Oligomeric State and Stoichiometry of p24 Proteins in the Early Secretory Pathway* [S]

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The p24 proteins belong to a highly conserved family of membrane proteins that cycle in the early secretory pathway. They bind to the coat proteins of COPI and COPII vesicles, and are proposed to be involved in vesicle biogenesis, cargo uptake, and quality control, but their precise function is still under debate. Most p24 proteins form hetero-oligomers, essential for their correct localization and stability. Functional insights regarding the mechanisms of their steady state localization and the role of interaction with coat proteins has been hampered by a lack of data on their concentration and state of oligomerization within the endoplasmic reticulum, the intermediate compartment, and Golgi complex. We have determined for all mammalian p24 family members the size of the oligomers formed and their stoichiometric relation in each of these individual organelles. In contrast to earlier reports, we show that individual members exist as dimers and monomers and that the ratio between these two forms depends on both the organelle investigated and the p24 protein. We find unequal quantities, with p23 and p27 building up concentration gradients, ruling out a simple 1:1 stoichiometry. In addition, we show differential cycling of individual p24 members. These data point to a complex and dynamic system of altering dimerizations of the family members.

The p24 family of type I transmembrane proteins consists of six members in mammalian cells which, referring to sequence homologies, can be divided in four subfamilies (1, 2). Mammalian cells contain three members of the p26 subfamily, namely p26, p27, and tp24 and only one member each of the p23, p24, and p25 subfamilies. According to studies in yeast, p24 proteins may function as cargo receptors or adaptors since in knock-out mutants distinct cargo proteins show delayed transport kinetics (3–5). Additional experiments in yeast and Caenorhabditis elegans implicate also an involvement of p24 proteins in ER quality control (4, 6, 7). For mammalian p23, it has been proposed that it serves as COPI receptor because it can bind to coat components (1, 8) and belongs to the minimal machinery needed to bud COPI vesicles from liposomes (9). In addition, a direct interaction of p24 family members has been shown with proteins involved in vesicle budding and cargo sorting, such as ARF-1 (ADP-ribosylation factor) (10, 11), ARF-GAP (GTPase-activating protein) (10) and Sar-1p (12). p24 proteins are also effectively enriched in COPI (8, 13) and COPII vesicles (3, 5), and some of them bear signals in their cytoplasmic tails, which when fused to a cargo protein direct them either anterogradely or retrogradely (14). Therefore, p24 proteins have been implicated to be involved in transport processes of the early secretory pathway, but their precise function is still unclear.

All p24 proteins are found in membranes of the early secretory pathway (1, 8, 13, 15, 16), and there is evidence that they cycle constitutively between these membranes (13, 14, 16, 17). Another outstanding property of p24 proteins is the formation of hetero-oligomers. As was shown in several immunoprecipitation studies, they interact with each other to a certain extent (3, 4, 13, 16). Moreover, overexpression of a single p24 protein leads to a mislocalization of all p24 proteins in ER-derived structures (13, 15, 16), and the only simultaneous overexpression of p24 proteins of all subfamilies results in a convincing perinuclear Golgi localization (15, 16). Furthermore, in yeast cells lacking one p24 protein (4) and in cells from mice lacking one allele of p23 (18) other family members are degraded. Thus, there is a strict dependence among p24 proteins in terms of stability, transport, and/or localization. Consequently, the formation of hetero-oligomers seems to be a prerequisite for their correct function.

To set a basis for understanding the molecular functionality of p24 proteins, we have undertaken a detailed study of their oligomeric behavior, their stoichiometric relation, and cycling in the membranes of the secretory pathway. We demonstrate that p24 proteins, in contrast to earlier results (1, 4), exist either as dimers or monomers with no higher oligomers observed. Moreover, we find different ratios between dimers and monomers depending both on a given p24 protein and on its subcellular localization. p23 and p27 build up concentration gradients, and p24 and p25 are distributed equally in the early secretory pathway, with differential cycling between these compartments. Therefore, we assume highly dynamic and complex interactions of these four p24 proteins. In contrast, p26 and tp24 do not seem to interact with the other four members as they occur exclusively either as monomer or as dimer, respectively, and their concentrations are vastly different from other p24 proteins.

EXPERIMENTAL PROCEDURES

Antibodies—Antibodies directed against p23, p24, p25, p26, tp24, and p27 were raised in rabbits, chicken, and guinea pigs with the peptides or recombinant proteins indicated in parentheses coupled to keyhole limpet hemocyanin (Sigma): Henriette (p23 recombinant lumi-
Subcellular Fractionation—HeLa cells (ATCC CCL-2.2) were grown in Spinner culture according to standard conditions up to 4–6 × 10^9 cells/ml. Homogenization and centrifugation were performed according to Ref. 16. After centrifugation, 18 instead of 9 fractions were taken from each gradient, and were diluted 1:1 with buffer and centrifuged for 1 h at 100,000 × g at 4 °C. Membrane pellets were used for further analysis.

Golgi vesicles and trans-Golgi vesicles were separated by density gradient centrifugation. As shown in Fig. 1, these membranes were found in light fractions (fractions 3–4), and are separated from plasma membrane vesicles in ER fractions can be disregarded as these membranes of the early secretory pathway are well separated. Golgi membranes (in nmol of phospholipid) were subjected to gel electrophoresis (25) and Western blotting with antibodies against transferrin receptor (190 kDa), calnexin (90 kDa), Myc (CD8-LT, 60 kDa) and cytochrome b5 (15 kDa). Only values of the membrane samples within these linear curves were considered. Standard curves with the data of the p24 standards were established. For each condition the distribution of marker enzymes was tested, and fractions were pooled accordingly. The amount of membrane in each pool was determined by nano-ESI-MS/MS (electrospray ionization-tandem mass spectrometry) (27), and identical amounts of phospholipids were subjected to Western blot analysis with p24 protein antibodies (Henriette, Elfriede, #2469R1, #2088SM2, #2087R2, #2501R2), and developed with the ECL detection system. The antibodies had been tested before to be specific for each p24 protein and not to cross-react with GST. The signals were quantified with the QuantityOne software, and the signals for each compartment were used to calculate relative protein amounts.

Quantitative Western Blot Analysis—GST-p24 tail constructs (p24, p25, p26, p27, and tp24) or the luminal domain of p23 were used as standard proteins. Increasing amounts of standard protein (in ng) and membranes (in nmol of phospholipid) were subjected to gel electrophoresis (28) and Western blotting with antibodies against p24 proteins (Henriette, Elfriede, #2469R1, #2088SM2, #2087R2, #2501R2), and developed with the ECL detection system. The antibodies had been tested before to be specific for each p24 protein and not to cross-react with GST. The signals were quantified with the QuantityOne software, and standard curves with the data of the p24 standards were established. Only values of the membrane samples within these linear curves were taken into account to calculate the concentration of each p24 protein in ng of protein/nmol of phospholipid (see supplemental data Figure I at http://www.jbc.org). For each protein, at least three independent estimations were made, each time with both the standards and the samples on the same blot. Independent experiments with different p24 protein antibodies in a second quantitative Western blot analysis were performed as an additional control, and comparable results were obtained (supplemental data Figure II).

RESULTS
Subcellular Fractionation of ER, IC, and Golgi—To fractionate ER, IC, and Golgi, HeLa cell membranes were subjected to density gradient centrifugation. As shown in Fig. 1, these membranes of the early secretory pathway are well separated. Golgi membranes were found in light fractions (fractions 3 + 4), characterized by galactosyltransferase activity and the KDEL receptor (KDEL-R.), and are separated from plasma membrane in fraction 2 as shown by alkaline phosphodiesterase (AP) activity. The second peak of the KDEL receptor in the intermediate density fractions represents the membranes of the IC, as this protein localizes to the cis-Golgi and the IC, respectively (29, 30). In contrast, ER membranes move to the high density region of the gradient (fractions 15–17), as shown by the distribution of PDI. The ER pool also contains mitochondria and peroxisomes as indicated by the distribution of p30 and PMP69. However, the amount of membrane according to peroxisomes in ER fractions can be disregarded as these mem-

Plasmic tail sequences of p24, p25, p26, p27, and tp24 with overhanging restriction sites for EcoRI and BamHI. After annealing of the oligonucleotides they were cloned into the EcoRI and BamHI site of the pGEX-2T vector (Amersham Biosciences). The GST-p24 tail constructs were overexpressed and purified according to manufacturers specifications. The sequence for the luminal domain of p23 was cloned into the BamHI and HindIII site of the pQE-30 vector (Qiagen), overexpressed, and purified (19). Rabbit polyclonal antibodies against cytochrome b5 were a kind gift of N. Borgese (University of Milan, Italy), monoclonal antibodies against ERGIC-53 were from H.-P. Hauri (Biotec, Basel, Switzerland) (20), and rabbit polyclonal antibodies against p30 (mitochondrial protein) and PMP69 (peroxisomal membrane protein) were provided by W. Just (Biochimie-Zentrum Heidelberg, Heidelberg, Germany). Further antibodies were: anti-protein disulfide isomerase (PDI, BD Transduction Laboratories), anti-KDEL-receptor (Stressgen Biotechnologies), anti-transferrin receptor (Zymed Laboratories Inc.), anti-rabbit-peroxidase (POD, BioRad Laboratories), anti-mouse-POD (Dianova), anti-guinea pig-POD (Dianova), anti-chicken-POD (Dianova), monoclonal anti-rabbit-POD (Sigma), anti-mouse-Alexa-488 (Molecular Probes), and anti-guinea pig-Alexa-466 (Molecular Probes).

In contrast to this study, the antibodies were covalently coupled to the protein A-Sepharose (19). GST-p24 Tail Constructs and Luminal Domain of p23—Sense and antisense oligonucleotides were synthesized corresponding to the cytochrome b5.
branes only constitute 1% of total membranes (31). In contrast, mitochondria represent about 32% of total cell membranes (estimated for baby hamster kidney cells, (31)) and thus only part of the membranes in the ER pool represents ER membranes. This was quantified and taken into account.

Solubilization and Oligomeric Behavior of p24 Proteins in ER, IC, and Golgi—Three different detergents were tested under the same buffer and salt conditions described before for the solubilization of p24 proteins (1, 4, 32). As shown in Fig. 2, only octylglucoside and 8POE were able to solubilize p23 almost quantitatively. Cholic acid, used previously to determine an oligomeric size of mammalian p24 proteins of 35S (1) in our hands emerged to be unsuited for solubilization. Since an extensive study of p24 proteins in yeast was performed with octylglucoside, and this detergent solubilizes p23 nearly quantitatively (Fig. 2) (4), it was used for solubilization in further experiments. Equal amounts of ER, IC, and Golgi with regard to their phospholipid content were solubilized in 4% octylglucoside and the p24 oligomers were separated by gel filtration. The fractions were subsequently analyzed by Western blotting with antibodies against all p24 proteins. As shown in Fig. 3, p24 proteins elute at two defined volumes with peaks in fractions 18 and 21. To determine the size of the p24 oligomers in these two peaks, we calibrated the gel filtration column with membrane proteins rather than soluble proteins, which, like p24 proteins, need a detergent micelle to stay in solution (Fig. 4A). With reference to this calibration, p24 proteins appear as dimers and monomers in the ER, IC, and Golgi pools. To assess the oligomeric state of p23 independently, i.e. without the use of detergent, (i) soluble recombinant luminal domain of p23 was analyzed by gel filtration in the absence of detergent and (ii) ER, IC, and Golgi membranes were treated with a cross-linker (DSG) and afterward analyzed by Western blotting with an anti-p23 antibody (supplemental data Figs. III and IV). In both detergent-independent experiments a dimer is the highest oligomer observed. This clearly shows that the dimer observed in the gel filtration analysis is physiological and is not a result of the disruption of higher oligomers by the use of detergent.

In addition, we observed a different ratio between dimer and monomer, depending on the organelle investigated. As depicted in Fig. 3B, p23 is predominantly found as a dimer in the ER, but in the IC and the Golgi there are about equal amounts of dimer and monomer. In contrast, p24 shows a similar ratio between monomer and dimer in all membrane fractions. p25 and p27 exist predominantly as monomers, with p25 showing the highest amount of dimer in the ER and p27 in the Golgi. However, p26 and tp24, which coimmunoprecipitate to a significantly lower extent with other p24 members (16), are present only as either monomers or dimers, respectively.

As shown in Fig. 5 an anti-p24 antibody coimmunoprecipitates p23 and p25, and an anti-p27 antibody p23 and p24 from
Cycling of p24 Proteins in the Early Secretory Pathway—All members of the p24 family cycle in the early secretory pathway (13, 14, 16, 17) and accumulate in the IC after incubation of cells at 15 °C (13, 16, 17). After release of this 15 °C block by shifting to 37 °C, p24 proteins start to move to their steady state localization. This system was used as a tool to compare all compartments of the early secretory pathway. Therefore, a part of p24 proteins always exists as heterodimers, but the amount of the coimmunoprecipitated p24 protein is different depending on the organelle. For example, when compared with the Golgi, p27 coimmunoprecipitates less p24 than p23 from the IC. This implies that in the different organelles certain heterodimers are preferentially formed.

Cycling of p24 Proteins in the Early Secretory Pathway—All members of the p24 family cycle in the early secretory pathway (13, 14, 16, 17) and accumulate in the IC after incubation of cells at 15 °C (13, 16, 17). After release of this 15 °C block by shifting to 37 °C, p24 proteins start to move to their steady state localization. This system was used as a tool to compare the cycling of all p24 proteins by immunofluorescence and Western blot analysis. For immunofluorescence studies, cells were fixed after a 15 °C block for 2 h and after various time periods of release at 37 °C. Then the cells were triple-labeled with antibodies against p27, ERGIC-53, and a second p24 member and analyzed by confocal microscopy. As shown in Fig. 6A, at 15 °C p24 and p27 colocalize with the ERGIC-marker ERGIC-53 in a perinuclear region (yellow arrowhead) and peripheral punctate structures (yellow arrows). After shifting the cells to 37 °C, the colocalization with ERGIC-53 in the perinuclear region and later in punctate structures decreases, as parts of p24 and p27 are redistributed to the Golgi (white arrowheads, Fig. 6A, I and II). In contrast, p23 and p25 (Fig. 6A, III and IV, for p25 supplemental data Fig. IV) at 15 °C are localized to the perinuclear region where they colocalize with p27 and ERGIC-53, but they are also found in reticular structures likely of the ER (blue arrowheads). After shifting to 37 °C, parts of p23 and p25, such as p24 and p27, are transported to the Golgi with some of p23 and p25 still found in reticular structures. This clearly shows that p24 proteins travel differentially in the early secretory pathway, with p24 and p27 mainly cycling between the IC and the Golgi and p23 and p25 accumulating in the IC and reticular structures of the ER, indicating that they are either transported retrogradely or that their appropriate partner for further transport (for example p24) is blocked elsewhere. p26 and tp24 behave similar to p24 and p27, but show a pronounced punctate staining pattern (data not shown).

To analyze the cycling of p24 proteins biochemically, subcellular fractionation was performed after a temperature block at 15 °C and at several time points after transferring the cells to 37 °C. As described under “Experimental Procedures,” the relative amounts of p24 proteins were compared by Western blot analysis (Fig. 6B). In agreement with the immunofluorescence studies, p23 and p25 are both constitutively exported from the ER (Fig. 6B). On the other hand, the amount in the ER of p27 increases with the release, and p24 shows no significant change of relative protein amounts in the ER during the time course. In the IC, p25 redistributes like p24 and p27, as all three members seem to be transported into this organelle. However, the amount of p23 first increases and then decreases in this compartment. In Golgi membranes, the relative amounts of p25, p24, and p27 decrease after a 5-min release and then increase after 15 min but, in every case, to a markedly different extent. In contrast, the relative amount of p23 decreases steadily. This demonstrates that p24 proteins cycle differentially, but at some transport steps also behave similarly, such as p23 and p25 in the ER.

Localization of p24 Proteins and Their Concentration in the Individual Organelles of the Early Secretory Pathway—Several independent studies show that all p24 proteins localize to the various membranes of the early secretory pathway (1, 8, 16, 17, 33), but it is still not clear to what extent p24 proteins reside within the same compartment. Therefore, we addressed both their localization and concentration by quantitative Western blot analysis. To this end, ER, IC, and Golgi were enriched by subcellular fractionation of HeLa cells as described above (Fig. 1). As exemplified with p27 (see supplemental data Fig. 1), defined amounts of membrane according to phospholipid content and p24 protein standards were subjected to Western blot analysis with their respective antibodies. After quantification of the signals, a standard curve with the data of the p24 protein standards was obtained, and only values of the membrane samples within the linear range of this curve were used to calculate the concentration of a p24 protein. In Fig. 7, the average concentrations of all p24 proteins in ER, IC, and Golgi are summarized. Two p24 members, namely p25 and p24, are equally distributed between ER, IC, and Golgi, whereas p23 and p27 built up opposing gradients across the early secretory pathway. In addition, p23 and p25, the only members with functional ER retrieval signals in their cytoplasmic tails (14, 34, 35), exist in a 5-fold lower concentration than p24 and p27. However, p27 is present in the ER at the same concentration as
FIG. 6. Analysis of p24 protein cycling in the early secretory pathway. A, immunofluorescence analysis. Vero cells cultivated on coverslips were fixed after 2 h of incubation at 15 °C or 15 min release at 37 °C after 15 °C block. Then cells were triple-labeled with antibodies against ERGIC-53 (green), p27-cy5 (blue), and p24 cy3 or p23-cy3 (red). The distribution of p24 (I + II) and p23 (III + IV) and simultaneously ERGIC-53

B, quantification of p23 and p24 proteins. Relative protein amount in ER, IC, and Golgi was analyzed after 2 h at 15 °C, 15 min at 37 °C, or 10 min at 37 °C.
p23 and p25, due to its concentration gradient along the early secretory pathway. In contrast, p26 exists in very high and tp24 in very low concentrations in all three organelles, again pointing to a less related function of these p24 proteins.

**DISCUSSION**

p24 proteins present a highly conserved protein family of small type I transmembrane proteins, all with small cytoplasmic domains. Although it is in general accepted that they play a role in transport processes of the early secretory pathway, their precise function is still unclear. Prominent properties of p24 proteins are their constitutive cycling in the early secretory pathway (14, 16, 17, 33) and the capability to form hetero-oligomers with each other (3, 4, 13, 16). Studies in yeast and in mammalian cells show that in vivo the amount of one p24 protein is regulated depending on other p24 proteins (4, 18). In addition, overexpression of other p24 proteins in ER-derived structures, and only the overexpression of at least one member of each subfamily leads to their clear-cut localization to the Golgi apparatus (15, 16). Thus, most of the p24 proteins depend on each other in terms of stability, localization, and/or transport. Hence, the formation of hetero-oligomers seems to be intimately coupled to their function.

**p26 and tp24 Seem Not to Require Hetero-oligomers for Their Function**—In contrast to other p24 proteins, p26 and tp24 do not seem to require the formation of hetero-oligomers for their function, as they do not significantly coimmunoprecipitate (16), and are not mislocalized when other p24 proteins are overexpressed (32). We demonstrate here that also their oligomeric behavior and stoichiometric relation, compared with other p24 proteins, are pointing to a different molecular functionality. p26, which occurs mainly as a monomer and in much higher concentrations than all other members, is distributed more or less equally among all membranes. Therefore, it might serve as a placeholder in budding zones building up a sorting and/or budding competent matrix by interacting with other p24 proteins only transiently. tp24 is set apart from other members by its occurrence only in miniscule concentrations.

**Oligomeric State of p24 Proteins in ER, IC, and Golgi Membranes**—We investigated the oligomeric behavior of all mammalian p24 proteins in the membranes of the early secretory pathway by gel filtration analysis. In an earlier study, a hetero-oligomer of p24 proteins with a sedimentation coefficient of 35S was determined in mammalian cells, which would correspond to a complex of around fifty p24 proteins (1). However, in our hands the detergent used in this study appeared to be unsuited for solubilization of p24 proteins, as after centrifugation at 100,000 × g for 30 min most of p23 remain insoluble. Therefore, the determination of a 35S complex is very likely caused by partly solubilized membranes. In further studies, a size of about 100 kDa for a p24 oligomer in yeast and p23 in mammalian cells was determined (4, 32), and therefore it was proposed that p24 proteins form tetramers. In contrast to these earlier studies, we demonstrate that p24 proteins form dimers and monomers in the membrane of ER, IC, and Golgi (Fig. 3). The differing results are due to the fact that we have calibrated the gel filtration with membrane proteins rather than with soluble proteins. Calibration with soluble proteins, as shown in Fig. 4B, leads to an overestimation of the oligomer size caused by an increase of the apparent molecular mass by the detergent micelle in the membrane protein samples. To challenge our results, the soluble luminal domain of p23 was also subjected to a gel filtration analysis calibrated with soluble standard proteins in the absence of detergent (supplemental data, Fig. III), and mainly a dimer was found. Moreover, in a cross-link experiment performed with p23, a band of around 45 kDa was observed, which corresponds to a dimer and confirms our results. Altogether this is taken as detergent-independent evidence that a p24 oligomer indeed is a dimer and not the result of detergent-induced disruption of higher oligomers.

Several independent studies show that p24 proteins can be coimmunoprecipitated with each other (3, 4, 13, 16). As p27 can coimmunoprecipitate p23, p24, and p25, it was concluded that these p24 proteins form a 1:1:1:1 tetramer (16). However, our data imply that there are heterodimers of p23, p24, p25, and p27 rather than the formerly proposed tetramer (4, 16), which is also in line with the coimmunoprecipitation data. In vitro studies show that p24 protein cytoplasmic tails can form tetramers (36), which seems to be functionally important as a tetramer of p23 cytoplasmic tails is able to induce a conformational change in coatomer (37). On the other hand, it has been reported that in vivo an interaction of p24 proteins is mediated by the coiled-coil domains in the luminal part of p24 proteins.
(15), and not by their cytoplasmic tails. Therefore, we conclude that tetrameric states of p24 proteins mediated by the cytoplasmic tails are of transient nature, and are obviously not stable in the detergent conditions used in the gel filtration analysis.

For p23, p24, p25, and p27, a different ratio of dimer to monomer was observed depending on the p24 protein and the organelle investigated (Fig. 3). In addition, the question arises of whether p24 proteins exist as homodimers or heterodimers. In Fig. 5 we show that heterodimers exist in all compartments of the early secretory pathway, whereas the ratio of communoprecipitated p24 proteins is different depending on the organelle. This implies an environment-specific binding preference of certain p24 proteins.

There is evidence from independent studies that p24 proteins might function as dimers as only the dimeric form of a peptide resembling the cytoplasmic tail of p23 inhibits the recruitment of ARF1-GDP to Golgi membranes (10) and only a dimer of p24 binds to Golgi matrix proteins (38). Therefore, it could be presumed that the dimeric form of these family members is a functional one. This then would signify that some of them, like p24, function at all levels of the secretory pathway as they always show the same ratio between monomeric and dimeric state. Others like p23, which exists mainly as a dimer in the ER and displays an equal ratio between monomer and dimer in the other compartments, would then play a role only at certain stages. This is also in line with a concentration gradient of p23 between ER and Golgi and the equal distribution of p24 to all compartments. As discussed above, by interaction of cytoplasmic domains of some p24 proteins transient formation of tetramers might occur.

**Stoichiometry of p24 Proteins in the Organelles of the Early Secretory Pathway**—As p24 proteins depend on each other in terms of stability (4, 18), localization, and/or transport (15, 16), a stoichiometric relationship of these proteins has always been presumed. To investigate this relationship, the concentration of all p24 proteins in the membranes of the early secretory pathway was determined by quantitative Western blot analysis (Fig. 7). We demonstrate that p24 proteins are present in unequal concentrations and localize to differing extents to membranes of ER, IC, and Golgi (Fig. 7), which excludes a simple 1:1 stoichiometry. p23 and p25, both containing functional ER retrieval signals in their cytoplasmic tails (14, 34, 35), exist in 5-fold lower concentration in the membranes of the early secretory pathway than the “anterograde” directed proteins p24 and p27 (1, 14). p24 and p25 localize equally to all membranes; p23 and p27 build up opposing gradients with p23 present in higher concentrations in the ER and IC than in the Golgi and p27 with higher amounts in the Golgi and the IC than in the ER. In the ER p27 exists in mass amounts equal to p23 and p25. In this regard the family members resemble the SNARE proteins, which also build up gradients (39). As p23 contains a functional retrieval signal in its cytoplasmic tail (14), it is probably active in the “anterograde” transport processes from IC to ER, and p27 as an anterograde p24 protein from the IC to the Golgi. In contrast, the equally distributed p24 and p25 seem to have a function in all organelles of the early secretory pathway. p25, carrying a classical ER retrieval signal may prevent other anterogradely directed p24 proteins from being transported to as far as the plasma membrane, and thus ascertain their localization to the membranes of the early secretory pathway. Then p24, with no functional retrieval signal (14), may serve in the opposite direction and facilitate the transport of retrograde members in the anterograde direction. To simplify the quantitative aspect of our data, the relative mass amounts found for p23, p24, p25, and p27 within the individual organelles of the early secretory pathway are depicted schematically in Fig. 8.

**Cycling of p24 Proteins within ER, IC, and Golgi Membranes**—The redistribution of p24 proteins after a 15 °C block and subsequent release at 37 °C implies that p24 proteins cycle differentially in the early secretory pathway but at certain transport steps seem to behave similarly. For example, p23 and p25 upon temperature block are distributed more retrogradely to ER membranes (Fig. 6A) and are transported out of the ER to the same extent after release of the block (Fig. 6B). On the other hand, they show unequal redistribution to the IC and the Golgi. Hence, it is impossible that they continuously form p23/p25 heterodimers. On the other hand, p25 is redistributed like p24 and p27 to the IC and Golgi and may then form heterooligomers with these p24 members. This altogether implies highly dynamic interactions of various family members.

**Molecular Functionality of p23, p24, p25, and p27**—p23, p24, p25, and p27 need each other for their correct localization and stability (4, 15, 18). Taking into account (i) their formation of heterodimers, (ii) differential cycling, and (iii) varying ratios of dimers and monomers in the membranes of the early secretory pathway, one explanation for this behavior would be a frequent alteration of binding partners, likely to depend on the transport step, and resulting in various dimeric combinations (heterodimers AB, AC, . . . and also possibly homodimers). Some of the p24 proteins contain functional retrieval signals in their cytoplasmic domains (14, 34, 35), and they are effectively incorporated into COPI (8, 13) and COPII vesicles (3). Therefore, it is tempting to speculate that the composition of a p24 dimer rules the direction of a vesicle. p23 and p27, which build up opposing gradients between ER, IC, and Golgi, could fulfill this function, and p24 and p25, which are equally distributed, would serve as anchoring partners to keep them in place until they are incorporated into vesicles or mediate their transport back to their place of function.

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