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Selective export of human GPI-anchored proteins from the endoplasmic reticulum

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Summary

Selective export of transmembrane proteins from the endoplasmic reticulum (ER) relies on recognition of cytosolic-domain-localized transport signals by the Sec24 subunit of the COPII vesicle coat. Human cells express four Sec24 isoforms, termed Sec24A, Sec24B, Sec24C and Sec24D that are differentially required for selective, signal-mediated ER export of transmembrane proteins. By contrast, luminally exposed glycosylphosphatidylinositol (GPI)-anchored membrane proteins cannot bind directly to Sec24 and must either use membrane-spanning cargo receptors or alternative mechanisms for ER export. Little is known about the mechanism underlying export of GPI-anchored proteins from the ER in higher eukaryotes. Using siRNA-based silencing, we identified that ER-to-Golgi transport of the human GPI-anchored protein CD59 requires Sec24, with preference for the Sec24C and Sec24D isoforms, and the recycling transmembrane protein complex p24-p23 that exhibited the same Sec24C-Sec24D isoform preference for ER export. Co-immunoprecipitation indicated unprecedented physical interaction of CD59 as well as a GFP-folate-receptor-GPI-anchor hybrid with a p24-p23 complex. Density gradient centrifugation revealed co-partitioning of CD59 and p24-p23 into biosynthetically early lipid raft fractions, and CD59 transport to the Golgi was cholesterol dependent. The results suggest that the 24p-23p complex acts as a cargo receptor for GPI-anchored proteins by facilitating their export from the ER in a Sec24-isoform-selective manner involving lipid rafts as early sorting platforms.

Key words: Cargo receptor, CD59, COPII, ER export signal, ERGIC-53, Folate receptor, Glycosylphosphatidylinositol-anchored proteins, p23, p24, Sec24 isoforms, Lipid rafts

Introduction

Exocytic proteins are synthesized in the endoplasmic reticulum (ER) and move along the secretory pathway by vesicular transport (Bonifacino and Glick, 2004; Lee et al., 2004). In higher eukaryotic cells, these proteins are packaged into coat protein complex II (COPII) vesicles that depart from ER exit sites to the ER-Golgi intermediate compartment (ERGIC) (Appenzeller-Herzog and Hauri, 2006; Lee et al., 2004). COPII is composed of the small GTPase Sar1, the inner Sec23-Sec24 protein complex and the outer Sec13-Sec31 complex. Numerous transmembrane proteins are selectively exported from the ER. Selective ER export is controlled by cytosolic domain-exposed transport motifs that interact with the Sec24 COPII subunit (Miller et al., 2003; Mossessova et al., 2003). A variety of ER export signals has been defined including di-acidic, di-aromatic and di-hydrophobic motifs (Barlowe, 2003; Dominguez et al., 1998; Kappeler et al., 1997; Nishimura and Balch, 1997; Nufer et al., 2002). Mammalian cells express the four Sec24 isoforms Sec24A, Sec24B, Sec24C and Sec24D (Pagano et al., 1999). The individual isoforms exhibit binding preferences for the different signals but there is also some overlap (Mancias and Goldberg, 2008; Wendeler et al., 2007). Thus, the mammalian Sec24 isoforms expand the repertoire of cargo proteins for signal-mediated ER export and are in part functionally redundant.

Unlike transmembrane proteins, glycosylphosphatidylinositol (GPI)-anchored proteins cannot interact directly with Sec24 since they lack a cytosolic domain. GPI-anchored proteins are synthesized as transmembrane precursor proteins in the ER. Still in the ER, their luminal domain is transferred to the preformed GPI-lipid anchor resulting in hybrid molecules in which the polypeptides face the ER lumen and the fatty acids of the GPI anchor reside in the luminal leaflet of the lipid bilayer (Chatterjee and Mayor, 2001; Orlean and Menon, 2007). The question arises therefore, of whether GPI-anchored proteins are exported passively from the ER by bulk flow or in an active way by a cargo receptor mechanism.

Some transport requirements for GPI-anchored proteins have been established in yeast (Mayor and Riezman, 2004). The yeast GPI-anchored protein Gas1p can be incorporated into ER-derived vesicles in vitro in the presence of purified COPII components (Doering and Schekman, 1996) and mutations in the COPII subunits Sec13p and Sec23p, abolish transport of Gas1p to the Golgi (Sutterlin et al., 1997), suggesting that ER export of GPI-anchored proteins in yeast is COPII dependent. In mutants that lack the transmembrane proteins Emp24p and Erv25p, Gas1p is delivered to the Golgi with reduced kinetics, and Gas1p can bind to Emp24p, consistent with the notion that Emp24p-Erv25p may act as a cargo receptor for Gas1p (Muniz et al., 2000; Schimmoller et al., 1995).

Sphingolipids, phospholipids and cholesterol are key components of specialized membrane domains, termed lipid rafts, which can act as platforms mediating protein sorting and signaling (Brown and London, 1998; Hoetzl et al., 2007; Simons and Vaz, 2004). The GPI anchor confers to proteins the ability to associate with lipid rafts which are insoluble at 4°C in the non-ionic detergent Triton X-100, and this property has been exploited for their isolation (Brown and London, 2000; London and Brown, 2000). In the

secretory pathway of mammalian cells protein partitioning into lipid rafts occurs in the Golgi complex (Brown and Rose, 1992; Simons and Ikonen, 1997), whereas in yeast the partitioning takes place in the ER (Bagnat et al., 2000).

Very little is known about the specific requirements for the transport of mammalian GPI-anchored proteins early in the secretory pathway. Even though the yeast system has provided meaningful insight into GPI-anchored protein transport, it is unable to provide a comprehensive picture for the mammalian system. The mammalian secretory pathway is considerably more complex than that of yeast, both in terms of membrane compartments and the number of expressed proteins, including multiple isoforms of coat proteins. We have explored mechanisms underlying ER-to-Golgi transport of GPI-anchored proteins in the human cell line HeLa and determined whether export of this class of membrane proteins from the ER is selective.

Results

ER-to-Golgi transport of CD59 protein is COPII-dependent and requires specific Sec24 isoforms

To study the transport of human GPI-anchored proteins, we used HeLa cells that endogenously express the GPI-anchored protein CD59 (Elortza et al., 2006; Nichols et al., 2001; Zheng et al., 2003). We first tested whether transport of CD59 is dependent on COPII, by taking advantage of a previously established siRNA-based silencing approach that was developed to study Sec24 isoform-selective transport of the cargo receptor ERGIC-53 to which various ER export motifs were appended (Wendeler et al., 2007). Human CD59 is an ~20 kDa GPI-anchored protein carrying a single *N*-glycan (Rudd et al., 1997). Its transport from ER to medial-Golgi can therefore be measured by the acquisition of endoglycosidase H (endo-H) resistance in [^{35}S]methionine-cysteine pulse-chase experiments. Fig. S1A in supplementary material shows that the rate of transport (half-maximal conversion of endo-H-sensitive to endo-H-resistant protein) of CD59 is about 30 minutes and complete endo-H resistance is apparent after a chase of 120 minutes. Based on this kinetics, the 30 minutes chase time was chosen for further experiments. To evaluate whether the transport of GPI-anchored proteins depends on the COPII machinery, which is unknown in mammalian cells, all four Sec24 isoforms were silenced. This quadruple knockdown led to a considerable impairment of transport of endogenous CD59 by a factor of about three, clearly indicating that transport of CD59 is mediated by COPII (Fig. 1A,B). Knockdown efficiency of the Sec24 isoforms in these experiments was about 90% as published previously (Wendeler et al., 2007). The remaining Sec24 isoforms may be responsible for the incomplete inhibition of CD59 transport.

Which Sec24 isoform mediates ER export of CD59? To address this question the four Sec24 isoforms were silenced, either individually or in different combinations. Fig. 1 shows that single knockdowns did not affect transport of CD59. However, double knockdowns revealed a dependence on the isoforms Sec24C and Sec24D, very much in contrast to all other double knockdown combinations which had no effect. Next, we tested if single Sec24 isoforms can maintain CD59 transport when the other three are silenced. Transport of CD59 was considerably affected in triple knockdown combinations that included knockdown of either Sec24C or Sec24D, whereas it remained unaffected in triple knockdowns that included Sec24A or Sec24B (Fig. 1). Interestingly, the profile of Sec24 isoform dependence of folate receptor alpha,

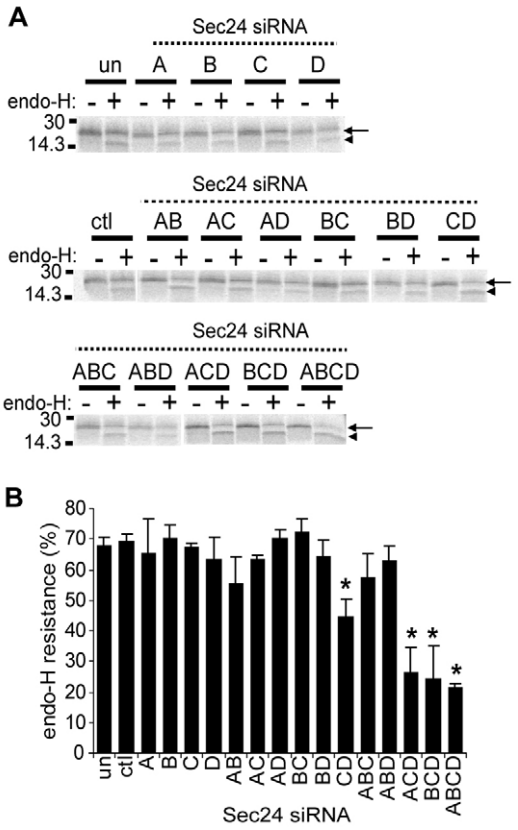

Fig. 1. Effect of Sec24 isoform silencing on transport of the endogenous GPI-anchored protein CD59 in HeLa cells. (**A**) Cells were transfected with siRNA against Sec24A, Sec24B, Sec24C and Sec24D, either individually or in different combinations as indicated. After 3 days, the cells were pulsed with [^{35}S]methionine-cysteine for 10 minutes, chased for 30 minutes, and immunoprecipitated with an antibody against CD59. Immunoprecipitates were treated with (+) or without (–) endo-H and separated by SDS-PAGE followed by autoradiography. Endo-H-resistant (arrow) and endo-H-sensitive (arrowhead) forms are indicated on the right and molecular masses (in kDa) on the left margin. (**B**) Quantification of endo-H resistance of CD59 from the pulse-chase experiments in A (means ± s.d., n=3 independent experiments). un, untransfected; ctl, control siRNA-treated. *Statistically significant difference from untransfected cells (P<0.05; Student's t-test).

another endogenously expressed GPI-anchored protein, was very similar (not shown). These results suggest a dominant role for the Sec24C and Sec24D in ER-to-Golgi transport of CD59 and folate receptor.

How does the Sec24 isoform preference of GPI-anchored proteins compare with that of transmembrane proteins that carry cytoplasmic-domain-exposed transport signals? Using a glycosylated retrieval-impaired variant of the type I membrane protein ERGIC-53 as a reporter, we previously showed that double knockdowns of Sec24 isoforms affect di-aromatic and di-hydrophobic signal-mediated transport in characteristic ways (Wendeler et al., 2007). In no case, however, was a narrow Sec24C-Sec24D dependence observed. To extend this signal analysis, we studied the requirement for Sec24 of the transport of the vesicular stomatitis virus glycoprotein (VSV-G). VSV-G is a model type I membrane protein carrying a cytoplasmically exposed DXE motif that is required for efficient ER-to-Golgi transport by interacting

with Sec24 (Mossessova et al., 2003; Nishimura and Balch, 1997; Sevier et al., 2000). HeLa cells were transfected with siRNA against Sec24 isoforms and 1 day later with VSV-G cDNA. After two more days, transport of VSV-G was studied by [^{35}S]methionine-cysteine pulse-chase. As shown in supplementary material Fig. S2, triple and quadruple isoform knockdowns strongly inhibited transport of VSV-G, confirming the known COPII dependence of ER exit of VSV-G (Aridor et al., 2001). Knockdown of single Sec24 isoforms had no effect on transport of VSV-G. By contrast, double knockdown of Sec24A and B (and to a lesser extent Sec24C and D) impaired transport of VSV-G, whereas the other double combinations had no effect. Similar results were obtained for the ER export of endogenously expressed non-raft-associated transferrin receptor, another DXE-signal-containing transmembrane protein (not shown). Thus, the dependence of DXE-signal-mediated ER export on the Sec24 isoforms also is considerably broader than for GPI-anchored proteins.

Transport of GPI-anchored proteins depends on p24 family proteins

The preference for Sec24C+Sec24D in the transport of CD59 suggests that in mammalian cells GPI-anchored proteins are actively recruited into COPII vesicles. This may occur by binding to a transmembrane protein that in turn binds to Sec24 and operates as a cargo receptor. In yeast, transport of the GPI-anchored protein Gas1p depends on the type I transmembrane protein Emp24p (Muniz et al., 2000; Muniz and Riezman, 2000). Emp24 is a member of the p24 protein family that comprises numerous ~24 kDa type I membrane proteins conserved from yeast to mammals (Carney and Bowen, 2004; Strating and Martens, 2009). We wondered if p24 proteins are involved in the transport of GPI-anchored proteins in HeLa cells. The major mammalian p24 family members are p23, p24 and p25. They form hetero-oligomeric complexes and cycle in the early secretory pathway (Blum et al., 1999; Rojo et al., 2000; Strating and Martens, 2009). p24 proteins are also major components of COPI-coated vesicles, acting in Golgi-to-ER retrograde traffic (Bethune et al., 2006a; Gommel et al., 1999). However, the precise function of the individual p24 proteins in mammals remains unclear.

To test for a potential role of p24 proteins in ER export of GPI-anchored proteins, we individually knocked down p23, p24 and p25 in HeLa cells by siRNA. The knockdown efficiency 3 days after transfection was 75% for each of the three family members as assessed by western blotting (supplementary material Fig. S3). Knockdown of p24 also silenced p23 dramatically. Inversely, knockdown of p23 also decreased the level of p24 considerably. Thus, the expression of p24 and p23 are interdependent. p25 levels were less dependent on p24 and p23 as silencing of p25 had a less dramatic effect on p24 and p23 levels.

Next, we examined the effect of the individual p24 family proteins on the transport of endogenous CD59. p24 silencing reduced the transport of CD59 considerably (Fig. 2A). p23 silencing reduced CD59 transport almost as efficiently. By contrast, p25 silencing had no statistically significant effect on CD59 transport, although p25 is believed to heterooligomerize with p24 and p23 (Jenne et al., 2002). A very similar dependence on p24 and p23 was observed for the GPI-anchored protein folate receptor alpha (Fig. 2B) that is also endogenously expressed in HeLa cells (Nichols et al., 2001; Zheng et al., 2003). Folate receptor is a ~32 kDa GPI-anchored protein, carrying three *N*-glycans (Roberts et al., 1998). Fig. S1B in supplementary material shows that the rate of transport

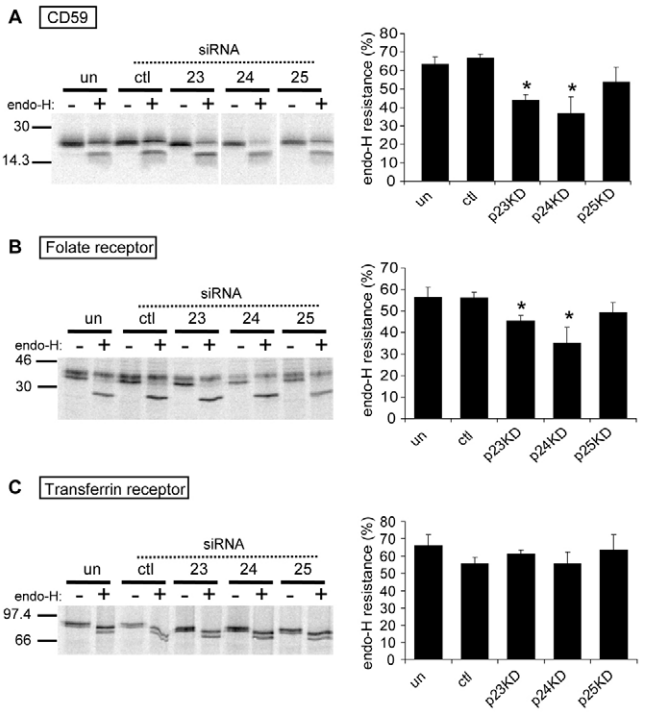

Fig. 2. Silencing p24 or p23 impairs transport of endogenous GPI-anchored proteins but not transferrin receptor. HeLa cells were transfected with siRNAs against p23 (23), p24 (24) or p25 (25). After 3 days, the cells were pulsed with [^{35}S]methionine-cysteine for 10 minutes, chased for 30 minutes (**A**), 90 minutes (**B**) or 40 minutes (**C**) and subjected to immunoprecipitation with antibodies against the indicated proteins. Immunoprecipitates were treated with (+) or without (–) endo-H and separated by SDS-PAGE followed by autoradiography (left panels). Quantification of endo-H resistance is given in the right panels (means ± s.d., n=3 independent experiments). *Statistically significant difference from untransfected cells (P<0.05; Student's t-test). Molecular masses in kDa are indicated on the left; un, untransfected; ctl, transfection with control siRNA.

of folate receptor, assessed by the pulse-chase with endo-H approach, is about 90 minutes, and this time point was chosen for analysis. Again, p25 was not important for transport. Thus, anterograde transport of the two endogenous GPI-anchored proteins exhibited very similar dependence on p24 proteins.

For comparison, we investigated whether the transport of endogenously expressed transferrin receptor is affected by the silencing of p24 family members. This type I transmembrane protein carries three *N*-glycans, one of which remains endo-H sensitive during passage through the Golgi (Enns et al., 1991). The rate of ER-to-Golgi transport of transferrin receptor in HeLa cells was found to be 30-40 minutes (supplementary material Fig. S1C). Fig. 2C clearly shows that silencing p23, p24 or p25 does not affect the transport of transferrin receptor. Overall, these findings suggest a selective function of p24 and p23 in ER-to-Golgi transport of human GPI-anchored proteins.

p24 and p23 interact with GPI-anchored proteins

The dependence of GPI-anchored proteins on p24 and p23 for transport may be due to an interaction of the two protein classes. To test this, co-immunoprecipitation experiments were performed with HeLa cells transiently expressing Myc-tagged p23 or HA-

tagged p24. Myc-p23 and HA-p24 proteins have been described previously (Emery et al., 2000; Rojo et al., 2000). We first examined whether the tags affect p24-p23 interaction. We found that endogenous p23 can be pulled down with anti-HA, and endogenous p24 with anti-Myc, indicating that the tags allow normal p24-p23 interaction (supplementary material Fig. S4). We then probed for interaction of endogenous CD59 and Myc-p23. Fig. 3 shows that endogenous CD59, as well as CD59-GFP, can pull down Myc-p23 and vice versa. Similar pull-down results were obtained for CD59 (or CD59-GFP) and HA-p24 (Fig. 3D). By contrast, CD59 did not pull down Myc-p25 (supplementary material Fig. S5), which is consistent with the unaltered transport of CD59 in p25 knockdown cells (Fig. 2A). Moreover, CD59 was not pulled down by Myc-p23ΔCC, a construct in which the coiled-coil domain, known to mediate oligomerization (Koegler et al., 2010), was deleted (not shown).

Since, at steady state, CD59 is predominantly associated with the plasma membrane (Kenworthy et al., 2000) and p24 and p23 with the ER-ERGIC-Golgi system [although some post-Golgi localization of p23 has been reported (Blum and Lepier, 2008; Chen et al., 2006)], it was important to determine if the interaction of CD59 and p24 proteins can take place early in the secretory pathway. To test this, HeLa cells expressing Myc-p23 or HA-p24 were pulse labeled with [^{35}S]methionine-cysteine for 15 minutes and cell lysates were immunoprecipitated with anti-HA, anti-Myc or anti-CD59. Fig. 3E,F shows that newly synthesized Myc-p23 and HA-p24 were able to pull down newly synthesized endogenous CD59 and vice versa.

To investigate whether p24 and p23 also interact with other GPI-anchored proteins, we performed pull-down experiments with anti-folate receptor which indeed resulted in specific co-immunoprecipitation with Myc-p23 (not shown). Since the antibody to folate receptor was only useful for immunoprecipitation but not for western blotting, the interaction of the folate receptor with p24 or p23 was not studied in greater detail. Instead, we used GFP-GPI, a hybrid reporter protein consisting of Myc-tagged GFP fused to the GPI anchor attachment signal of the folate receptor (Paladino et al., 2004). Cells were transfected with GFP-GPI together with Myc-p23 or HA-p24 and subjected to immunoprecipitation. Fig. 4A,B shows that GFP-GPI can pull down Myc-p23 as well as HA-p24. The interaction of the GFP-GPI with p24 occurs early after synthesis as shown by a 15-minute [^{35}S]methionine-cysteine pulse-labeling experiment (Fig. 4C). Taken together, these results suggest physical interactions of p24 and p23 with GPI-anchored proteins.

p24 and GPI-anchored proteins share the same Sec24 isoform preference for ER export

If p24 and p23 are indeed required for efficient packaging of GPI-anchored proteins into COPII transport vesicles, one would expect that ER-to-Golgi transport of p24 and p23 is also preferentially dependent on Sec24C and Sec24D. This transport cannot be studied by pulse-chase and endo-H experiments because p24 and p23 lack *N*-glycans. We therefore monitored the effect of knockdown of Sec24 isoforms on the distribution of endogenous p24 using immunofluorescence microscopy. This approach is based on our previous findings which showed that a quadruple knockdown of Sec24 isoforms leads to redistribution of ERGIC-53 from the ERGIC to the ER (Wendeler et al., 2007) (also see Fig. 5H). In control cells, p24 localized to the Golgi and to peripheral ERGIC dots (Fig. 5A). Double knockdown of Sec24C+Sec24D clearly led to an ER pattern of p24 in addition

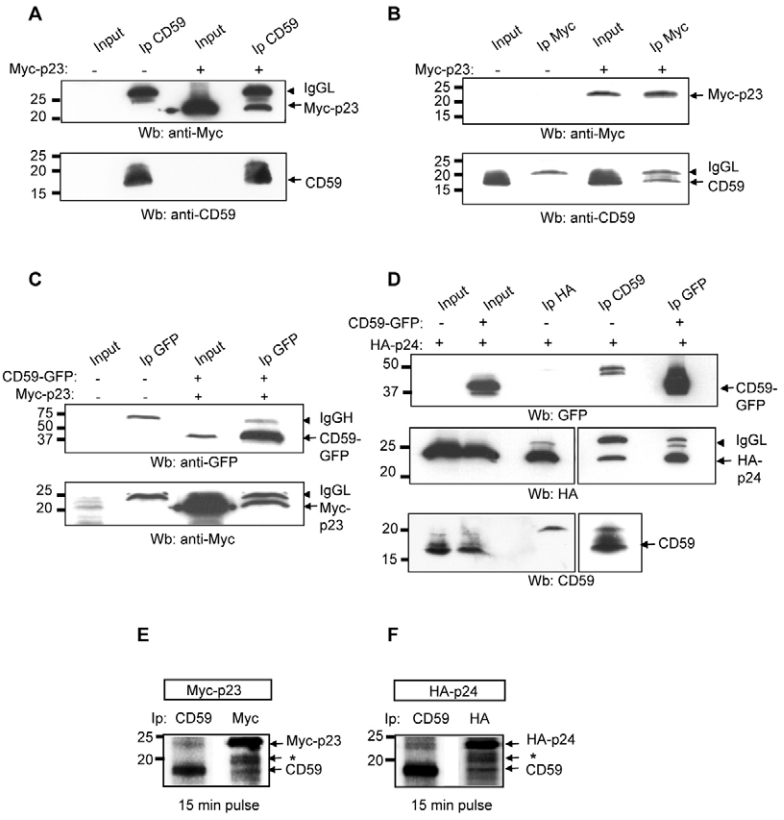

Fig. 3. CD59 interacts with p24 and p23. HeLa cells were either untransfected (–) or transfected (+) with Myc-p23 (**A**,**B**,**E**), Myc-p23 and CD59-GFP (**C**), HA-p24 (**D**,**F**), HA-p24 and CD59-GFP (D), as indicated. After 48 hours the cells were subjected to immunoprecipitation (Ip), SDS-PAGE, and western blotting (Wb) using the indicated antibodies. (E,F) Cells were pulsed with [^{35}S]methionine-cysteine for 15 minutes and subjected to immunoprecipitation with the indicated antibodies followed by SDS-PAGE and autoradiography. Note that newly synthesized Myc-p23 and HA-p24 pulled down newly synthesized endogenous CD59 and vice versa. *Bands that probably correspond to endogenous p24 and p23. IgGH, heavy chain of immunoglobulins; IgGL, light chain of immunoglobulins. Positions of molecular mass markers are indicated on the left.

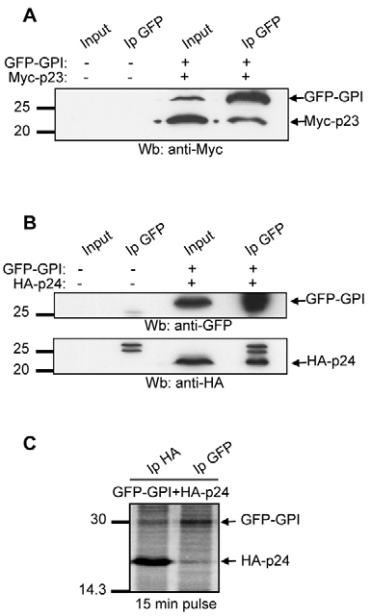

Fig. 4. GFP fused to the GPI anchor of folate receptor (GFP-GPI) interacts with p24 and p23. HeLa cells were either untransfected (–) or transfected (+) with GFP-GPI and Myc-p23 (**A**), or GFP-GPI and HA-p24 (**B**,**C**). (A,B) After 48 hours, the cells were subjected to immunoprecipitation (Ip) and western blotting (Wb) using the indicated antibodies. (C) Cells were pulsed with [^{35}S]methionine-cysteine for 15 minutes and subjected to immunoprecipitation with the indicated antibodies followed by SDS-PAGE and autoradiography. Note that newly synthesized Myc-p23 and HA-p24 pulled down newly synthesized GFP-GPI and vice versa. Positions of molecular mass markers are indicated on the left.

to the Golgi signal (Fig. 5C). However, such an ER pattern was not seen for the Sec24A+Sec24B double knockdown (Fig. 5B). By contrast, the Sec24A+Sec24B knockdown increased the ER pattern of endogenous ERGIC-53 (Fig. 5F), consistent with its transport dependence on Sec24A+Sec24B (Wendeler et al., 2007). No ER increase was seen for ERGIC-53 after knockdown of Sec24C+Sec24D (Fig. 5G) which does not reduce ERGIC-53 transport (Wendeler et al., 2007). Consistent with the biochemical data, a quadruple knockdown of all Sec24 isoforms blocked p24 in the ER (Fig. 5D). Whether p23 transport is also dependent on Sec24C+Sec24D could not directly be assessed since no appropriate antibody was available for its localization using immunofluorescence microscopy, but given the transport interdependence of p24 and p23, it is highly likely that p23 transport is also Sec24C+Sec24D dependent.

Peptides corresponding to cytoplasmic tails of p24 family members have been shown to bind to COPII in vitro in a diphenylalanine-signal-dependent manner (Dominguez et al., 1998), but interactions with Sec24 isoforms have not been studied. To investigate such potential interactions, lysates of cells expressing Myc-p23 or HA-p24 were immunoprecipitated with anti-Myc or anti-HA. Western blot analysis with isoform-specific antibodies revealed specific interaction of Sec24C and Sec24D with both Myc-p23 and HA-p24, Sec24C exhibiting the strongest signal (Fig. 5I,J). Co-immunoprecipitation with anti-Sec24A and anti-Sec24B was considerably less prominent. These biochemical results are in accord with the morphological data. Together, the results indicate that Sec24C and Sec24D are required for efficient export of p24 and p23 from the ER.

Co-partitioning of GPI-anchored proteins and p24-p23 into lipid rafts

An important feature of GPI-anchored proteins is their association with detergent-resistant membrane domains termed lipid rafts. Lipid rafts are specialized, dynamic lipid assemblies enriched in cholesterol and sphingolipids (Brown and London, 1998; Simons and Ikonen, 1997). Since p24 and p23 interact with the GPI-anchored CD59, we examined whether they partition into lipid rafts by using a cold Triton X-100 extraction procedure in conjunction with equilibrium sucrose gradient centrifugation (see Materials and Methods). Fractions were collected and probed by western blotting. As expected, CD59 was present in the low density lipid raft fraction and co-partitioned with the typical raft marker caveolin-1 (Fig. 6A). By contrast, the endosomal marker transferrin receptor, the ER marker BAP-31, the cis-Golgi marker GPP-130 and the ERGIC marker ERGIC-53 were entirely recovered in high-density fractions (Fig. 6A). Interestingly, a fraction of endogenous p24 and p23 co-partitioned with CD59 into low-density fractions, although the proteins were most prominent in the high-density fractions at the bottom of the gradient (Fig. 6A). Partial co-distribution with raft fractions was also seen for HA-p24 and Myc-p23, and the two proteins could be co-precipitated with CD59 from pooled raft fractions (Fig. 6B,C). The partial presence of p24 and p23 in lipid rafts hinted at a possible role of lipid microdomains in selective ER export of GPI-anchored proteins.

Since the analysis of lipid rafts from total membranes at steady state does not indicate in which compartment of the secretory pathway this partitioning occurs, we used a 10-minute pulse with [^{35}S]methionine-cysteine which makes it possible to study newly synthesized proteins that have not reached the Golgi complex. HeLa cells transfected with HA-p24 or Myc-p23 were pulse labeled and subjected to sucrose density gradient centrifugation. Fig. 6D shows that 22.3% of CD59 floated to the 15-25% fractions unlike transferrin receptor and BAP-31, used as non-raft markers. CD59 in this early lipid raft fraction was completely endo-H sensitive. Of note, 18% of HA-p24 and 14.4% of Myc-p23, but not ERGIC-53, co-partitioned with CD59 in the raft fractions. Pull-down experiments with pooled raft fractions (Fig. 6E) and non-raft fractions (supplementary material Fig. S6A) prepared from 15-minute [^{35}S]methionine-cysteine pulse-labeled cells showed interaction of HA-p24 with CD59. These results indicate that the interaction of GPI-anchored proteins with p24-p23 and the partitioning of this complex into lipid rafts occur early in the secretory pathway, most probably in the ER.

To validate the association of the complex with rafts, we used methyl-β-cyclodextrin (MβCD), which is known to remove cholesterol from membranes. MβCD disrupts sphingolipid- and cholesterol-based microdomains and renders detergent-insoluble complexes detergent soluble (Scheiffele et al., 1997). After a 30-minute MβCD treatment, caveolin-1 and CD59 appeared in detergent-soluble high density fractions rather than raft fractions, as expected (Fig. 7A). Similarly, p24 and p23 were no longer associated with low density fractions (Fig. 7A). Does cholesterol removal interfere with the binding of GPI-anchored proteins to p24-p23? To address this question, HeLa cells expressing Myc-p23 or HA-p24 were treated with MβCD prior to immunoprecipitation. As shown in Fig. 7B, MβCD reduced the interaction of Myc-p23 and HA-p24 with CD59. We also tested

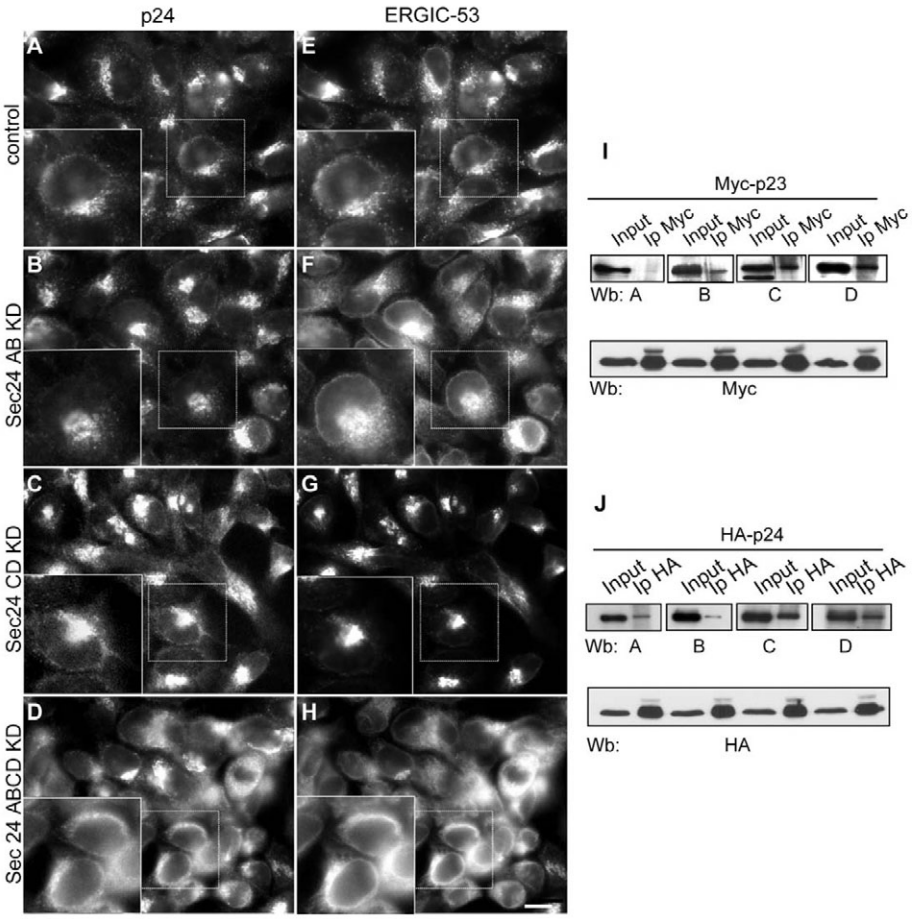

Fig. 5. Sec24 isoform selectivity of p24 and p23. (**A-H**) HeLa cells were analyzed by double immunofluorescence microscopy using antibodies against p24 and ERGIC-53 3 days after transfection with control siRNA (control), or siRNAs against Sec24A and B, Sec24C and D, and Sec24A, B, C and D. Scale bar: 10 μm. Insets show higher magnifications of boxed regions. (**I**,**J**) Cells were transfected with Myc-p23 or HA-p24 and 48 hours later subjected to immunoprecipitation (Ip) with anti-Myc or anti-HA followed by immunoblotting with antibodies against Sec24A (A), Sec24B (B), Sec24C (C) or Sec24D (D) (upper panels). Molecular masses of the Sec24A-D isoforms (in kDa) are 113, 137, 121 or 110, respectively. Lower panels: blots with anti-Myc or anti-HA of the samples shown in the upper panel.

whether the early interaction of CD59 and HA-p24 requires cholesterol. Cholesterol depletion was found not to disturb the CD59–HA-p24 interaction in 15-minute pulsed cells (supplementary material Fig. S6B). The results suggest that complex maintenance but not complex initiation requires cholesterol.

We also monitored the effect of cholesterol depletion on the intracellular transport of CD59, transferrin receptor, and a Myc-tagged glycosylated construct of ERGIC-53 that lacks the dilysine ER-retrieval signal (Myc-ERGIC-53) (Itin et al., 1995). MβCD treatment strongly affected ER to medial-Golgi transport of CD59 but not that of transferrin receptor and Myc-ERGIC-53 (Fig. 7C,D). Reducing the concentration of MβCD to 10 mM gave similar results (supplementary material Fig. S7), arguing against a non-specific effect of the drug.

Finally, we analyzed the localization of the different cargo receptors and cargo proteins after cholesterol depletion. HeLa cells were treated with MβCD, immunostained, and analyzed by fluorescence microscopy. MβCD treatment led to retention of CD59 in the ER (Fig. 8E), although some CD59 was still present at the cell surface; this was most probably molecules that had not turned over within the time of MβCD treatment. Likewise, p24 redistributed to the ER (Fig. 8G). By contrast, ERGIC-53 did not redistribute to the ER upon MβCD treatment (compare Fig. 8F and B). Cholesterol depletion led to some concentration of ER exit sites in the juxtanuclear region as indicated by Sec24C staining (Fig. 8H). The data indicate a block in trafficking of CD59 and p24 but not ERGIC-53 in the ER in the absence of cholesterol and are in agreement with the pulse-chase experiments shown in Fig. 7.

Discussion

The results of this study establish a critical role for COPII in the export of human GPI-anchored proteins from the ER and show that this process preferentially involves the Sec24 isoforms Sec24C and Sec24D. Efficient anterograde transport depends on the p24-p23 transmembrane protein complex that binds to GPI-anchored proteins and also largely depends on Sec24C+Sec24D. Moreover, we find that newly synthesized GPI-anchored proteins together with a fraction of p24-p23 partition into a biosynthetically early raft-like fraction.

COPII dependence of GPI-anchored protein transport

The mechanism by which newly synthesized proteins enter COPII vesicles has not generally been solved, although good examples exist for selective signal-mediated export of transmembrane and soluble proteins. ER export signals of transmembrane proteins typically reside in their cytoplasmic domain and bind to the Sec24 subunit of the COPII coat (Barlowe, 2003; Mancias and Goldberg, 2005; Miller et al., 2002; Wendeler et al., 2007). Such proteins can act as cargo receptors for soluble proteins, as best illustrated for ERGIC-53 (Baines and Zhang, 2007; Hauri et al., 2000; Nufer et al., 2003; Nyfeler et al., 2006). Since GPI-anchored proteins cannot directly interact with Sec24, the simplest mechanism of ER export

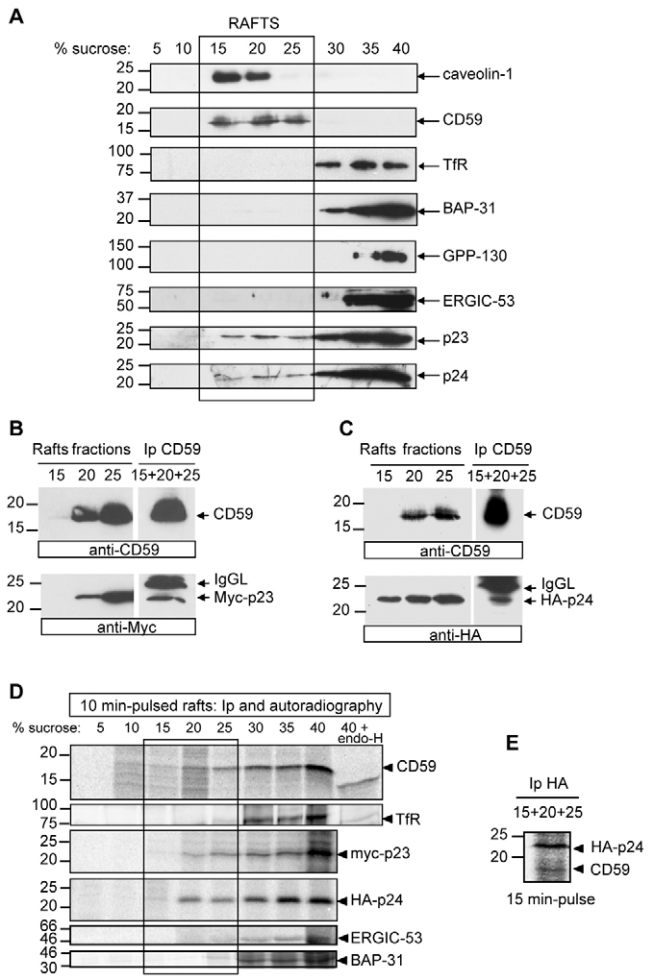

Fig. 6. Co-partitioning of GPI-anchored proteins and p24-p23 into lipid rafts. HeLa cells were untransfected (**A**) or were transfected with Myc-p23 (**B**) or with HA-p24 (**C**). Cells were lysed in MBS-Triton X-100 at 4°C and analyzed by sucrose gradient centrifugation (see Materials and Methods). Fractions were collected and separated by SDS-PAGE followed by western blotting with antibodies against the indicated proteins. In parallel, the lipid raft fraction (15%+20%+25% sucrose) was collected, immunoprecipitated with anti-CD59 antibody (B and C), separated by SDS-PAGE, and immunoblotted with anti-CD59 and anti-Myc antibodies (B) or with anti-CD59 and HA antibodies (C). IgGL: light chain of immunglobulins. (**D**) Untransfected cells [to detect endogenous CD59, transferrin receptor (TfR), ERGIC-53, and BAP-31] or cells transfected with Myc-p23 or HA-p24 were pulsed for 10 minutes with [^{35}S]methionine-cysteine and subjected to sucrose density gradient centrifugation (as in A). Fractions were immunoprecipitated with antibodies against the indicated proteins or Myc and HA, and separated by SDS-PAGE followed by autoradiography. 40+endo-H, immunoprecipitated CD59 and TfR were digested with endo-H (+endo-H) before analysis. (**E**) Lipid raft fractions (15+20+25) from 15-minute pulse-labeled cells were pooled, immunoprecipitated with anti-HA and analyzed by SDS-PAGE followed by autoradiography. Note co-immunoprecipitation with CD59. Positions of molecular mass markers (in kDa) are shown on the left.

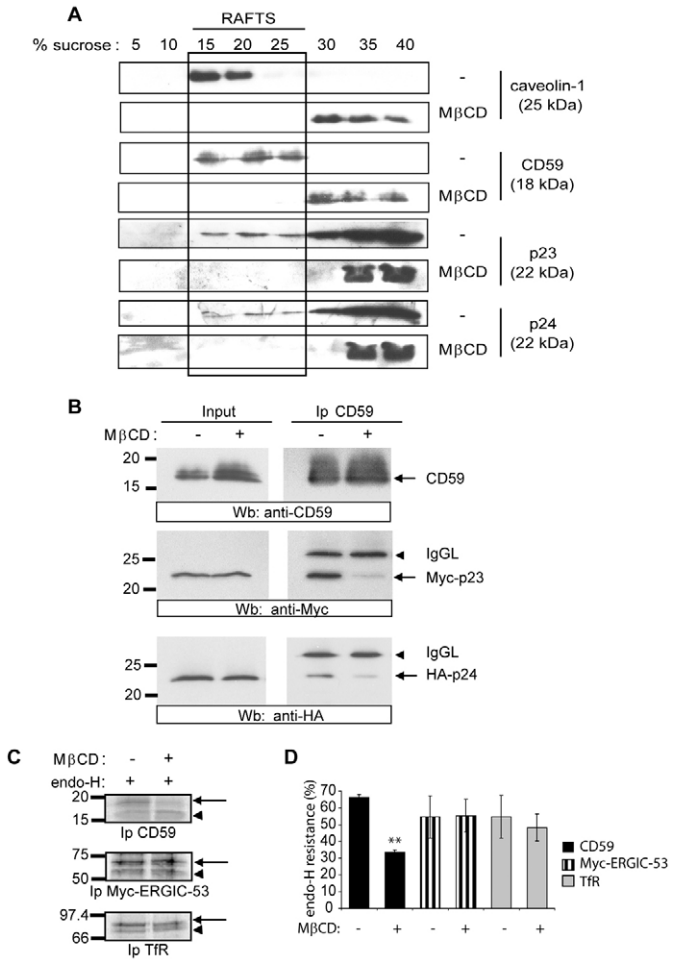

Fig. 7. Cholesterol depletion by MβCD disrupts the interaction of GPI-anchored proteins and p24-p23. HeLa cells either untransfected (**A**,**C**,**D**) or transfected with Myc-p23 or with HA-p24 (**B**) or with glycosylated retrieval-impaired Myc-ERGIC-53 (Myc-ERGIC-53) (C,D) were treated (+) or not (–) with 20 mM MβCD for 30 minutes at 37°C. (A) Cells were lysed in MBS-Triton X-100 at 4°C, run through 5-40% sucrose gradients, subjected to SDS-PAGE and immunoblotted with antibodies against the indicated proteins. (B) Cells were lysed in 1% Triton X-100 and immunoprecipitated with anti-CD59. The immunoprecipitates were separated by SDS-PAGE and western blotting was performed with anti-CD59, anti-Myc or anti-HA. IgGL, light chain of immunoglobulins. (C,D) Cells were pulsed with [^{35}S]methionine-cysteine for 10 minutes, chased for 30 minutes (for CD59), 40 minutes (for transferrin-receptor) or 60 minutes (for Myc-ERGIC-53), lysed in 1% Triton X-100, and subjected to immunoprecipitation with antibodies against CD59, Myc or transferrin receptor. Immunoprecipitates were treated with endo-H (+) and separated by SDS-PAGE followed by autoradiography. Endo-H-resistant (arrow) and endo-H-sensitive (arrowhead) forms of CD59, transferrin receptor (TfR) and Myc-ERGIC-53 (Myc-ERGIC-53) are indicated. MβCD, methyl-β–cyclodextrin.

would be bulk flow, that is, non-selective inclusion into transport vesicles, and such a mechanism may not strictly depend on COPII. Our data show, however, that export of the GPI-anchored protein CD59 from the ER in human cells is a COPII-dependent process, since a quadruple knockdown of the Sec24 isoforms considerably impairs its ER-Golgi transport, like that of known COPII-dependent transmembrane proteins. This Sec24 requirement preferentially involves Sec24C+Sec24D, indicating that ER export of GPI-anchored proteins is selective rather than by bulk flow. Interestingly, this isoform selectivity is rather narrow in comparison to that of the type I transmembrane proteins VSV-G and transferrin receptor, both of which contain a cytosolic di-acidic ER export signal and is

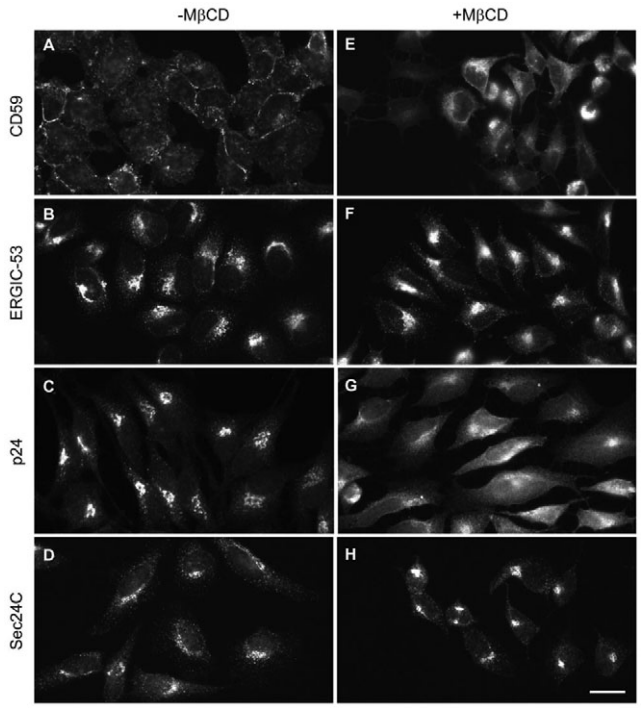

Fig. 8. Effect of cholesterol depletion by MβCD on the localization of endogenous CD59, ERGIC-53, p24 and Sec24C. HeLa cells were untreated (**A-D**) or treated (**E-H**) with 20 mM MβCD for 30 minutes at 37°C and analyzed by immunofluorescence microscopy using antibodies against CD59, ERGIC-53 p24 and Sec24C. Note that CD59 and p24 localize to the ER in MβCD-treated cells, whereas ERGIC-53 does not redistribute to the ER. Scale bar: 10 μm.

different from that of ERGIC-53 whose ER export from the ER preferentially depends on Sec24A+Sec24B (Wendeler et al., 2007) (this study). In the absence of Sec24C+Sec24D there may be some compensatory action by the other Sec24 isoforms as indicated by the fact that a knockdown of all four Sec24 isoforms further reduces transport.

Role of p24 proteins in selective ER export of GPI-anchored proteins

Conceptually, selective export of human GPI-anchored proteins may be achieved by an indirect mechanism in which these proteins bind to transmembrane proteins carrying ER export signals that can selectively interact with Sec24 isoforms. Alternatively, sorting may be driven by a lipid-based mechanism in which the proteins partition into a lipid subdomain of the ER that forms COPII vesicles preferentially carrying Sec24C+Sec24D. Inspired by previous work in yeast, showing that the transmembrane protein Emp24p is required for efficient ER export of the GPI-anchored protein Gas1 (Muniz et al., 2000), we uncovered a role of the p24 family members p24 and p23 in GPI-anchored protein export from the ER in HeLa cells. p24 family proteins form heteromeric complexes that continuously cycle between ER, ERGIC and Golgi (Carney and Bowen, 2004; Jenne et al., 2002; Strating and Martens, 2009). Owing to their abundance in COPI vesicles (Stamnes et al., 1995) and their capacity to bind COPI in vitro (Dominguez et al., 1998; Sohn et al., 1996), p24 members are assumed to play an important role in COPI-vesicle biogenesis and retrograde transport (Aguilera-Romero et al., 2008; Beck et al., 2009; Bethune et al., 2006a; Bethune et al., 2006b; Mitrovic et al., 2008). Although mammalian p24 proteins also bind COPII in vitro (Dominguez et al., 1998), little is known about their role in anterograde ER to Golgi transport. Our knockdown experiments revealed a role of p24 and p23 in ER export of human GPI-anchored proteins. Both p24 and p23 silencing impaired ER-to-Golgi transport of CD59 and folate receptor but not that of the transmembrane protein transferrin receptor. Most likely, GPI-protein-mediated transport involves both p24 and p23, given that p24 forms a complex with p23 and a knockdown of p24 also drastically reduced p23 protein levels. By contrast, p25 does not seem to participate in selective ER export of GPI-anchored proteins, although numerous studies suggest that p25 can oligomerize with p24 and p23 (Denzel et al., 2000; Dominguez et al., 1998; Emery et al., 2003; Jenne et al., 2002). Conceivably, p25 binds to p24 and p23 in the Golgi and thereby facilitates retrograde rather than anterograde transport of the protein complex (Jenne et al., 2002).

While our work was in progress, Takida et al. reported that a knockdown of p23 results in delayed plasma membrane appearance of a transfected VSV-G-GPI hybrid molecule and of transfected decay-accelerating factor, a GPI-anchored protein (Takida et al., 2008). This work is consistent with our data. However, in our study we have gone considerably further into the mechanism underlying anterograde transport of mammalian GPI-anchored proteins by studying early events and also including endogenously expressed proteins. Importantly, we discovered an unprecedented interaction of GPI-anchored proteins and p24-p23, early in the secretory pathway, most probably in the ER. Together with the shared Sec24C+Sec24D isoform preference of the p24 and GPI-anchored proteins, this finding suggests that the p24-p23 complex acts as a cargo receptor, facilitating export of GPI-anchored proteins from the ER. Sec24 binding of p24 and p23 is most likely mediated by their diphenylalanine ER-export motif in the cytoplasmic domain at position –7 and –8 from the C-terminus (Dominguez et al., 1998). Of note, ERGIC-53 also carries a diphenylalanine ER-export motif (at position –1 and –2) (Kappeler et al., 1997), but this signal determines a Sec24A+Sec24B and Sec24A+Sec24C preference (Wendeler et al., 2007). Obviously the two diphenylalanine ER export motifs are decoded differently in a Sec24 isoform-selective manner.

Lipid raft-based early sorting of GPI-anchored proteins

Lipid domains can influence cargo sorting into budding vesicles. This is particularly well known for the sorting of GPI-anchored proteins into the apical pathway of polarized epithelial cells at the level of the Golgi complex (Schuck and Simons, 2004). Apical sorting is determined by the partitioning of GPI-anchored proteins into lipid rafts enriched in cholesterol and sphingolipids, which form apical sorting platforms (Simons and Ikonen, 1997). We found a fraction of p24 and p23 to co-partition with the GPI-anchored protein CD59 into lipid rafts of HeLa cells. Although rafts are believed to form in the Golgi in mammalian cells, this biochemically determined partitioning was already apparent in 10-15 minutes in [^{35}S]methionine-cysteine pulse-labeled cells, consistent with an ER event. Further evidence for an ER event is the observation that ER-to-Golgi transport of CD59 but not transferrin receptor is impaired in cholesterol-depleted cells, as assessed by pulse-chase experiments, and that cholesterol depletion leads to a selective accumulation of CD59 and p24 in the ER, as visualized by immunofluorescence microscopy.

There are some previous reports on the association of proteins with biosynthetically early rafts. For instance, Browman et al. reported the existence of lipid-raft-like domains in the ER, into which the members of the prohibitin family erlin-1 and erlin-2 partition (Browman et al., 2006). Moreover, GPI-anchor intermediates (Sevlever et al., 1999) and ER-confined enzymatic activities involved in the synthesis and transfer of the GPI anchor (Pielsticker et al., 2005) have been observed in rafts of subcellular compartments that include the ER. The precise composition of these lipid-raft-like microdomains remains to be determined, but it is clear that the ER membrane possesses sterols and sphingolipids, although at low concentrations (van Meer and Lisman, 2002), which are likely to form the lipid rafts into which GPI-anchored proteins and the p24-p23 complex partition. Consistent with this notion, the association of newly synthesized cholesterol with lipid rafts was detectable already after a 5-minute chase following a 5-minute pulse (Heino et al., 2000). Interestingly, the interaction of CD59 and p24-p23 in 15-minute pulsed cells was not affected by MβCD, although ER export of these proteins was impaired, suggesting that the initial interaction of CD59 with its cargo receptor after biosynthesis is cholesterol independent, but cholesterol is required for the partitioning of the CD59-p24-p23 complex to ER rafts and ER export.

We propose the following model for ER export of newly synthesized mammalian GPI-anchored proteins. Newly synthesized GPI-anchored proteins interact with p24-p23, and the triple complex partitions into raft-like lipid domains in the ER. Binding of Sec24C+Sec24D to the cytosolic ER export signals of p24 and p23 then clusters the rafts to larger entities, and COPII coat completion induces vesicle budding (Fig. 9). This pathway excludes non raft-associated proteins, such as ERGIC-53, which are packaged into non-raft-derived COPII vesicles. The non-raft-derived COPII vesicles may be heterogenous regarding their Sec24 isoform composition. The partitioning of GPI-anchored proteins to ER rafts would be mediated by their anchor. The detailed mechanism underlying co-partitioning of the p24-p23 complex to ER rafts remains to be elucidated. One possibility is transmembrane domain-mediated co-partitioning resulting from protein-lipid interaction, but such a mechanism does not appear to be responsible for the initial interaction. Alternatively, the p24-p23 complex may indirectly partition into the lipid rafts by binding to the GPI-anchored proteins. The known common denominator of GPI-anchored proteins is their anchor although its composition is quite diverse. Owing to the considerable size of the luminal domains of the GPI-anchored proteins and the p24-p23 complex, a direct interaction of the lipid anchor with the transmembrane domain of p24-p23 is precluded. Likewise, a direct luminal protein-protein interaction of GPI-anchored proteins with p24-p23 seems unlikely since GPI-anchored proteins are non-homologous proteins. A speculative possibility is, however, that p24-p23 acts as a lectin by binding to the glycan part of the GPI anchor. Alternatively, the interaction of GPI-anchored proteins and p24-p23 is mediated by a linker protein. Such a function has been proposed for VIP17/MAL in sorting GPI-anchored proteins to apical transport vesicles in polarized cells (Magal et al., 2009; Schuck and Simons, 2004). Perhaps PGAP5 is such a linker in the ER. PGAP5 catalyzes the remodeling of the glycan moiety on GPI-anchored proteins, interacts with p23, and is required for GPI-anchored protein export from the ER (Fujita et al., 2009).

ER raft-derived COPII vesicles would also require SNARE proteins for their subsequent docking and fusion with ERGIC membranes. We note that in semi-permeable cells the SNARE proteins syntaxin 5 and membrin are specifically packaged into COPII vesicles by Sec24C and Sec24D, but not Sec24A and Sec24B (Mancias and Goldberg, 2008). Thus, these SNAREs may be included in ER-raft-derived COPII vesicles. It is worth noting, however, that a Sec24C+Sec24D selectivity per se is not sufficient for a protein to partition into lipid-raft-derived vesicles. It must be combined with a corresponding lipid selectivity, which remains to be investigated for these SNAREs.

Raft-based co-sorting of GPI-anchored proteins and p24-p23 in the ER provides a means of transporting tail-less membrane proteins together with long chain lipids by the general COPII machinery. The overall preference of this pathway for Sec24C and Sec24D most probably reflects an abundance of Sec24C- and Sec24D-selective transmembrane proteins in ER lipid rafts of which p24 and p23 are major contributors.

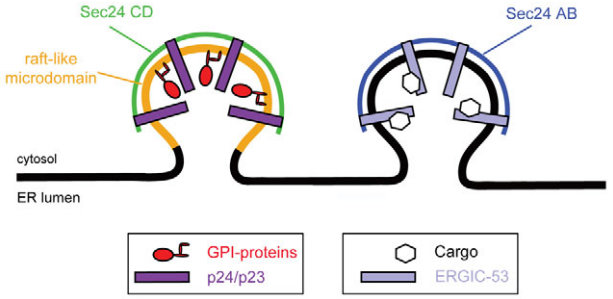

Fig. 9. Model of selective ER export of GPI-anchored proteins and of the transmembrane protein ERGIC-53 (see Discussion). GPI-anchored proteins and the p24-p23 complex co-partition into raft-like microdomains of ER membranes. The cytosolic domains of p24/p23 preferentially interact with the Sec24 isoforms Sec24C and Sec24D of COPII. In contrast, upon binding its soluble glycoprotein cargo, ERGIC-53 preferentially interacts with Sec24A and Sec24B in non-raft subdomains of the ER. After full assembly of the COPII coat, different vesicles are formed, defined by a combined action of lipid partitioning and Sec24 isoform selectivity.

Materials and Methods

Antibodies, siRNAs and cDNAs

Mouse monoclonal antibodies: G1/93 against human ERGIC-53 (Enzo Life Sciences, Lausen, Switzerland) (Schweizer et al., 1988), G1/221/12 against human transferrin-receptor (Vollenweider et al., 1998), IgG1 against human folate receptor (Abcam, Cambridge, UK), IgG2a against human CD59 (AbD Serotec, Dusseldorf, Germany), IgG2a against human anti-tubulin (kind gift from Karl Matter, University College London, UK), A1/182/5 against human BAP31 (Enzo Life Sciences, Lausen, Switzerland), A1/118/4 against human GPP130 (Linstedt et al., 1997), 9E10.2 against the Myc epitope (ATCC #CRL 1729), 12CA5 against the hemagglutinin (HA) epitope, IgG1 against green fluorescent protein (GFP; Roche, Rotkreuz, Switzerland), anti-VSV-G (clone P5D4, kind gift from Kai Simons, Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany). Rabbit polyclonal antibodies: anti-human ERGIC-53, anti-human Sec24A, Sec24B, Sec24C, Sec24D (Pagano et al., 1999; Wendeler et al., 2007), anti-human p23, p24, p25 (Jenne et al., 2002) (kind gifts from Felix Wieland, University of Heidelberg, Germany), anti-caveolin-1 (Santa Cruz, Biotechnology Inc., Heidelberg, Germany), Alexa-Fluor-488-, Alexa-Fluor-568- (Molecular Probes Europe, Leiden, The Netherlands); and horseradish-peroxidase-coupled antibodies (The Jackson ImmunoResearch Laboratories, Newmarket, UK) were used as secondary antibodies. siRNAs: control siRNA and siRNA oligonucleotides against human Sec24A, Sec24B, Sec24C and Sec24D were previously described (Wendeler et al., 2007). 5′-AAGCATTTACTAATTGGAAA-3′ and 5′-CACCTCAGAATCACAG-TGTTA-3′ were target sequences for human p24 (NM_006815) and human GP25L (p25, BC001123), respectively (Qiagen, Hilden, Germany). The siRNA oligonucleotide against human TMP21 (p23) was from Dharmacon RNA technologies (SI-GENOMESMART pool reagent NM_006827). cDNAs: plasmids for the expression of N-terminally tagged Myc-p23, HA-p24 and Myc-p25 [pCB6-

Myc-p23 (Rojo et al., 2000); pCB6-HA-p24 and pCBA-Myc-p25 (Emery et al., 2000) were kind gifts from Jean Gruenberg, University of Geneva, Geneva, Switzerland]; pCB6-Myc-p23ΔCC (Koegler et al., 2010), pCDM8-CD59-GFP [(Nichols et al., 2001) kind gifts from Anne Kenworthy, Vanderbilt University, Nashville, USA], pJB20-GFP-GPI [(Paladino et al., 2004), possessing a Myc tag, a kind gift from Chiara Zurzolo, Pasteur Institute, Paris, France], pCDNA 3.1-VSV-G a kind gift from Gert Zimmer (Institute of Virology, Hannover, Germany). Myc-ERGIC-53, a myc-tagged, N-glycosylated variant of ERGIC-53 in which the di-lysine ER retrieval signal was mutated to di-alanine was described previously (Itin et al., 1995).

Cell culture, transfection, metabolic labeling and western blotting

HeLa cells (obtained from ATCC) were grown at 37°C in DMEM supplemented with 10% fetal bovine serum, 1% non-essential amino acids (Sigma-Aldrich, Buchs, Switzerland), 1% penicillin-streptomycin (Sigma-Aldrich). Twenty-four hours before transfection, HeLa cells were seeded in 6 cm or 10 cm plates. Incubation of 6 µl of Hiperfect transfection reagent (Hiperfect, Qiagen) with siRNA at a final concentration of 5 nM in 100 µl of serum-free DMEM was performed for 15 minutes at room temperature and the transfection complexes were then added drop-wise onto the cells. After 72 hours, the cells were pulsed with [^{35}S]methionine-cysteine for 10 minutes and chased in the presence of unlabeled methionine in excess for 30 minutes (for CD59), 40 minutes (for transferrin-receptor) or 90 minutes (for folate receptor) and immunoprecipitated with the corresponding antibodies. After endo-H digestion (Wendeler et al., 2007), proteins were separated by SDS-PAGE, visualized by autoradiography, and quantified by phosphor imaging using ImageQuantTM software (Molecular Dynamics, Sunnyvale, CA, USA). Percentage endo-H resistance refers to the ratio of the signal of the endo-H-resistant form to the combined signal of the endo-H-resistant plus the endo H-sensitive form expressed as a percentage. For western blotting, protein extracts were separated by SDS-PAGE, transferred to nitrocellulose and the blots were incubated with appropriate primary antibodies at 4°C overnight. Blots were developed using the ECL (GE Healthcare Europe GmbH, Otelfingen, Switzerland) or Chemiglow (Witec AG, Littau, Switzerland) chemiluminescent detection systems.

Immunoprecipitation

HeLa cells were washed twice in PBS and solubilized in 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl (lysis buffer) and protease inhibitors: pepstatin A (Axon Lab AG, Baden-Dättwil, Switzerland); leupeptin (Roche, Rotkreuz, Switzerland), phenylmethylsulfonyl fluoride (PMSF; Promega AG, Wallisellen, Switzerland); aprotinin, papain inhibitor and benzamidine (Sigma-Aldrich); for 1 hour at 4°C, followed by centrifugation at 17,600 ***g*** for 30 minutes at 4°C. The input samples (1/30 of the lysates) were directly collected after the centrifugation step and boiled for 3 minutes at 95°C after addition of three times concentrated, reducing Laemmli buffer. The supernatant was added to antibodies adsorbed to protein-A–Sepharose beads (GE Healthcare Europe GmbH) for anti-CD59 or to protein-G–Sepharose beads (Sigma-Aldrich) for anti-GFP, anti-Myc and anti-HA. Beads were washed three times in lysis buffer, three times in 50 mM Tris-HCl, pH 7.4 and 150 mM NaCl, and three times in 50 mM Tris-HCl, pH 7.4. Proteins were eluted by the addition of reducing Laemmli buffer followed by boiling at 95°C for 3 minutes. Proteins were separated by SDS-PAGE.

Sucrose density gradient centrifugation

Density gradient analysis of Triton X-100-extracted cells was performed as follows. Cells were grown to confluence in three 100-mm dishes, rinsed with PBS, and lysed for 30 minutes in MBS-Triton X-100 1% buffer [25 mM Mes (pH 6.5), 150 mM NaCl, 1% Triton X-100 and proteases inhibitors] on ice. The lysate was centrifuged at 17,600 ***g*** or 30 minutes at 4°C and the supernatant was brought to 40% sucrose, placed at the bottom of a Beckman centrifuge tube, overlaid with a discontinuous sucrose gradient (5–35% in MBS), and centrifuged at 26,000 rpm (56,800 ***g***) for 18-20 hours in an ultracentrifuge (model SW41; Beckman Instruments). Fractions of 1 ml were harvested from top to bottom and used for western blotting or immunoprecipitation.

MβCD treatment

Unless stated otherwise, HeLa cells were incubated in the absence or presence of 20 mM methyl-β-cyclodextrin (Sigma-Aldrich) in DMEM, 25 mM HEPES for 30 minutes at 37°C and subjected to Triton X-100 extraction followed by sucrose density gradient analysis as described above.

Immunofluorescence microscopy

HeLa cells were seeded in 8-well glass chamber slides (Lab-Tek, Nunc; New York, USA). Three days after transfection with siRNAs, the cells were fixed with 3.5% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 4 minutes, and incubated with antibodies against ERGIC-53 (1:1000) and p24 (1:800) for 60 minutes. Primary antibodies were diluted in PBS containing 3% bovine serum albumin (BSA). In the cholesterol depletion experiment, anti-CD59 was diluted 1/200. For Sec24C staining, the cells were fixed with methanol at –20°C for 4 minutes, followed by 30 minutes blocking with PBS containing 3% BSA, followed by a 60-minute incubation with anti-Sec24C (1/100). After three washes with PBS the cells were incubated for 30 minutes with anti-rabbit Alexa Fluor 568 and anti-mouse Alexa Fluor 488 secondary antibodies (Molecular Probes, Leiden, The Netherlands), embedded in Mowiol and examined with a Polyvar fluorescence microscope using CamWare software.

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Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/123/10/1705/DC1

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