p24 and p23, the major transmembrane proteins of COPI-coated transport vesicles, form hetero-oligomeric complexes and cycle between the organelles of the early secretory pathway

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Abstract COPI-coated vesicles that bud off the Golgi complex contain two major transmembrane proteins, p23 and p24. We have localized the protein at the Golgi complex and at COPI-coated vesicles. Transport from the intermediate compartment (IC) to the Golgi can be blocked at 15°C, and under these conditions p24 accumulates in peripheral punctated structures identified as IC. Release from the temperature block leads to a redistribution of p24 to the Golgi, showing that p24, similar to p23, cycles between the IC and Golgi complex. Immunoprecipitations of p24 from cell lysates and from detergent-solubilized Golgi membranes and COPI-coated vesicles show that p24 and p23 interact with each other to form a complex. Transient transfection of p23 in HeLa cells shows that p23 and p24 colocalize in structures induced by the overexpression of p23. Taken together p24 interacts with p23 and constitutively cycles between the organelles of the early secretory pathway.

Key words: p24 family; COPI-coated vesicle; Cycling; Secretory pathway; Golgi complex

1. Introduction

COPI-coated vesicles mediate transport within the early secretory pathway. Their coat consists of cytosolic proteins: coatomer, a complex of seven different polypeptides [1], and of ARF [2], a small GTPase. As a prerequisite for coat recruitment, the GTP-bound form of ARF has to bind to a Golgi membrane, before coatomer is able to bind [3,4]. In recent years membrane constituents were investigated that are involved in this budding process. Earlier studies have shown that coatomer is able to interact with resident transmembrane proteins of the endoplasmic reticulum that posses a sorting signal in their short cytoplasmic domains, with a double lysine motif at their C-termini. This motif is thought to interact with coatomer in the Golgi apparatus when such proteins have escaped from the endoplasmic reticulum (ER) [5], resulting in an efficient retrieval back to the ER of these proteins [6,7]. More recently a novel family of type I transmembrane proteins has been described: the so-called p24 family [8], members of which were also shown to interact with coat proteins in vitro via their short cytoplasmic C-termini, either with coatomer [9–11], or with COPII coat proteins [11], and were localized to COPI- and COPII-coated vesicles [8,10,12,13]. These findings led to the proposal that these transmembrane proteins could function as coat receptors during vesicle budding [8–10]. On the other hand, evidence from yeast suggests a function for these proteins as cargo receptors. Deletion of the gene coding for two yeast members of the p24 family, Emp24 and Erv25, resulted in delayed transport kinetics of some secretory proteins [12,13]. However, it is not yet clear whether these transport defects were due directly to missing cargo sorting proteins [12,13], or to impaired budding of transport vesicles [8].

To date, only two mammalian members of this protein family, p23 and p25, have been localized at the ultrastructural level [10,11,14]. Both proteins reside in the Golgi and the *cis*-Golgi network. It was shown that p23 cycles between Golgi and intermediate compartment [14,15], and is present in COPI-coated vesicles [10]. For mammalian p24, a detailed study of its intracellular localization is missing. This paper describes the localization of mammalian p24 as well as intracellular cycling of this protein between the organelles of the early secretory pathway. We show that the intracellular localization of p24 depends on p23 and that both proteins interact with each other. We propose that this interaction is the basis for the localization and cycling of these proteins.

2. Materials and methods

2.1. Antibodies

Antibodies directed against p24 were raised in rabbits and chickens with the following peptide corresponding to the cytoplasmic tail of p24 and coupled to KLH: CYLKRFFEVRRVV. IgYs directed against p23 were raised in chickens by immunizing with the peptide KITDSAGHILYSK also coupled to KLH. Immunizations and affinity purifications were performed according to standard protocols [16]. Monoclonal antibodies directed against ERGIC-53 were a kind gift of Hans-Peter Hauri (Biocenter Basel, Switzerland), antibodies against KDEL receptor were generously provided by Hans-Dieter Söling (University of Göttingen, Germany) and monoclonal antibodies against coatomer, CM1 [4], were from James E. Rothman (Sloan-Kettering Cancer Center, New York, USA). For localization of p23 by immunofluorescence microscopy rabbit polyclonal antibodies #1402 [10] were used.

2.2. Cycling of p24, immunofluorescence and electron microscopy

Where indicated HeLa cells were incubated at 15°C (using HEPES-buffered medium) to inhibit anterograde IC to Golgi transport [17]. HeLa cells were then prepared for indirect immunofluorescence according to standard protocols including fixation with 3% paraformaldehyde and permeabilization with Triton X-100. After the incubation of primary and secondary antibodies (Dianova, Hamburg, Germany)

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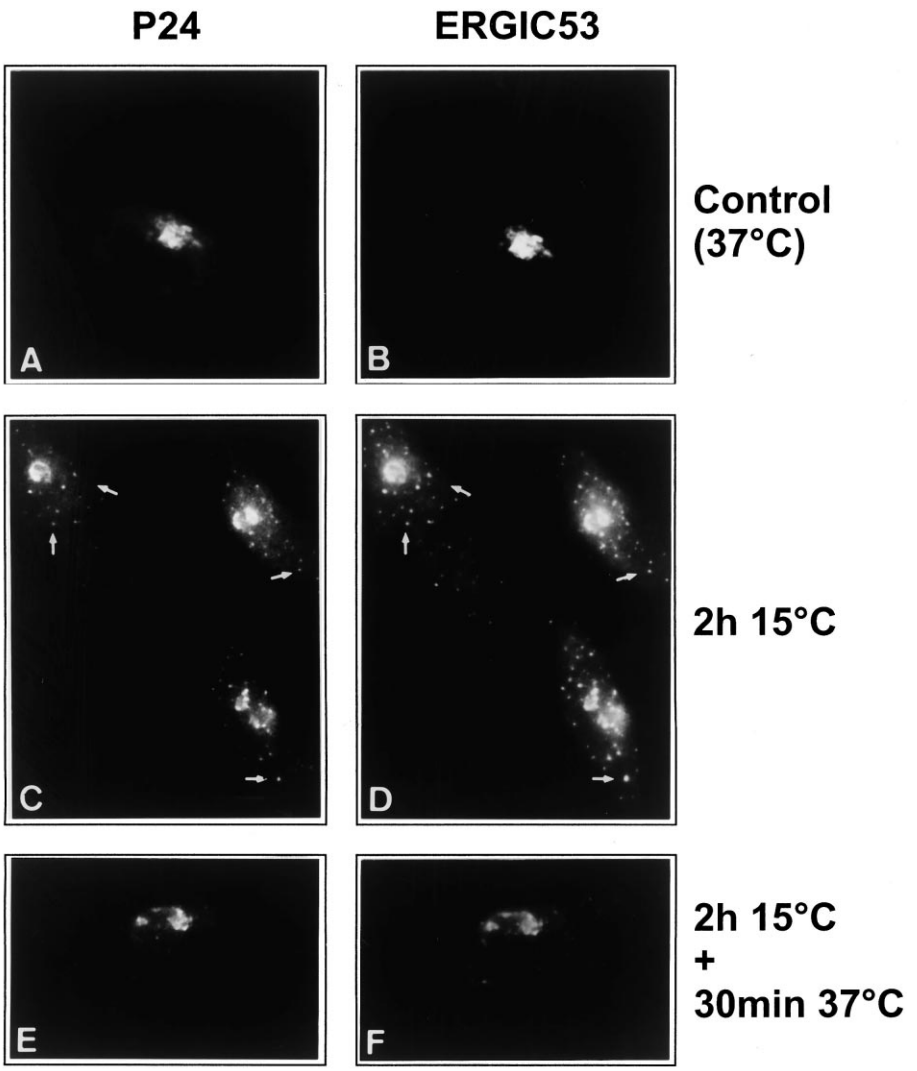

Fig. 1. Cycling of p24 between IC and Golgi. HeLa cells were incubated either at 37°C (A,B), 2 h at 15°C to block anterograde transport from the intermediate compartment to the Golgi (C,D) or 2 h at 15°C and subsequently 30 min at 37°C (E,F). Cells were then fixed for immunofluorescence using antibodies directed against p24 ('Elfriede', A,C,E) or ERGIC-53 (B,D,F). The block of transport at 15°C leads to an accumulation of p24 in peripheral, punctated structures which colocalize with ERGIC-53 (C,D). Upon shifting the temperature back to 37°C, p24 is released from these peripheral structures and is found in compact perinuclear structures typical for Golgi (E,F).

cells were washed and embedded in Fluoromount G (Biozol, Eching, Germany). Samples were viewed using a Zeiss Axiovert 35 microscope or a Leica TCS^{4D} laser scanning confocal microscope using a 63×1.4 NA plan apo objective (Leica Lasertechnik, Heidelberg, Germany). Each image is a single optical section taken midway between the top of the cell and the coverslip. For immunoelectron microscopy, thin Lowicryl sections [18], of purified rabbit liver COPI-coated vesicles were labelled with p24 antibody (dilution 1:5) revealed by gold (15 nm)-tagged, goat anti-rabbit antibody.

2.3. cDNA constructs and transfection of HeLa cells

$p23_{wt}$ construct: the cDNA of p23 from a full length clone (accession number: X98303) of a rabbit liver cDNA library (λgt10; Clontech, Palo Alto, CA, USA) was amplified by PCR and inserted into the eukaryotic expression vector p_{bi-5} [19], via a *Hin*dIII and *Eco*RV restriction site.

$p23_{myc}$ construct: the cDNA coding for the c-myc epitope EQKLISEEDL was inserted between signal sequence and N-terminus of the mature p23 molecule (Ile^{32}) by PCR-based splicing [20]. $p23_{wt}$ and $p23_{myc}$ constructs were verified by DNA sequencing.

For transient expression of constructs HeLa HtTA cells were grown on coverslips to 30–40% confluence according to [21]. DNA transfer was performed with 1 μg of plasmid DNA per coverslip using the calcium phosphate precipitation method according to [22]. 24 h after transfection cells were fixed and processed for immunofluorescence.

2.4. Subcellular fractionation and enrichment of p23 and p24

Golgi-enriched fractions from rat liver were isolated according to [23], without performing the final salt wash. COPI-coated transport vesicles were generated in vitro by incubating purified Golgi membranes with bovine brain cytosol in the presence of GTPγS and isolated according to [24].

Lipid extraction of Golgi membranes and COPI-coated vesicles was performed according to Bligh and Dyer [25]. The amounts of phospholipid were determined as described by Rouser et al. [26] using phosphatidyl choline as a standard. Equal amounts with respect to phospholipid content of purified COPI-coated vesicles and rat liver Golgi membranes were analyzed by Western blotting for the presence of p23 and p24.

2.5. Metabolic labelling of CHO cells, preparation of lysates and immunoprecipitations

Prior to labelling CHO cells were cultured for 2 h in a α-DMEM medium lacking methionine/cysteine (Sigma, St. Louis, MO, USA). After 2 h 300 μCi of PRO-MIX in vitro cell labelling mix (Amersham, Braunschweig, Germany) was added per petri dish and cells were

further incubated for 3 h at 37°C. Then cells were washed twice with PBS and pelleted to prepare the lysate.

For the preparation of cell lysates about $0.5–1\times10^7$ unlabelled or metabolically labelled CHO cells were pelleted and solubilized for 30 min on ice in 1 ml of IP buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.5% NP-40). Insoluble material was removed by a first centrifugation at $13\,000\times g$ for 10 min and by a second centrifugation at $100\,000\times g$ for 30 min. The supernatant was used as the lysate in subsequent immunoprecipitation studies.

For solubilization of proteins from Golgi membranes or COPI-coated vesicles under mild detergent conditions, purified Golgi membranes or COPI-coated vesicles from rat liver were pelleted ($100\,000\times g$, 30 min) and solubilized with 1 ml of IP buffer as described for CHO cells. To solubilize Golgi membranes under denaturing conditions, 1 ml of 1% SDS was added to 1 mg of pelleted Golgi membranes and samples were incubated at 95°C for 5 min. Afterwards SDS was diluted with 10 vol. of IP buffer and lysates were further prepared as described above.

For immunoprecipitations, antibodies directed against the cytoplasmic tail of p24 ('Elfriede') or control antibodies were bound to protein A-Sepharose 4B (Pharmacia, Uppsala, Sweden). Beads were incubated with lysate for 2 h at 4°C. Then beads were washed four times with IP buffer and once with PBS. Finally, bound proteins were eluted with a sample buffer and analyzed by SDS-PAGE and autoradiography or by Western blotting using chicken antibodies directed against p23 and p24 for detection.

3. Results

3.1. Localization and cycling of p24 in mammalian cells

Localization of p24 was first analyzed by immunofluorescence microscopy. As shown in Fig. 1A, p24 localizes to perinuclear structures, which are typical for Golgi. It has been shown earlier that p23 cycles between the intermediate compartment (IC) and Golgi complex [15]. When anterograde transport from the IC to Golgi is blocked by incubating cells at 15°C [17], p23 is trapped in peripheral structures that colocalize with ERGIC-53 or VSV-G protein [14,15]. We analyzed whether p24, like p23, also cycles between these compartments of the early secretory pathway. Incubation of HeLa cells at 15°C for 2 h led to an accumulation of ERGIC-53, previously described as a marker for the IC [27], in peripheral, punctated structures (Fig. 1D), which were not visible in control cells incubated at 37°C (Fig. 1B). Under these conditions resident Golgi proteins remain in a compact perinuclear localization [17]. The peripheral punctated structures accumulated at 15°C colocalize with p24 (Fig. 1C). In cells incubated at 15°C and subsequently shifted back to 37°C for 30 min, p24 is released from these peripheral structures and redistributes to a perinuclear localization (Fig. 1E). This indicates that p24, similar to p23, cycles between the organelles of the early secretory pathway, at least between the IC and the Golgi.

As originally p24 was described as constituent of COPI-coated vesicles [8], we used electron microscopy to morphologically analyze the presence of p24 in COPI-coated vesicles. Fig. 2A shows a thin Lowicryl section of a highly purified vesicles fraction, with strong immunogold labelling of the vesicular membranes.

Earlier studies have revealed that p23 is concentrated in COPI-coated vesicles compared to Golgi membranes [10]. This view was recently challenged [14]. To analyze whether p23 and p24 are enriched in this type of vesicle the relative amounts of both proteins were determined in purified Golgi membranes and COPI-coated vesicles from rat liver. Equal amounts with respect to phospholipid content of partially purified Golgi membranes and purified vesicles were analyzed by Western blotting for the presence of p23 and p24 (Fig. 2B). As a result both proteins are enriched about 4–5-fold in COPI-coated vesicles (Fig. 2B, compare lanes 1, 2 and 4) which for p23 is in good agreement with morphological data [10]. Although a highly purified Golgi fraction was used for the comparison of the relative amounts of p23 and p24, this subcellular fraction still contains to some extend contaminating membranes (e.g. endoplasmic reticulum, endosomes). Therefore the exact factor for the enrichment of p23 and p24 varies with the purity of the fractions. However, it is obvious that p23 and p24 are enriched in COPI-coated vesicles. This finding is in agreement with Stamnes et al. [8] and Sohn et al. [10], but in contradiction to Rojo et al. [14] who reported that p23 is not present in COPI-coated vesicles to a significant extent. We do not ultimately know the reasons for these different results, but we cannot exclude the hypothesis that by using a different source of membranes and a protocol for the purification of COPI-coated vesicles different from ours, Rojo et al. have isolated a vesicle fraction with lower purity, therefore containing less p23.

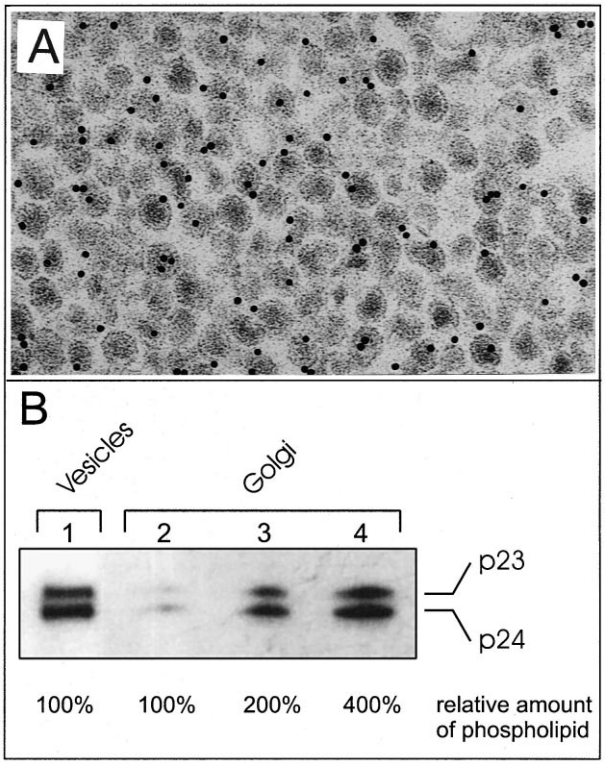

Fig. 2. Enrichment of p24 in COPI-coated vesicles. A: A thin Lowicryl section of a purified preparation of rabbit liver COPI-coated transport vesicles immunolabelled for p24. Magnification: ×52 000. B: Various amounts with respect to phospholipid content of purified COPI-coated vesicles (lane 1) and rat liver Golgi membranes (lanes 2–4) are analyzed for the presence of p23 and p24. Proteins were separated by Tricine SDS-PAGE [29], blotted and developed with antibodies directed against p23 and p24.

3.2. p24 forms a complex with p23

Immunoprecipitation studies with the antibodies directed against p24 were performed to identify proteins that specifically interact with p24. Lysates of metabolically labelled CHO cells were used for immunoprecipitation with anti-p24 antibodies bound to protein A-Sepharose. Bound immune com-

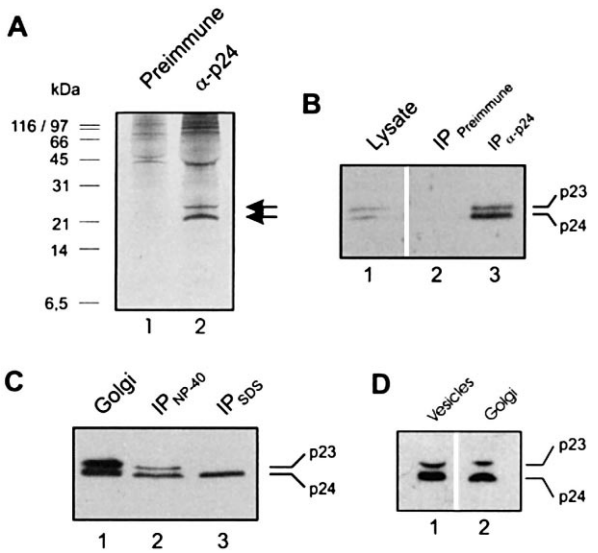

Fig. 3. Immunoprecipitations with anti-p24 antibodies. A: Lysate from metabolically labelled CHO cells was incubated either with preimmune serum (lane 1) or with antibodies directed against the cytoplasmic tail of p24 ('Elfriede' lane 2). Immune complexes were collected with protein A-Sepharose and bound proteins were eluted with sample buffer. Proteins were separated by Tricine SDS-PAGE (16.5% acrylamide) and analyzed by autoradiography. Arrows at the right indicate bands around 23 and 24 kDa which specifically precipitate with anti-p24 antibodies and which were not observed using preimmune serum. B: Immunoprecipitations with anti-p24 antibodies using lysate from unlabelled CHO cells. Input used for precipitation (lane 1), immunoprecipitates using preimmune serum (lane 2) or anti-p24 antibodies (lane 3) were separated by Tricine SDS-PAGE and analyzed by Western blotting using chicken antibodies directed against p23 and p24. C: Purified Golgi membranes from rat liver were solubilized with a buffer containing either NP-40 or SDS as detergent. Immunoprecipitations were performed using anti-p24 antibodies. Input (lane 1), immunoprecipitates from membranes solubilized with NP-40 (lane 2) or from membranes solubilized with SDS were analyzed by Tricine SDS-PAGE and subsequent Western blotting using chicken antibodies directed against p23 and p24. D: Immnuprecipitations from purified COPI-coated vesicles (lane 1) and Golgi membranes (lane 2). Golgi membranes and COPI-coated vesicles from rat liver were solubilized with buffer containing NP-40. Immunoprecipitations were carried out with anti-p24 antibodies and resulting immune complexes were analyzed as described above (B,C).

plexes were eluted and analyzed by SDS-PAGE and autoradiography. Two metabolically labelled bands with apparent molecular masses of around 23 and 24 kDa were precipitated (marked with arrows in Fig. 3A, lane 2). These bands were not precipitated using a preimmune serum (Fig. 3A, lane 1).

To identify these proteins, immunoprecipitations were carried out with unlabelled lysate from CHO cells. Immune complexes were analyzed by Western blotting using antibodies directed against p23 and p24. Fig. 3B shows that the upper band corresponds to p23, the lower one to p24. Immunoprecipitations of membranes solubilized under denaturing conditions using SDS instead of NP-40 reveal that only p24 is recognized by the antibodies during immunoprecipitation (Fig. 3C, lanes 2 and 3). These results demonstrate that under mild detergent conditions (NP-40) p23 and p24 can be coprecipitated as a hetero-oligomeric complex. As both proteins were found to be enriched in COPI-coated vesicles we analyzed whether this complex is also present in these carriers. Purified COPI-coated vesicles were solubilized under mild detergent conditions (NP-40) and immunoprecipitations for p24 were carried out as described for Golgi membranes. Analysis of the resulting immune complexes by Western blotting revealed that a similar hetero-oligomeric complex is also present in COPI-coated vesicles (Fig. 3D, lane 1).

3.3. Localizations of p23 and p24 depend on each other in vivo

In order to perform in mammalian cells double labelling of both p23 and p24, two different constructs of p23 were made for transient transfection. One construct was homologous to wild-type p23 (named $p23_{wt}$). Another construct was made by inserting a myc-epitope [28] between signal sequence and N-terminus of the mature p23 molecule (named $p23_{myc}$). Transient overexpression of both the wild-type and the myc-tagged constructs resulted in a localization at steady state different from the distribution of endogenous p23. It has been shown earlier that endogenous p23 resides in compact perinuclear structures that colocalize with coatomer (Fig. 4A–C) [10] and with the KDEL receptor [14]. Interestingly, overexpressed $p23_{wt}$ and $p23_{myc}$ molecules do not colocalize with coatomer (Fig. 4D–F,G–I) or with KDEL receptor (Fig. 4J–L). Overexpression of constructs induces the formation of structures derived from membranes of the ER positive for calreticulin (data not shown), but devoid of coatomer or KDEL receptor. These structures are unique for HeLa cells overexpressing p23, as they are not present in wild-type cells. In this context, it is not clear whether they reorganize the structures of the secretory pathway. Analysis of the distribution of endogenous p24 of cells transfected with the $p23_{myc}$ construct revealed that these structures that are positive for $p23_{myc}$ also contain endogenous p24 (Fig. 4M–O). Although p24 in wild-type cells is localized to compact perinuclear structures, p23 seems to redistribute p24 to structures induced by its overexpression.

As we have shown that p23 and p24 form complexes which can be isolated by immunoprecipitation, redistribution of p24 most likely is due to formation of a complex containing p23 and p24 in vivo. Thus the steady state localization in wild-type cells of both molecules would depend on their physical interaction.

4. Discussion

p24 is localized to organelles of the early secretory pathway and cycles continuously between these structures. Similar to

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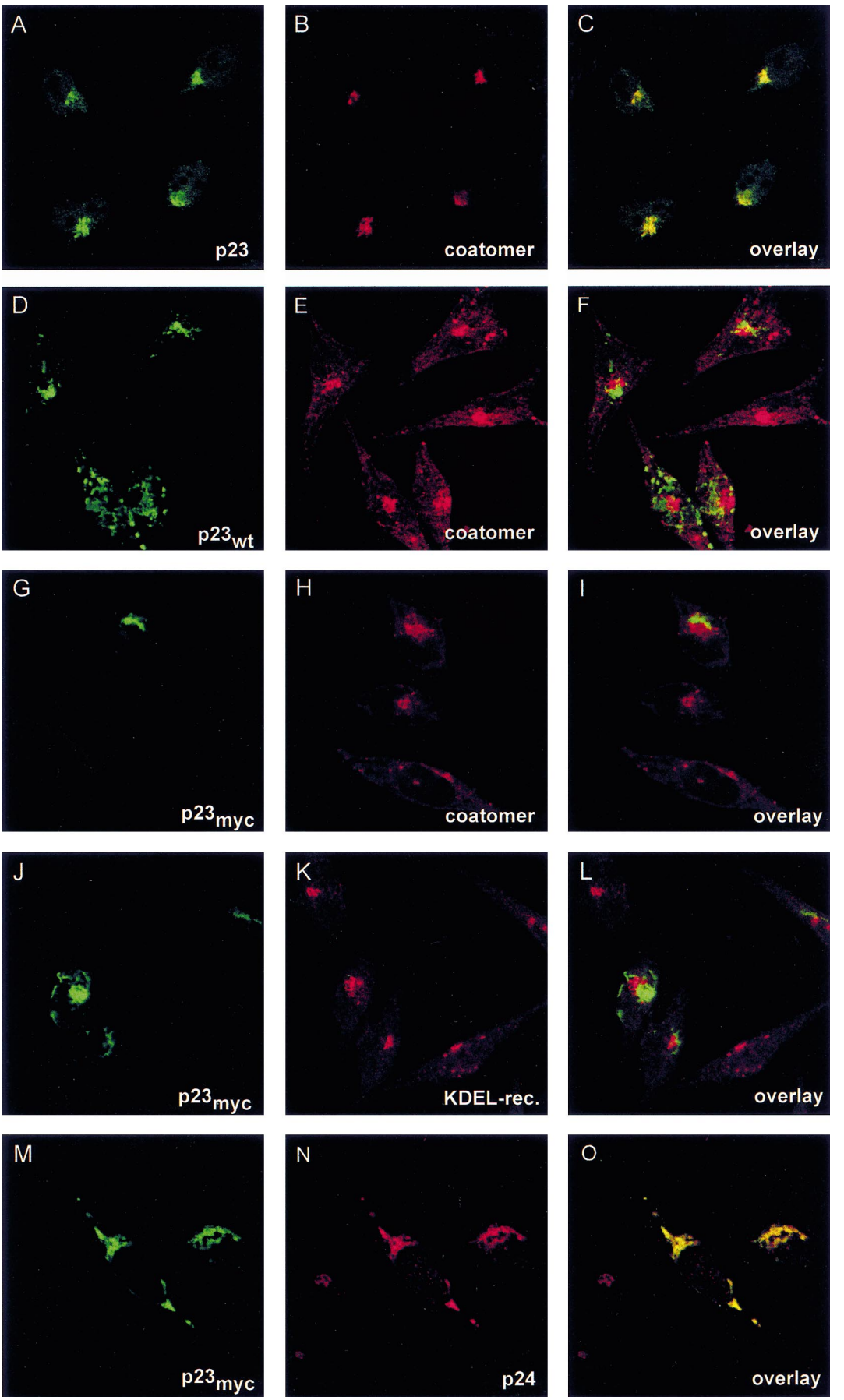
Fig. 4. Localization of p23 and p24 in wildtype and transiently transfected HeLa cells. Endogenous p23 (A,C) and coatomer (B,C) colocalize in wildtype HeLa cells. In contrast to wild-type cells double immunofluorescence stainings of p23 (D,F and G,I) and coatomer (E,F and H,I) in HeLa cells expressing $p23_{wt}$ construct (D–F) or $p23_{myc}$ construct (G–I) reveal different distributions for these proteins. Moreover, $p23_{myc}$ construct (J,L) does not colocalize with KDEL receptor (K,L) upon transient transfection. However, endogenous p24 (N,O) resides in structures positive for $p23_{myc}$ construct (M,O). Endogenous p23 and expressed $p23_{wt}$ construct were visualized using rabbit antibodies directed against the cytoplasmic tail of p23 (#1402) [10], $p23_{myc}$ constructs were stained with monoclonal anti-myc antibodies [28]. For localization of endogenous p24 rabbit polyclonal antibodies directed against the cytoplasmic tail of p24 ('Elfriede') were used. The right panel (C,F,I,L,O) shows superimpositions of the left and middle panel figures.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p23, p24 can be trapped in peripheral structures by incubating cells at 15°C. These structures were identified as the so-called intermediate compartment by colocalization of p24 with ERGIC53. Shifting the temperature back to 37°C allows p24 to move in anterograde direction to the Golgi complex. Therefore cycling of p24 extends at least between intermediate compartment and Golgi apparatus, but it cannot be excluded that both p23 and p24 cycle via the endoplasmic reticulum. Our data on overexpression of p23 indicates that this cycling may also involve the endoplasmic reticulum, because under these conditions endogenous p24 is trapped in ER-derived structures (data not shown). Localization of p23 and p24 to COPI-coated vesicles suggests that both cycle in this type of carrier. However, p23 and p24 are most likely not restricted to COPI-coated vesicles, as cycling via the endoplasmic reticulum would involve also COPII-coated vesicles. We have localized p24 to vesicular structures in the rough ER transitional region in rat acinar pancreatic cells treated with BFA (data not shown). Under these conditions COPI coats are dispersed and only COPII coats remain unaffected. This indicates that both proteins are also present in COPII-coated vesicles which is in good agreement with the findings that the putative yeast homologues of p24 and p23, Emp24p and Erv25p, were also found in COPII-coated vesicles [13].

Previously it has been shown that p23 and p24 contain different localization signals in their cytoplasmic domains. In p23 the cytoplasmic tail functions as a retrieval signal in vivo that is able to direct a reporter molecule towards the ER. Replacing the cytoplasmic tail of the marker molecule CD8 normally localized to the plasma membrane at steady state, with the cytoplasmic tail of p23, directs this construct to the ER [15]. In contrast, a CD8 construct bearing the cytoplasmic tail of p24 was localized at the plasma membrane, arguing that this tail contains a localization signal for the opposite direction [9], or no signal at all. However, neither is endogenous p23 found in the ER, nor p24 at the plasma membrane. Moreover, as shown here, both proteins form a complex, most likely by an interaction of p23 with p24 via their lumenal domains, containing coiled-coil structures predicted by sequence analysis [8,10]. This complex has the p23 tail as an ER localization signal at its cytoplasmic side. p24 now may prevent trapping of p23 in the ER by its interaction with p23, thus overcoming its retrieval signal. Likewise, p24 may not be able to escape from the organelles of the early secretory pathway because its interaction with p23 leads to an efficient transport of the p23/p24 complex in retrograde direction.

In a simple view, the steady state distribution of this complex between the ER and Golgi may reflect the relative kinetics of its anterograde versus retrograde transport. Kinetic differences may, for example, be caused by different relative affinities of the two cytoplasmic domains to anterograde and retrograde coats. In fact, the p23 domain efficiently interacts with COPI [10,11], whereas the p24 domain binds to COPII with higher affinity than p23 [11].

In vivo evidence that p23 and p24 influence their respective localization also comes from our experiments with tagged p23 molecules expressed in HeLa cells (Fig. 4). Earlier studies proposed that upon simultaneous overexpression of wild-type and mutant forms of mammalian p23, p24, p25 and p26/p27 (also named hp24$_{\alpha-\delta}$) [11], all proteins colocalize by forming a hetero-oligomeric complex that moves within the cell. Here we cannot exclude that structures positive for p23 and p24 also contain additional members of the p24 family, but our immunoprecipitation experiments suggest that p23 and p24 are able to form a minimal oligomeric complex. A similar complex was found in yeast where the putative homologues of p23 and p24, Erv25p and Emp24p, form a hetero-oligomeric complex in microsomes [13].

The involvement of members of the p24 family in protein secretion was first described in yeast where the deletion of Emp24 and Erv25 resulted in delayed kinetics of transport for some secretory proteins. Therefore these proteins were thought to function as cargo receptors [12,13]. In a more recent study anterograde transport of VSV-G protein was shown to be impaired when antibodies directed against the cytosolic tail of p23 were microinjected into mammalian cells, also demonstrating a functional role of this protein in secretory transport [14].

The deletion of Emp24 in yeast also resulted in the generation of fewer transport vesicles, indicating that Emp24 is involved in the budding of vesicles [8]. As mammalian p23 and p24 were both found in COPI-coated vesicles [8,10], and both proteins were shown to interact with coat proteins in vitro [9–11], they could function as coat receptors during vesicle budding. It is not yet clear whether these proteins function as coat or cargo receptors or whether they represent a kind of adaptor molecule that interacts with both cytosolic coat proteins and lumenal cargo. The ability of mammalian p23 and p24 to cycle between organelles of the secretory pathway as components of COPI-coated vesicles is consistent with these possible functions. More detailed studies of the interaction between p23/p24 and coat proteins at the cytosolic side as well as a functional characterization with respect to cargo recognition of their lumenal domains are necessary for a better understanding of the functions of these proteins.

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