mercaptoethanol at 37°C (Fig. 1D,E). This permeabilization procedure allowed us to immunolocalize p23 and p24A in perinuclear structures of COS-1 cells, typical for the Golgi complex. The p23-labeled structure is similar to the structure labeled by the Golgi marker protein 58K (Ktistakis et al., 1991) (see Fig. 1F).

### p24A localizes to membranes of the cis-Golgi/intermediate compartment and has the cycling characteristic of an intermediate compartment protein

Costaining of p24A and p23 (Fig. 2) and of p24A and ERGIC-53 (Fig. 3) was possible using their respective antibodies. However, the strong reduction and denaturation of proteins fixed with paraformaldehyde destroyed the epitopes of the following marker proteins: mannosidase II for the cis/medial Golgi cisternae,  $\gamma$ -adaptin for the trans-Golgi network and AP1-clathrin-coated vesicles,  $\beta$ -COP for the cis/medial-Golgi and COPI vesicles, and calnexin for the ER. Therefore it was impossible to costain p24A with these markers. To overcome this methodical problem, we fused p24A to the C terminus of *Aequorea victoria* 'green fluorescent protein' (GFP; Prasher et al., 1992). The GFP part of the construct served as a stable fluorescent tag for p24A. The expression product GFP-p24A was integrated into ER-derived membranes by an N-terminal signal peptide. Endogenous p24A and GFP-p24A showed identical localization (compare Fig. 2 with Figs 3, 4, 'steady state'). For comparison, intracellular localization of the marker proteins rat-mannosidase II for the cis/medial Golgi cisternae and of  $\gamma$ -adaptin for the trans-Golgi network and AP1-clathrin-coated vesicles is demonstrated in Fig. 4.

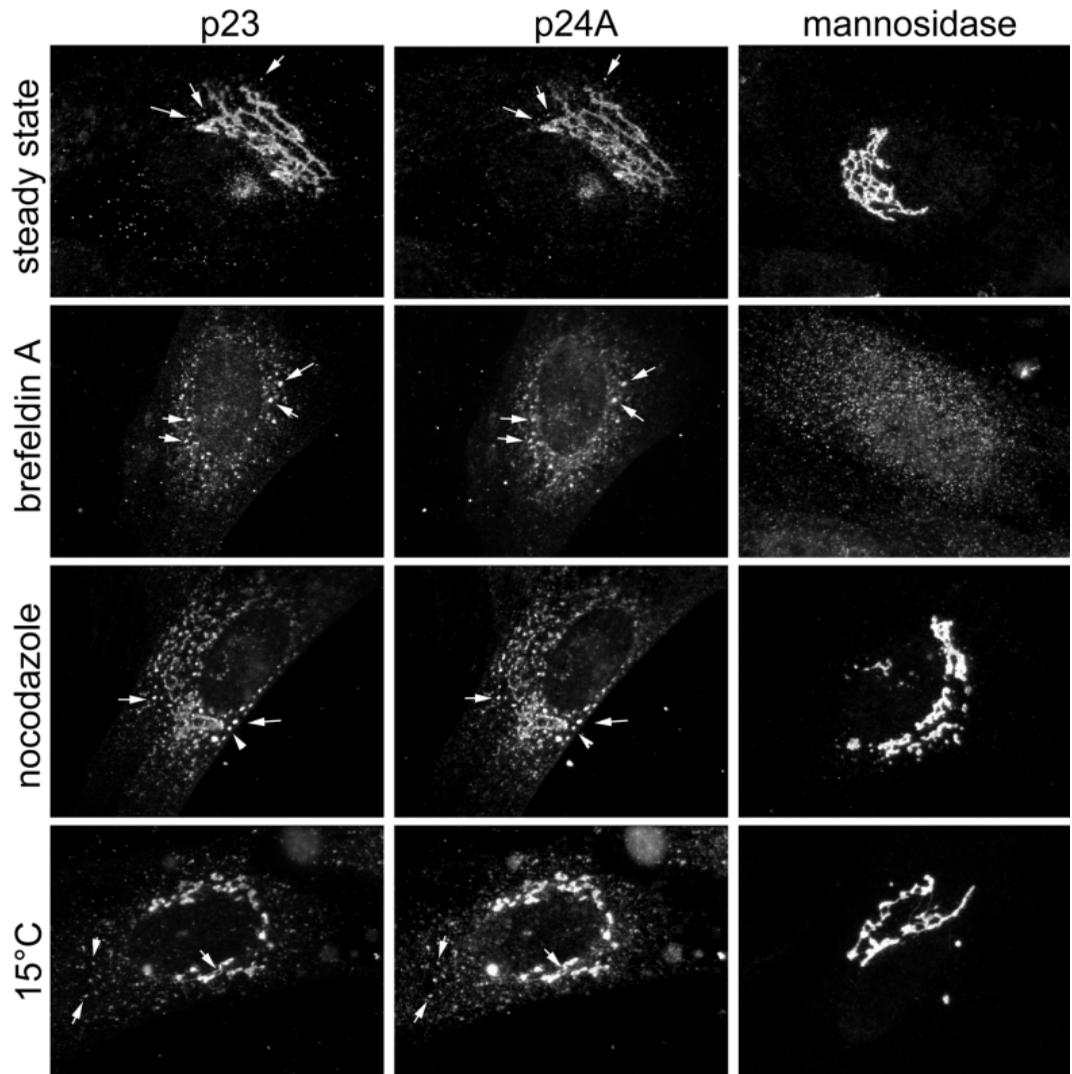
### Localization of p24A and p23 at steady state

Immunofluorescence with anti-p24A, GFP-p24A, anti-p23 and GFP-p23 revealed localization of both proteins in perinuclear Golgi-like structures of different cell types (pancreatic acinar cells, Fig. 2, 'steady state', COS-1 cells, Figs 3, 4, 'steady state', NRK-52E cells, Fig. 4, 'steady state'). Anti-p24A-



**Fig. 1.** Immunolocalization of p24 proteins in COS-1 cells using different denaturation conditions. COS-1 cells were fixed with 4% paraformaldehyde, permeabilized (A-F), denatured (B-E) and reduced (B-E) with the indicated substances in PBS-buffer and 10% FCS. Finally, cells were labeled with anti-MAL-p23 (A-D) and anti-p24A-NT (E). For a control, cells were labeled with the Golgi marker 58K protein (F). As a secondary antibody, a Cy3-coupled antibody was used.

**Fig. 2.** Costaining of p23 and p24A in pancreatic acinar cells under steady-state conditions, after short-term treatment with brefeldin A or nocodazole, and after incubation at reduced temperature (15°C). The distributions of the cis/medial Golgi marker mannosidase II, p23 and p24A were examined in rat pancreatic acinar primary culture cells. Cells were treated for 30 minutes at 37°C with 5 µg/ml brefeldin A to redistribute Golgi proteins, for 20 minutes with 5 µg/ml nocodazole to disrupt microtubules, or incubated for 3 hours at reduced temperature, 15°C, in HEPES-buffered medium. Cells were then prepared for indirect immunofluorescence microscopy with the indicated antibodies. For the costaining experiments the antibodies MAL-p23 and MAL-p24A were used and labeled with anti-rabbit DTAF-conjugated and anti-mouse Texas Red-conjugated antibodies. The images of the p23 and the p24A label were recorded with a video camera (see Materials and methods). Mannosidase II was labeled with a Cy3-coupled secondary antibody. Images are from the same cell population, grown, treated and fixed together in the same Petri dish.



labeled pre-Golgi elements and the perinuclear Golgi area were in most cases costained by anti-ERGIC-53, a marker for the intermediate compartment (Schweizer et al., 1990) that cycles between the ER and the cis-side of the Golgi complex (Lippincott-Schwartz et al., 1990) (Fig. 3, yellow overlay elements). However, peripheral structures containing ERGIC-53 did not overlap completely with anti-NT-p24A and GFP-p24A (see thick arrowhead for separate p24A and thin arrow for separate ERGIC-53 (Fig. 3, overlay). Similar results were obtained with anti-MAL-p23 and GFP-p23 (not shown). The cis/medial Golgi marker mannosidase II overlapped with GFP-p24A in the cisternal Golgi elements but not in the periphery (Fig. 4, 'steady state').

To further investigate if p24A exhibit the same distribution characteristic as p23, we treated cells with the microtubule-disrupting agent nocodazole, the fungal metabolite brefeldin A (BFA) and with a reduction in temperature (15°C).

#### Effect of brefeldin A (BFA)

BFA is known to induce the release of COPI from Golgi membranes and to induce redistribution of proteins of the Golgi complex (Lippincott-Schwartz et al., 1989; Donaldson et al., 1990, 1992; Orci et al., 1991). Treatment of cells with

BFA induced the redistribution of p23 and p24A from the periphery to IC elements, marked by anti-MAL-p23 (see arrows in Fig. 2, 'steady state' and 'brefeldin A') and by anti-ERGIC-53 (Fig. 3, yellow overlay elements). This was in contrast to the distribution of the cis/medial-Golgi resident mannosidase II, which was present in Golgi cisternae at steady state (Fig. 2) and spread to the ER-like periphery following treatment with brefeldin A (Fig. 2).

#### Effect of nocodazole

In cells treated with nocodazole to depolymerize microtubules, p24A-containing membranes were rapidly dispersed (within 7.5-20 minutes) into large, peripheral structures of varying size (Fig. 3, 'nocodazole'), which also contained p23 (Fig. 2, arrows) and ERGIC-53 (Fig. 3, yellow overlay elements). Mannosidase II remained in the cisternae-like structures, where it was colocalized with a minor portion of GFP-p24A (see Fig. 4, yellow overlay elements). p24A did not colocalize with the trans-Golgi network marker  $\gamma$ -adaptin (Fig. 4, bottom). The overlapping signals of both in the perinuclear area (yellow) are due to superimposition of close but separate fluorescent signals. Peripheral elements were never costained.

### Effect of reduced temperature (15°C)

To investigate if p24A travels through peripheral elements of the intermediate compartment, we kept cells for 3 hours at 15°C. This allows entry of proteins into the intermediate compartment, but prevents their anterograde delivery to the Golgi cisternae (Saraste and Kuismanen, 1984; Kuismanen and Saraste, 1989). After a 3 hour incubation period at 15°C, mannosidase II was detected in large tubular, cisternae-like structures (Fig. 2). In addition to cisternae-like structures, p24A and p23 were also localized in peripheral membrane components (Fig. 2). As shown in Fig. 4, reduced temperature leads to an accumulation of GFP-p24A in the periphery, while the cisternal, mannosidase-overlapping portion of p24A was reduced (Fig. 4, 15°C). The peripheral structures were positive for the IC-marker ERGIC-53 (Fig. 3, yellow overlay elements) and the p24A-homologue p23 (Fig. 2, arrows).

Depolymerization of the actin cytoskeleton with cytochalasin D (5 µM) or latrunculin B (5 µM) for 2 hours led to rounding up of treated cells, but had no effect on the steady-state localization of p24A and p23 (not shown).

In summary, our localization experiments imply that p24A resides in membranes of the intermediate- and the cis-Golgi compartment. Results obtained with BFA, nocodazole and treatment at 15°C lead us to conclude that p24A behaves like a protein of the intermediate compartment and neither like a cis/medial-Golgi-resident nor a trans-Golgi network protein. The localization and distribution of p24A and p23 suggest a constitutive anterograde IC to Golgi mechanism for both proteins. To further characterize the anterograde transport we have performed *in vivo* trafficking experiments.

### *In vivo* trafficking of GFP-p24A and GFP-p23

To visualize the anterograde movement of p24A and p23 from the periphery to the cisternae, we transfected COS-1 cells transiently with GFP-p23 or GFP-p24A and stopped protein translation 16–20 hours after transfection by addition of cycloheximide. 2 hours later, cells were incubated for 3 hours at 15°C to allow accumulation of GFP-p24A/p23 in the IC. Localization of the GFP-p24A and GFP-p23 was imaged with a confocal laser scanning system and movement of the proteins was documented *in vivo*. Following rewarming to 30–32°C, peripheral IC elements of GFP-p24A and GFP-p23 translocated rapidly to the perinuclear Golgi area. The moving structures had the characteristics of the recently described pre-Golgi carriers, which use microtubules as tracks to move into the Golgi complex area (Presley et al., 1997; Scales et al., 1997). The carriers were of varying size (0.2–1 µm) and translocated as discrete entities *en masse* from the periphery to the perinuclear Golgi region with a maximal speed of up to 1.6 µm s<sup>-1</sup> (Fig. 5). Occasionally, they traveled in a ‘stop-and-go’ fashion. Sometimes, carriers in the periphery stopped, separated into two carriers and moved into opposite directions or stayed in the periphery. These characteristics argue against transport via small, COP-like vesicles, which have a diameter of about 100 nm and are thought to diffuse from their donor compartment to the acceptor compartment. A discussion of vesicle diffusion versus microtubule-dependent movements of membranes has recently been published by Bloom and Goldstein (1998).

Disruption of microtubules in cells which had been kept at 15°C did not influence the IC-localization of endogenous p24A

and p23 (immunofluorescence data, not shown). Rewarming of cells, which had been kept at 15°C, to 37°C in the presence of nocodazole induced the rapid formation of large vesicular and tubular membranes, which were not comparable to those labeled by the cis/medial marker mannosidase II (immunofluorescence data, not shown).

Our data demonstrate that p24A and p23 undergo a retrograde path to peripheral IC elements, from where they travel by anterograde orientated, microtubule-dependent carriers to the perinuclear Golgi complex.

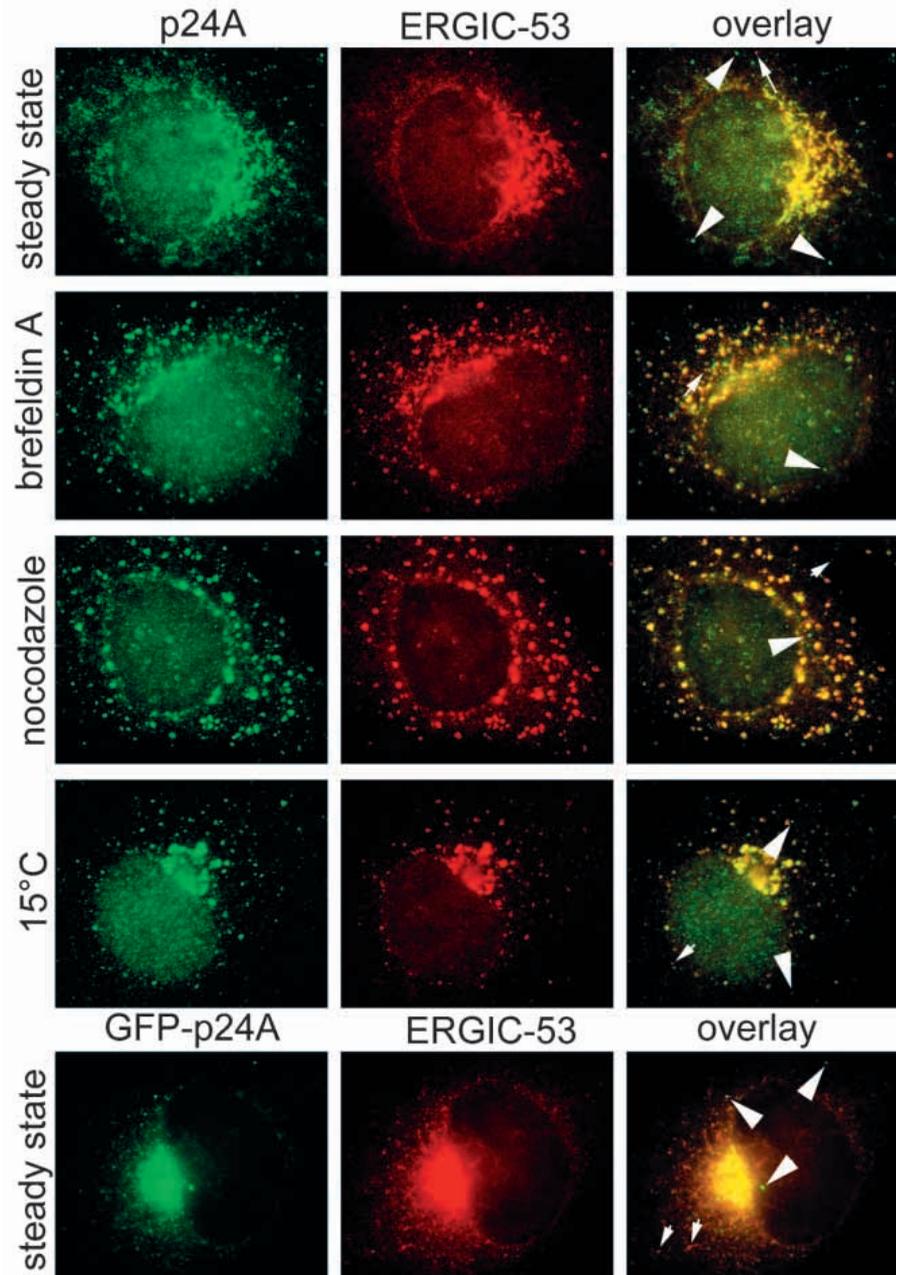
### p24A and p23 trafficking is completely blocked by aluminum fluoride

To further characterize movement of GFP-p24A, we bleached part of the perinuclear Golgi area in which GFP-p24A was localized at steady state with a high-energy laser scan of our confocal system. Recovery of the fluorescence in the bleached area was then monitored at lower laser intensities. Following bleaching of a part of the Golgi area, fluorescence recovery in this area occurred within seconds (Fig. 6, left panels). Changes in the perinuclear GFP-fluorescence intensities indicate a continuous movement of GFP-p24A labeled Golgi structures. Whereas single pre-Golgi carriers translocate rapidly between the periphery and the perinuclear Golgi area, the main portion of peripheral elements travel through the cytoplasm (Fig. 6, left panels). The same cell was then incubated for 30 minutes with AlF<sub>4</sub><sup>-</sup>, a general activator of heterotrimeric G-proteins (for a review, see Helms, 1995), which is known to lock COPI-coat proteins on Golgi membranes in a manner similar to guanosin-5'-O-(3'-thio-triphosphate) (GTPγS) (Donaldson et al., 1991). AlF<sub>4</sub><sup>-</sup> is also known to inhibit lateral movements of GFP-chimera in cis/medial-Golgi membranes by disruption of their continuity, while it has no effect on movements of ER-localized GFP-chimera (Cole et al., 1996). When the same perinuclear region was bleached in the presence of AlF<sub>4</sub><sup>-</sup>,

**Table 1. The effect of GFP-p24A/p23 overexpression on the integrity of the Golgi apparatus as estimated by localization of ERGIC-53**

GFP-construct	Cells showing normal ERGIC-53 localization at various times after transfection of GFP-chimera (%)		
	7 hours	24 hours	48 hours
Luminal-GFP	97	97	98
	96	98	96
GFP-KDEL	98	93	90
	96	93	89
GFP-p24A	93	60	36
	92	55	35
GFP-p23	92	69	34
	90	60	38

COS-1 cells were grown on coverslips and transfected with luminal-GFP, GFP-KDEL, GFP-p24A and GFP-p23. 7, 24 and 48 hours after transfection, cells were fixed and labeled with anti-ERGIC-53 (primary) and anti-mouse Texas Red-conjugated secondary antibody. Then the colocalization pattern of ERGIC-53 and GFP was investigated. Cells expressing an intact distribution of ERGIC-53 were compared to those with an altered ERGIC-53 localization (see Fig. 7) and shown as a percentage of all counted cells. In each individual experiment 500 transfected cells correspond to 100%. Values are the data from two independent experiments.



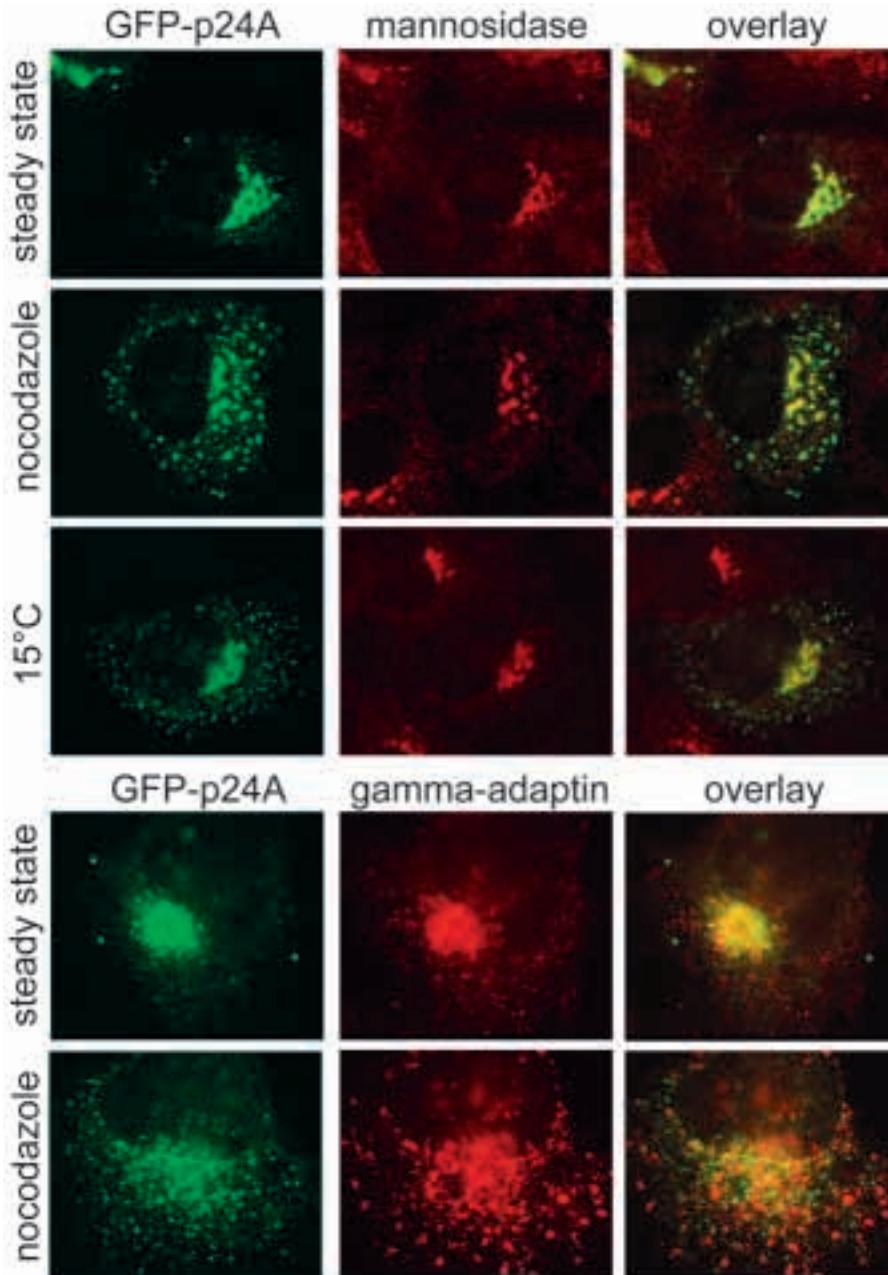
**Fig. 3.** Costaining of p24A, GFP-p24A and ERGIC-53 in COS-1 cells. Cells were pretreated and prepared for immunostaining as described in Fig. 2. The images of p24A (green) and ERGIC-53 (red) were recorded with a video camera and the pictures were combined by the Adobe photoshop program, resulting in the yellow color of overlapping localization (overlay). Brefeldin A and nocodazole were added to the cells to induce the formation of dispersed Golgi complex elements. The p24A signal overlapped largely with the intermediate compartment marker ERGIC-53. Single, vesicle-like elements were either p24A-positive (thick arrowheads) or ERGIC-53-positive (thin arrows). Similar results were obtained in cells kept for 3 hours at 15°C. This result was confirmed with the fluorescence signal of GFP-p24A, transiently expressed for 24 hours in COS-1 cells and compared with the indirect ERGIC-53 label (bottom).

fluorescence recovery was dramatically reduced. The pre-Golgi movements of GFP-p24A/p23 in the periphery were completely abolished in the presence of AIF<sub>4</sub><sup>-</sup> (Fig. 6, right panels, arrows). (Time-lapse sequences demonstrating the dynamic and in vivo morphology of GFP-p24A/p23-labeled transport intermediates of the IC-cis-Golgi area of NRK-cells and the consequences of AIF<sub>4</sub><sup>-</sup> treatment on p24-trafficking are available as Quicktime movies at [http://www.med-rz.uni-sb.de/med\\_fak/physiol2/schulz/blum/pb1.html](http://www.med-rz.uni-sb.de/med_fak/physiol2/schulz/blum/pb1.html), section: *Additional movies*.)

The in vivo trafficking confirms our localization data demonstrating that p24A and p23 behave like typical Golgi proteins and not like ER proteins. Furthermore, the observations suggest that a trimeric G-protein is involved in the process of pre-Golgi movements (see Discussion).

### Overexpression of GFP-p24A and GFP-p23 alters the morphology of the Golgi complex

In cells overexpressing GFP-p24A/p23, these proteins were localized to the perinuclear Golgi area. However, the morphology of the IC-Golgi complex area was destroyed. The effect of p24A/p23 overexpression on the morphology of COS-1 cells was investigated 7 hours, 24 hours and 48 hours after transient transfection of GFP-chimera. 'Destroyed' Golgi areas were distinguished from 'intact' Golgi-structures by the loss of the typical colocalization pattern of GFP-p24A/p23 and ERGIC-53 (Fig. 7, GFP-p24A, A versus D). 48 hours after transfection GFP-p23 and GFP-p24A were about 5- to 6-fold overexpressed as compared to endogenous p24A/p23 (transfection rate in these experiments >70%). In cells expressing high amounts of GFP-p24A/p23, the GFP-chimera

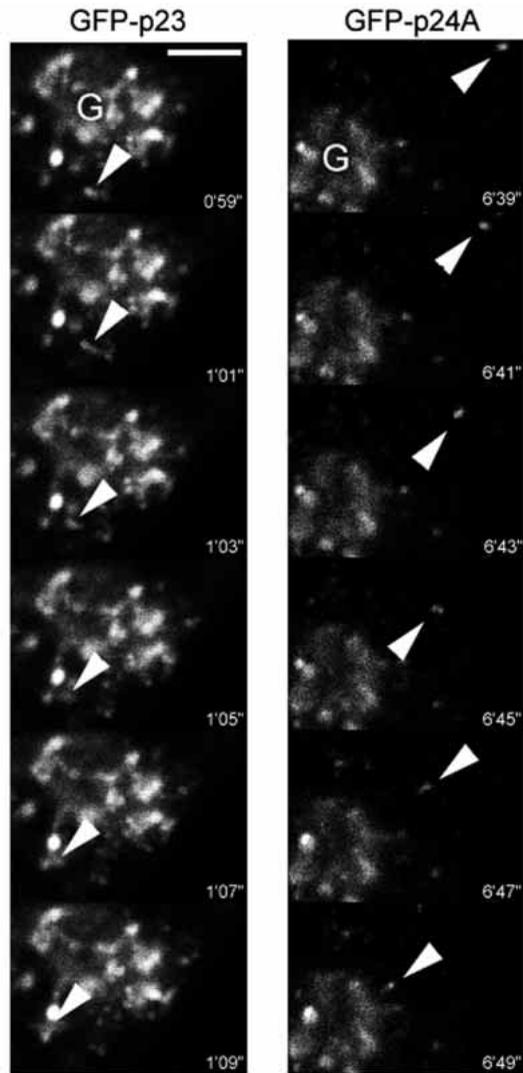


**Fig. 4.** Immunolocalization of GFP-p24A at steady state, after microtubule disruption and after incubation at reduced temperatures (15°C). For mannosidase II-costaining, GFP-p24A was transiently expressed in normal rat kidney (NRK-52E) cells for 20 hours. For labeling with  $\gamma$ -adapain COS-1 cells were used. Cycloheximide (10  $\mu$ g/ml) was then added to stop the protein translation. Nocodazole (5  $\mu$ g/ml) was added to the cells 20 minutes before fixation. Keeping the cells in HEPES-buffered medium for 3 hours at 15°C induced the accumulation of GFP-p24A into the intermediate compartment. Immunolocalization experiments and image-processing were performed as described above. Microtubule disruption by nocodazole segregates the main portion of GFP-p24A from cis/medial-Golgi (mannosidase) and trans-Golgi proteins ( $\gamma$ -adapain) as demonstrated in the overlay. Reduced temperature, blocking the anterograde IC-to-Golgi protein transport, accumulates GFP-p24A in the IC-like periphery (see also Figs 2 and 3).

formed large membranous perinuclear structures. The typical cysternal Golgi-elements were destroyed (Fig. 7C,I). Additionally, the IC-cis-Golgi marker ERGIC-53 lost its typical perinuclear localization and appeared in a peripheral, punctate staining (Fig. 7F). A similar effect was observed for the trans-Golgi network marker  $\gamma$ -adapain (not shown). The cis/medial Golgi marker mannosidase II, which is normally localized to perinuclear cysternal Golgi elements (Fig. 7K), appeared in a peripheral punctate staining or in fragmented Golgi elements (see Fig. 7L). It is presently unclear to which membranes the markers ERGIC-53,  $\gamma$ -adapain and mannosidase II were distributed.

7 hours after transfection only about 7% of GFP-p24A/p23-expressing cells showed an altered Golgi complex, but after 48 hours Golgi morphology of more than 60% of the cells was

destroyed (see Table 1). Cells containing a soluble GFP-protein, which was translocated into the ER lumen using the signal peptide of p23 (luminal GFP, see Materials and methods), showed no significant structural modifications of intracellular membranes. This indicates that neither GFP nor the ER-translocation process nor any influence by high levels of CMV-promoter-mediated expression were responsible for destruction of the Golgi complex (Table 1). 48 hours after transient transfection, cells expressing high amounts of GFP-p24-chimera were only rarely stained in Trypan Blue cell viability exclusion tests, indicating that the predominant portion of overexpressing cells was still alive. To determine if the concentration of GFP in the ER-to-Golgi trafficking area affects the IC/cis-Golgi morphology, we used a luminal GFP-chimera containing a KDEL motif. The KDEL motif functions



**Fig. 5.** In vivo traveling of GFP-p24A and GFP-p23 from the intermediate compartment to the Golgi complex. GFP-p24A- and GFP-p23-expressing cells were incubated for 3 hours at 15°C in Hepes-buffered medium to localize both proteins in the intermediate compartment. The in vivo traveling of the GFP-proteins after rearming the cells to 30–32°C was imaged using a confocal laser scanning system (Bio-Rad, MRC 600). Images were collected at low laser intensities to reduce the fluorescence bleaching rate. Image series showing the movement of typical highly motile pre-Golgi structures (marked by arrowheads) are shown for both proteins. The perinuclear fluorescence known to be the Golgi complex is marked by G. The pre-Golgi carrier structure, marked by GFP-p24A fluorescence, moved over an estimated distance of 9.7  $\mu\text{m}$  in a time of 10 seconds with varying speeds (speed maximum up to 1.6  $\mu\text{m s}^{-1}$ ). The carrier length changed within the movement, but ranged in the area of about 0.2–0.5  $\mu\text{m}$ . For the GFP-p23-marked carrier, a length of about 1  $\mu\text{m}$  was estimated. It should be mentioned that the size of small structures heavily loaded with fluorescent proteins may be overestimated. Bar, 5  $\mu\text{m}$ .

as retention and retrieval motif for luminal ER-proteins (Munro and Pelham, 1987; Lewis and Pelham, 1992). The strongly expressed GFP-KDEL construct appeared in the ER network and in perinuclear IC elements (Fig. 7G), where it partially colocalized with ERGIC-53 (Fig. 7J). As shown in Table 1,

overexpression of GFP-KDEL had only a weak destructive effect on the ER-to-Golgi trafficking pathway.

An obvious difference between GFP-p23- and GFP-p24A-expressing cells could be seen in these experiments. 7 hours after transient transfection, 8–9% of GFP-p24A-expressing cells with an intact ERGIC-53 distribution pattern showed, in addition to IC-cis-Golgi localization, a typical reticular staining of the ER. 24 hours after transfection, in 31–35% of the ‘intact’ cells, GFP-p24A appeared in the ER, while after 48 hours GFP-p24A was observed in the ER of 75–79% of the cells (see Fig. 7B, compared with E, ERGIC-53). GFP-p23-expressing cells showed no ER-like structures after transient overexpression in COS-1 cells. These results show that localization and trafficking behavior as well as the morphology of intracellular membranes can be significantly altered by recombinant overexpression of p24-members.

## DISCUSSION

### p24A travels through the early protein transport pathway

We have investigated the intracellular localization and in vivo trafficking of p24A and p23, two integral membrane proteins of the p24 protein family. The only mammalian p24 member for which localization has been described is p23. Sohn et al. (1996) localized p23 to the Golgi cisternae and Nickel et al. (1997) showed that p23 appeared in the intermediate compartment (IC) after incubation of cells at 15°C. Rojo et al. (1997) showed, however, that p23 is a major component (30% of the total protein) of IC/cis-Golgi network membranes.

We show that p24A and p23 are parts of IC/cis-Golgi network membranes and cycle constitutively between peripheral elements of the intermediate compartment and the cis-Golgi network. In vivo trafficking using GFP-chimera identifies p24A and p23 as integral membrane components of pre-Golgi carriers, which move in a microtubule-dependent manner from the IC to the Golgi complex. Results obtained with BFA, nocodazole and temperature lowering to 15°C indicate that p24A and p23 behave like proteins of the intermediate compartment and not like a cis/medial-Golgi-resident or a trans-Golgi network protein. Localization and trafficking of p24A are similar to that of p23. p24A and p23 reside in membranes that seem to connect the IC and the cis-Golgi network.

### IC-to-cis-Golgi transport of p24A and p23 by microtubule-dependent pre-Golgi carriers

Recent studies suggested that p24A and p23 travel by COPI vesicles through the early protein transport pathway (Fiedler et al., 1996; Sohn et al., 1996). It has been shown that the cytoplasmic tail of p24 members was able to bind in a bimodal manner to COPI proteins in vitro (Fiedler et al., 1996). p23 has been shown to concentrate into COPI-coated buds and vesicles (Sohn et al., 1996) and its cytoplasmic tail is also able to bind to COPI proteins. Several publications in recent years have focused on the question whether COPI vesicles mediate either anterograde or retrograde ER to Golgi transport or whether COPI vesicles move in both directions (for a review see Schekman and Mellman, 1997).

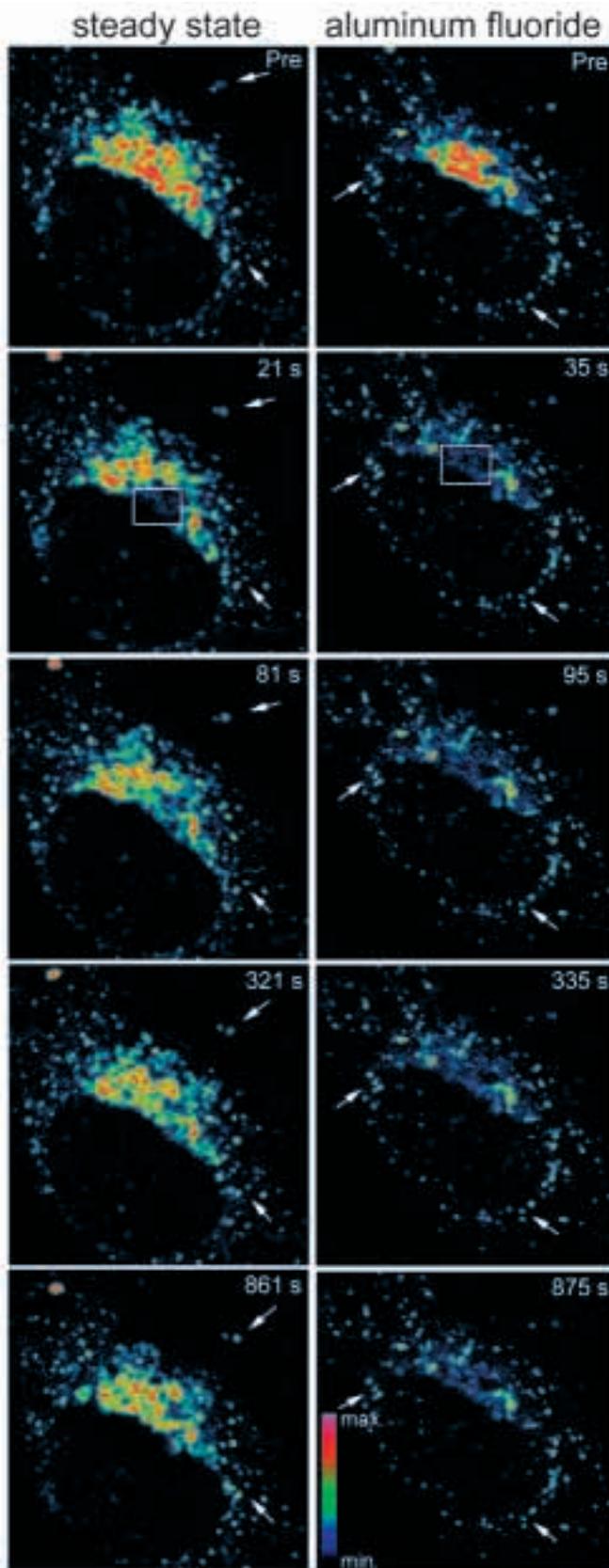
Our data suggest that p24A and p23 do not travel by small

vesicles, like COP vesicles, from the peripheral IC to the Golgi complex. The anterograde movement of both proteins occurs in a microtubule-dependent manner by pre-Golgi carriers of

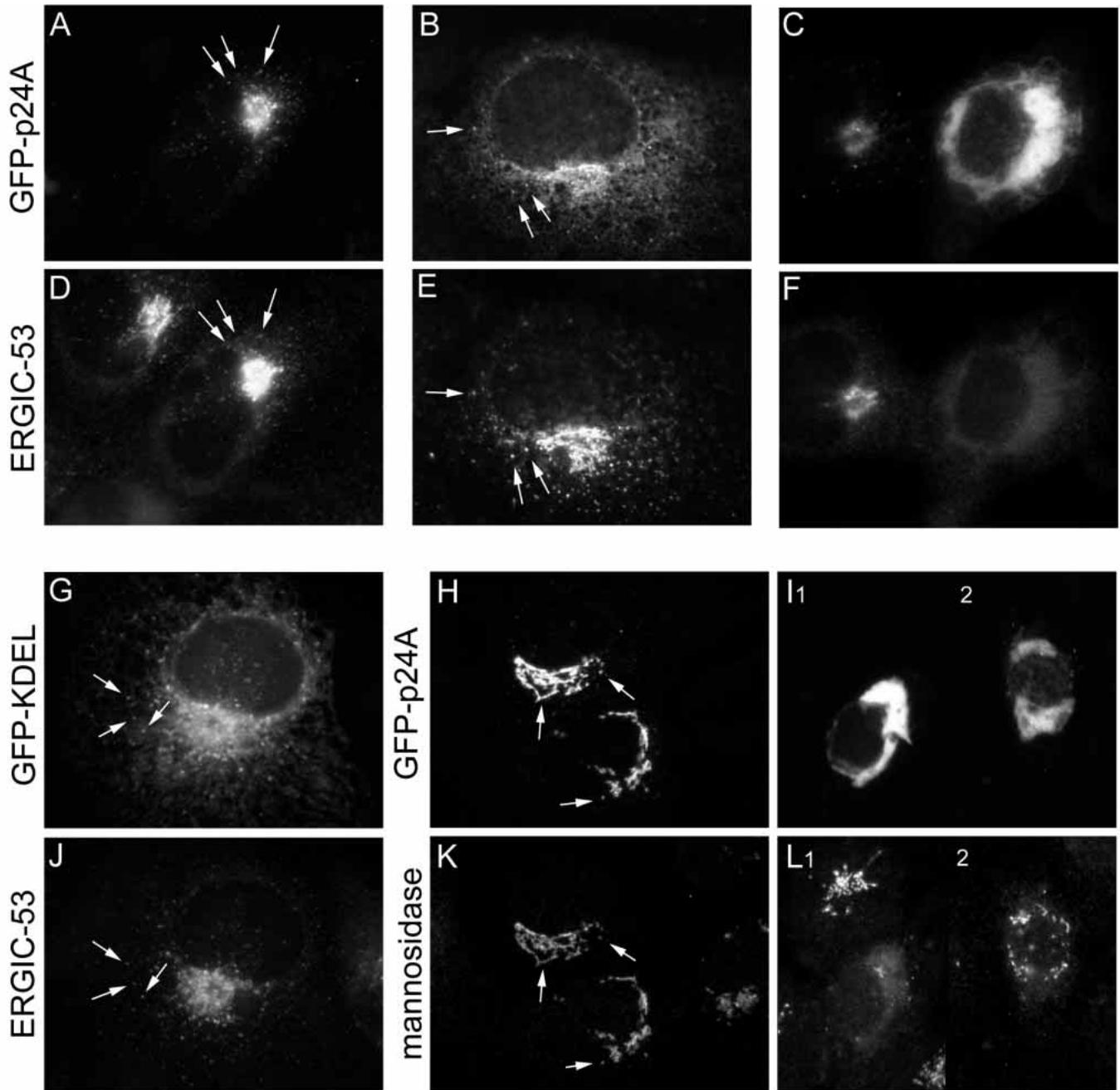
varying size. Our main argument against COP-vesicle-mediated IC-to-Golgi transport of GFP-p23/p24A are the characteristics by which the IC-cis-Golgi movement occurs (velocity, 'en masse' movement, 'stop and go' fashion, and size variations of the GFP-labeled structures). There is no inconsistency between biochemical data on the interaction of the p24-cytosolic tails with COPI proteins (Fiedler et al., 1996; Sohn et al., 1996) and our trafficking and localization data. Anterograde ER to Golgi vesicles and/or carriers are coated by COPI proteins (Aridor et al., 1995) and pre-Golgi carriers can be labeled by anti- $\beta$ -COP antibodies (Presley et al., 1997). The exact function of COPI coats is not known yet, but COPI coats could support the formation of pre-Golgi carriers for which the interaction with p24 proteins is necessary. The p24-COPI interaction could also be essential for intra-Golgi and/or retrograde transport mechanisms.

However, small vesicles with very low fluorescence intensity might not be detected using confocal imaging of GFP-chimera. Therefore we cannot completely exclude the presence of small vesicles, like COP vesicles, in the IC-to-Golgi transport pathway. The recently introduced 'vesicle on string theory' (Orci et al., 1998) should also be considered. This theory argues that transport vesicles neighboring cis- or trans-Golgi stacks are tethered to the stacks by flexible fibrous elements described as strings. These strings seem to fix COPI vesicles locally near their origin and therefore prevent free diffusion of budded vesicles. Such small 'vesicles on strings' emitting GFP-p24A- and/or GFP-p23-derived fluorescence would be masked by the strong fluorescence derived from the Golgi stacks to which they are fixed, and would therefore not be detectable.

Helms et al. (1998) describe an  $\text{AlF}_4^-$ -sensitive, membrane-bound Golgi-specific factor, a putative heterotrimeric G protein, which inhibits the fusion of COPI-coated vesicles. Such a mechanism could explain the complete block of p24A-trafficking in the presence of  $\text{AlF}_4^-$  in our experiments. The small GTP-binding protein Ras is



**Fig. 6.** Fluorescence recovery after photobleaching (FRAP) of transiently expressed GFP-p24A localized to intermediate compartment and cis-Golgi membranes. Image sequence of a cell under steady-state conditions and after aluminum fluoride treatment before (pre) and after photobleaching. The indicated area was bleached by scanning it with a high energy laser scan. To ensure that the optical plane remained adequately stable, the aperture of the confocal imaging system was maximally opened and several fluorescent points in the periphery served as intrinsic focal plane controls (see arrows). Recovery of the fluorescence was monitored with low laser power. Cells were then treated with aluminum fluoride ( $\text{AlF}_4^-$ ; 30 mM NaF, 50  $\mu\text{M}$   $\text{AlCl}_3$  for 30 minutes in HEPES-buffered medium) and the indicated area of the same cell was bleached and recorded as described above. To keep an impression of the Golgi structure, the indicated area was not bleached completely. At steady state, intensive fast movements of pre-Golgi structures were observed and fluorescence in the bleached area reappeared within seconds. In the presence of  $\text{AlF}_4^-$  fluorescence recovery in the Golgi area was inhibited. Peripheral movements of pre-Golgi intermediate compartment structures were completely blocked (see arrows, right panel). The experiment is shown by pseudocolor pictures. Red color corresponds to high fluorescence intensities, as indicated in the scale at the bottom of the right panel. Quicktime movie sequences are available at: [http://www.med-rz.uni-sb.de/med\\_fak/physiol2/schulz/blum/pb1.html](http://www.med-rz.uni-sb.de/med_fak/physiol2/schulz/blum/pb1.html)



**Fig. 7.** Overexpression of GFP-p24A alters the Golgi morphology. GFP-chimera were transiently expressed in COS-1 (A-G,J) and NRK-52E cells (H,I,K,L) and colocalization experiments were performed as described in Materials and methods. Cells expressing GFP-chimera for 48 hours were examined for their Golgi morphology. Cells expressing high amounts of GFP-chimera could be distinguished from cells expressing low amounts of GFP, by the relative intensity of the GFP-derived fluorescence. (In C, the left cell expresses low amounts of GFP-derived fluorescence and can be compared with the right cell expressing high amounts of GFP-p24A). The integrity of the typical ERGIC-53 distribution was observed in cells which contained low GFP-p24A amounts (compare A, GFP-p24A, with D, ERGIC-53). Cells expressing high amounts of GFP-p24A (C, right cell and I1 and I2) altered the morphology of ERGIC-53-labels (F, right cell) and mannose II-labels (L1 and L2). High amounts of GFP-KDEL, a luminal-orientated, soluble GFP-derivate, appeared in an ER-like reticular staining and in the perinuclear IC/cis-Golgi area (G). GFP-KDEL colocalized partially with ERGIC-53 (J) and did not alter the Golgi morphology. Overexpression could also induce delocalization of GFP-p24A to ER-like elements (B, compared with ERGIC-53, E). Arrows denote alignment of costained structures in A and D, B and E, G and J, and H and K.

also able to interact with  $\text{AlF}_4^-$  when it associates with GTPase-activating proteins (Mittal et al., 1996). Inhibition of p24-trafficking by  $\text{AlF}_4^-$  could therefore also involve small GTP-binding proteins.

#### **p24A and p23 exhibit the same traveling characteristics**

As shown here, p24A and p23 reside both in IC/cis-Golgi areas and exhibit the same traveling characteristics. Both proteins are

mainly colocalized. However, each small p24A-mediated fluorescence label did not colocalize with each p23 label (Fig. 2). The immunofluorescence technique does not provide high enough resolution to determine whether these small differences in the staining pattern of both proteins were significant.

p24A does not contain any functional ER-retrieval motif in its cytosolic tail (Fiedler et al., 1996). However, at relative high expression rates, which did not destroy the Golgi morphology, p24A also appeared in ER-like elements of COS-1 cells (see Fig. 7B). An explanation for this p24A-specific effect could be that the anterograde biosynthetic ER to Golgi transport mechanism for p24A limits transport efficiency. It could also be that overexpression of p24A saturates its Golgi retention mechanism and therefore p24A is delocalized to the ER by a retrograde Golgi to ER pathway. The study opens further questions about the mechanisms which are responsible for the proper IC-Golgi localization of p24A and p23.

We thank Prof. Dr Bernd Lindemann, Dr David Stevens and Dr Martin Jung for discussions. We also thank Frau Christel Waschow for her excellent technical assistance. We are grateful to Dr H. P. Hauri (Biocenter Basel, Switzerland) for his gift of ERGIC-53 antibodies. This work was supported by grants from the Deutsche Forschungsgemeinschaft to I. S. (Schu 429/10-1).

## REFERENCES

- Anderson, K. L. and McNiven, M. A. (1995). Vesicle dynamics during regulated secretion in a novel pancreatic acinar cell *in vitro* model. *Eur. J. Cell Biol.* **66**, 25-38.
- Aridor, M., Bannykh, S. I., Rowe, T. and W. E. Balch (1995). Sequential coupling between COPII and COPI vesicle coats in endoplasmic reticulum to Golgi transport. *J. Cell Biol.* **131**, 875-893.
- Balch, W. E., McCaffery, J. M., Plutner, H. and Farquhar, M. G. (1994). Vesicular stomatitis virus glycoprotein is sorted and concentrated during export from the endoplasmic reticulum. *Cell* **76**, 841-852.
- Barlowe, C., Orci, L., Yeung, T., Hosobuchi, M., Hamamoto, S., Salama, N., Rexach, M. F., Ravazzola, M., Amherdt, M. and Schekman, R. (1994). COPII: a membrane coat formed by sec proteins that drive vesicle budding from the endoplasmic reticulum. *Cell* **77**, 895-907.
- Bednarek, S. Y., Ravazzola, M., Hosobuchi, M., Amherdt, M., Perrelet, A., Schekman, R. and Orci, L. (1995). COPI- and COPII-coated vesicles bud directly from the endoplasmic reticulum in yeast. *Cell* **83**, 1183-1196.
- Belden, W. J. and Barlowe, C. (1996). Erv25p, a component of COPII-coated vesicles, forms a complex with Emp24p that is required for efficient endoplasmic reticulum to Golgi transport. *J. Biol. Chem.* **271**, 26939-26946.
- Bloom, G. S. and Goldstein, L. S. B. (1998). Cruising along microtubule highways: How membranes move through the secretory pathway (Commentary). *J. Cell Biol.* **140**, 1277-1280.
- Blum, R., Feick, P., Puype, M., Vandekerckhove, J., Klengel, R., Nastainczyk, W. and Schulz, I. (1996). Tmp21 and p24A, two type I proteins enriched in pancreatic microsomal membranes, are members of a protein family involved in vesicular trafficking. *J. Biol. Chem.* **271**, 17183-17189.
- Cole, N. B., Smith, C. L., Sciaky, N., Terasaki, M., Edidin, M. and Lippincott-Schwartz, J. (1996). Diffusional mobility of Golgi proteins in membranes of living cells. *Science* **273**, 797-801.
- Cosson, P. and Letourneur, F. (1994). Coatamer interaction with di-lysine endoplasmic reticulum retention motifs. *Science* **263**, 1629-1631.
- Donaldson, J. G., Lippincott-Schwartz, J., Bloom, G. S., Kreis, T. E. and Klausner, R. D. (1990). Dissociation of a 110-kDa peripheral membrane protein from the Golgi apparatus is an early event in brefeldin A action. *J. Cell Biol.* **111**, 2295-306.
- Donaldson, J. G., Lippincott-Schwartz, J. and Klausner, R. D. (1991). Guanine nucleotides modulate the effects of brefeldin A in semipermeable cells: regulation of the association of a 110-kD peripheral membrane protein with the Golgi apparatus. *J. Cell Biol.* **112**, 579-588.
- Donaldson, J. G., Finazzi, D. and Klausner, R. D. (1992). Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. *Nature* **360**, 350-352.
- Fiedler, K., Veit, M., Stammes, M. A. and Rothman, J. E. (1996). Bimodal binding of coatamer with the p24 family of putative cargo receptors. *Science* **273**, 1396-1399.
- Helms, J. B. (1995). Role of heterotrimeric GTP binding proteins in vesicular protein transport: indications for both classical and alternative G protein cycles. *FEBS Lett.* **369**, 84-88.
- Helms, J. B., Helms-Brons, D., Brügger, B., Gkantiragas, I., Eberle, H., Nickel, W., Nürnberg, B., Gerdes, H. H. and Wieland, F. T. (1998). A putative heterotrimeric G-protein inhibits the fusion of COPI-coated vesicles. *J. Biol. Chem.* **273**, 15203-15208.
- Kistakis, N. T., Roth, M. G. and Bloom, G. S. (1991). PtK1 cells contain a nondiffusible, dominant factor that makes the Golgi apparatus resistant to brefeldin A. *J. Cell Biol.* **113**, 1009-1023.
- Kuismanen, E. and Saraste, J. (1989). Low temperature-induced transport blocks as tools to manipulate membrane traffic. *Methods Cell Biol.* **32**, 257-274.
- Letourneur, F., Gaynor, E. C., Hennecke, S., Demolliere, C., Duden, R., Emr, S. D., Riezman, H. and Cosson, P. (1994). Coatamer is essential for retrieval of dilysine-tagged proteins to the endoplasmic reticulum. *Cell* **79**, 1199-1207.
- Lewis, M. J. and Pelham, H. R. (1992). Ligand-induced redistribution of a human KDEL-receptor from the Golgi complex to the endoplasmic reticulum. *Cell* **68**, 353-364.
- Lippincott-Schwartz, J., Yuan, L. C., Bonifacino, J. S. and Klausner, R. D. (1989). Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. *Cell* **56**, 801-813.
- Lippincott-Schwartz, J., Donaldson, J. G., Schweizer, A., Berger, E. G., Hauri, H. P., Yuan, L. C. and Klausner, R. D. (1990). Microtubule-dependent retrograde transport of proteins into the ER in the presence of brefeldin A suggests an ER recycling pathway. *Cell* **60**, 821-836.
- Mittal, R., Ahmadian, M. R., Goody, R. S. and Wittinghofer, A. (1996). Formation of a transition-state analog of the Ras GTPase reaction by Ras-GDP, tetrafluoroaluminate and GTPase-activating proteins. *Science* **273**, 115-117.
- Munro, S. and Pelham, H. R. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**, 899-907.
- Nickel, W., Sohn, K., Bünning, C. and Wieland, F. T. (1997). p23, a major COPI-vesicle membrane protein, constitutively cycles through the early secretory pathway. *Proc. Natl. Acad. Sci. USA* **94**, 11393-11398.
- Orci, L., Glick, B. S. and Rothman, J. E. (1986). A new type of coated vesicular carrier that appears not to contain clathrin: its possible role in protein transport within the Golgi stack. *Cell* **46**, 171-184.
- Orci, L., Tagaya, M., Amherdt, M., Perrelet, A., Donaldson, J. G., Lippincott-Schwartz, J., Klausner, R. D. and Rothman, J. E. (1991). Brefeldin A, a drug that blocks secretion, prevents the assembly of non-clathrin-coated buds on Golgi cisternae. *Cell* **64**, 1183-1195.
- Orci, L., Perrelet, A. and Rothman, J. E. (1998). Vesicles on strings: Morphological evidence for processive transport within the Golgi stack. *Proc. Natl. Acad. Sci. USA* **95**, 2279-2283.
- Palade, G. E. (1975). Intracellular aspects of the process of protein synthesis. *Science* **189**, 347-358.
- Pepperkok, R., Scheel, J., Horstmann, H., Hauri, H. P., Griffiths, G. and Kreis, T. E. (1993).  $\beta$ -COP is essential for biosynthetic membrane transport from the endoplasmic reticulum to the Golgi complex *in vivo*. *Cell* **74**, 71-82.
- Peter, F., Plutner, H., Zhu, H., Kreis, T. E. and Balch, W. (1993). Beta-COP is essential for transport of protein from the endoplasmic reticulum to the Golgi *in vitro*. *J. Cell Biol.* **122**, 1155-1167.
- Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergarst, F. G. and Cormier, M. J. (1992). Primary structure of the *Aequorea victoria* green fluorescent protein. *Gene* **111**, 320-323.
- Presley, J. F., Cole, N. B., Schroer, T. A., Hirschberg, K., Zaal, J. M. and Lippincott-Schwartz, J. (1997). ER-to-Golgi transport visualized in living cells. *Nature* **389**, 81-85.
- Rojo M., Pepperkok, R., Emery, G., Kellner, R., Stang, E., Parton, R. G. and Gruenberg, J. (1997). Involvement of the transmembrane protein p23 in biosynthetic protein transport. *J. Cell Biol.* **139**, 1119-1135.
- Rothman, J. E. and Wieland, F. T. (1996). Protein sorting by transport vesicles. *Science* **272**, 227-234.
- Saraste, J. and Kuismanen, E. (1984). Pre- and post-Golgi vacuoles operate

- in the transport of Semliki Forest virus membrane glycoproteins to the cell surface. *Cell* **38**, 535-549.
- Scales, S. J., Pepperkok, R. and Kreis, T. E.** (1997). Visualization of ER-to-Golgi transport in living cells reveals a sequential mode of action for COPII and COPI. *Cell* **90**, 1137-1148.
- Schekman, R. and Mellman, I.** (1997). Does COPI go both ways? *Cell* **90**, 197-200.
- Schimmöller, F., Singer-Krüger, B., Schröder, S., Krüger, U., Barlowe, C. and Riezman, H.** (1995). The absence of Emp24p, a component of ER-derived COPII-coated vesicles, causes a defect in transport of selected proteins to the Golgi. *EMBO J.* **14**, 1329-1339.
- Schweizer, A., Fransen, J. A., Matter, K., Kreis, T. E., Ginsel, L. and Hauri, H. P.** (1990). Identification of an intermediate compartment involved in protein transport from the endoplasmic reticulum to Golgi apparatus. *Eur. J. Cell Biol.* **53**, 185-196.
- Sohn, K., Orci, L., Ravazzola, M., Amherdt, M., Bremser, M., Lottspeich, F., Fiedler, K., Helms, J. B. and Wieland, F. T.** (1996). A major transmembrane protein of Golgi-derived COPI-coated vesicles involved in coatamer binding. *J. Cell Biol.* **135**, 1239-1248.
- Stammes, M. A., Craighead, M. W., Hoe, M. H., Lampen, N., Geromanos, S., Tempst, P. and Rothman, J. E.** (1995). An integral membrane component of coatamer-coated transport vesicles defines a family of proteins involved in budding. *Proc. Natl. Acad. Sci. USA* **92**, 8011-8015.