The α-Helical Region in p24γ2 Subunit of p24 Protein Cargo Receptor Is Pivotal for the Recognition and Transport of Glycosylphosphatidylinositol-anchored Proteins*

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Background: Glycosylphosphatidylinositol-anchored proteins (GPI-APs) depend on p24 cargo receptors for intracellular trafficking.

Results: The luminal α-helical but not GOLD domain of p24γ2 was required for efficient GPI-AP transport.

Conclusion: A p24 complex containing p24γ2 recognizes GPI-AP cargo using the α-helical region of p24γ2.

Significance: The α-helical regions of p24γ2 proteins are involved in complex formation and cargo recognition.

Glycosylphosphatidylinositol-anchored proteins (GPI-APs) are group of proteins that depend on p24 cargo receptors for their transport from the endoplasmic reticulum to the Golgi apparatus. The GPI anchor is expected to act as a sorting and transport signal, but so far little is known about the recognition mechanism. In the present study we investigate the GPI-AP transport in cell knockdown of p24γ2, the most diverse p24 subfamily. Knockdown of p24γ2 but not of other p24γ family members impaired the transport of a reporter GPI-AP. Restoration of the knockdown-induced phenotype using chimeric constructs between p24γ2 and the related p24γ1 further implied a role of the α-helical region of p24γ2 but not its GOLD domain in the specific binding of GPI-APs. We conclude that motifs in the membrane-adjacent α-helical region of p24γ2 are involved in recognition of GPI-APs and are consequently responsible for the incorporation of these proteins into coat protein complex II-coated transport vesicles.

Specific protein trafficking is essential to maintain the unique functions of different cellular compartments in eukaryotic cells. Coat protein complex I (COPI)1,2 and COPII-coated vesicles are implicated in the bidirectional transport between the endoplasmic reticulum (ER) and the Golgi apparatus. COPII assembles at the ER exit sites, and generated vesicles are transported from the ER toward the ER-Golgi intermediate compartment (ERGIC) (1, 2). The role of COPII vesicles includes the retrieval of proteins from the Golgi to the ER (3). Sec24 proteins, components of the multisubunit COPII complex, harbor several motifs for cargo binding. In contrast to transmembrane proteins, which are capable of interacting directly with the Sec24 subunits, luminal proteins depend on cargo receptors for their specific sorting into COPIII vesicles.

A distinct set of cargo receptors, recognizing either peptide and/or carbohydrate signals, have been described (4). Among them is a family of type I transmembrane proteins of approximately 24 kDa, termed p24 proteins, which can be divided into four subfamilies (p24α, β, γ, and δ) (5). Because of the expansion of two of the subfamilies (p24γ and p24α) containing three and five members, respectively, 10 p24 proteins are present in most vertebrates. However, tissue expression studies in mice revealed restricted expression patterns for p24α and p24γ, and due to an in-frame stop codon, p24α is a pseudogene in humans (6). Furthermore, an alignment of p24α and p24γ excluding the signal sequences reveals 96% similarity of the two proteins. The largest variety is hence provided by the p24γ subfamily containing four different and widely expressed proteins, whereas the other subfamilies exhibit no or very limited diversity.

All p24 proteins share a similar domain organization, consisting of a GOLD domain and an α-helical region, followed by a transmembrane region and a short C-terminal tail. Whereas Jenne et al. (7), propose the occurrence primarily of monomers and dimers, several studies describe the building of heterotetrameric or larger complexes involving one member from each of four subfamilies (8–11).

Glycosylphosphatidylinositol (GPI) anchoring is a post-translational modification, exerted to a wide variety of proteins in eukaryotic cells. Early steps of the GPI anchor assembly occur on the cytosolic side of the ER, and following flipping of
Role of p24γ2 in GPI-anchored Protein Transport

an intermediate product, the synthesis is completed in the ER lumen. After this multistage assembly process, the GPI anchor is transferred en bloc to targeted proteins and after further remodeling reactions, GPI-anchored proteins (GPI-APs) are transported via the Golgi to the cell surface. Several studies revealed a role of p24 proteins in the sorting of GPI-APs into COPII vesicles in yeast (12–15). The impaired transport of GPI-anchored, but not other cargo molecules upon knockdown of p24δ2 or p24δ4 in mammalian cells, supported these findings in yeast (16, 17). Fujita et al. reported that p24γ2, p24β1, p24γ2, and p24δ2 are associated with GPI-APs in the ER, supporting a model of heterotetrameric or larger complex of p24 cargo receptors (10). They further showed that two GPI anchor remodeling reactions in the ER, occurring after the transfer to proteins, are crucial for the interaction with these p24 proteins and efficient sorting into the ER exit sites. Hence, the GPI anchor is expected to act as a sorting and transport signal in the ER although little is known so far about the recognition mechanism.

Due to the largest variability, it is likely that the respective p24γ subunit determines the cargo specificity in the receptor complexes. Here, we demonstrate that knockdown of p24γ2, but not knockdown of other p24γ subfamily members, results in delayed GPI-AP transport. Using chimeric and mutant constructs, we define the region required for GPI anchor recognition and further confirm the results by a binding assay.

EXPERIMENTAL PROCEDURES

Cells—FCAT5 is a cell line obtained as a result of three separate stable transfections of CHO-K1 cells. In a first step 3B2A cells were established by stably transfecting CHO-K1 cells with pME-NEO plasmid expressing DAF and CD59, human GPI-APs, under the control of an SRα promoter, and selecting by cell sorting a clone expressing DAF and CD59 at high levels (18). 3B2A cells were stably transfected with pTRE2-puro-VSVG-sensitive—FF-mEGFP-GPI in conjunction with pUHrT62-1, an expresion plasmid for reverse tetracycline-controlled transactivators (16, 19) to obtain FF8 cells. Finally, for use in a retrovirus system, FF8 cells were stably transfected with a plasmid, expressing mouse CAT1, a receptor for ectopic retroviruses to generate FCAT5 cells. FCAT5 cells stably expressing p24γ2 shRNA or p24γ2 shRNA in combination with various restoration constructs were established by infection with a retrovirus produced in PLAT-E packaging cells (a gift from T. Kitamura, University of Tokyo, Tokyo, Japan), followed by selection with 7 μg/ml blasticidin (BSD). FCAT5 cells and their derivatives were maintained in Ham’s F-12 medium (Sigma-Aldrich) supplemented with 10% FCS, 600 μg/ml G418, 800 μg/ml hygromycin, 6 μg/ml puromycin, and if necessary 7 μg/ml BSD.

Reagents and Antibodies—Lipofectamine 2000 and Lipofectamine RNAiMAX were purchased from Invitrogen. Rabbit anti-p24γ2 antibody was provided by H. Hauri and H. Farhan (University of Basel, Basel, Switzerland). Rabbit anti-p24α2, rabbit anti-p24β1, rabbit anti-p24γ1, rabbit anti-p24γ2, and guinea pig anti-p24γ2 antibodies were generous gifts from F. Wieland and A. Herrmann (Heidelberg University, Heidelberg, Germany). Anti-p24γ2 antibody was obtained by immunizing rabbit with the peptide LRRFFKAKKILF followed by affinity purification on a peptide column. These are antibodies against the C-terminal tails of human p24 proteins, but they recognized mouse p24 proteins as well. Mouse anti-FLAG (clone M2), rabbit anti-ERGIC-53 and mouse anti-α tubulin (clone DM1A) were purchased from Sigma-Aldrich and mouse anti-GFP from Roche Applied Bioscience. Horseradish peroxidase (HRP)-conjugated sheep anti-mouse IgG and donkey anti-rabbit IgG were from GE Healthcare. HRP-conjugated goat anti-guinea pig IgG H&L was purchased from Abcam and phycoerythrin (PE)-conjugated goat anti-mouse IgG from BD.

Generation of Stable p24γ2 Knockdown and Chimeric Constructs—To generate stable p24γ2 knockdown cell lines, modified (capital) sense and antisense (bold) sequences of RNAi p24γ2-1 separated by a loop (underlined) (gggagTtaggtgGttaggtgCtactaatccagcaagcattgt) were cloned into a modified version of pSiNsi-hU6 (TaKaRa) containing a BSD instead of a neomycin resistance gene. From the obtained construct (pSiNsi-hU6-307-BSD), a fragment containing hU6 promoter, knockdown (307), and BSD resistance gene sequences was subsequently integrated into pLIB2 (pLIB vector (Clontech) harboring modifications in the multiple cloning site), to obtain pLIB2-307-BSD.

For generating full-length restoration constructs mouse p24γ2 cDNA was cloned into the EcoRI site of pLIB2. The introduction of an MtuI site and mutation of the EcoRI site in the forward primer allowed us to retain the EcoRI as a single cutting site downstream of the insert. hU6 promoter, knockdown (307), and BSD resistance gene sequences were subsequently integrated between the EcoRI and XhoI sites. pLIB2-p24γ1-307-BSD was cloned in a similar way to pLIB2-p24γ2-307-BSD after amplifying full-length p24γ1 from a mouse testis cDNA library. To ensure the recognition of p24γ1 by rabbit anti-p24γ2 antibody, the C-terminal part of the protein was subsequently changed from RFFHDKRPVPT (p24γ2) to SLFEKRKRSRT (p24γ2) to have an epitope recognized by the anti-p24γ2 antibody. Chimeric constructs were prepared by using pLIB2-p24γ2-307-BSD and pLIB2-p24γ1-307-BSD as PCR templates. See Table 1 for a list of primers used.

RNAi Oligonucleotides—RNAi oligonucleotides used to knock down p24δ1 and the p24γ family members in FCAT5 cells were designed using the BLOCK-it™ RNAi Designer and purchased from Invitrogen. Sequences were as follows: scrambled control siRNA (Invitrogen; 46-2000), p24δ1, 5′-ggccatattcttgtagcagaaag-3′; p24γ1_1, 5′-caagccgcttggcggagc-aga-3′; p24γ1_2, 5′-cactcaagctctttctcatgacaa-3′; p24γ1_2, 5′-ggagactacatgtgctgttgatgacyt-3′; p24γ2_2, 5′-cataacaaactgtgctttgatgacyt-3′; p24γ3_2, 5′-cagagccgctttgctttgatgacyt-3′; p24γ4_2, 5′-cactcaagctctttctcatgacaa-3′; p24γ4_1, 5′-gccgactaagtctgctttgatgacyt-3′; p24γ5_2, 5′-cataacaaactgtgctttgatgacyt-3′; p24γ5_3, 5′-cagagccgctttgctttgatgacyt-3′; p24γ6_2, 5′-cagagccgctttgctttgatgacyt-3′; p24γ6_3, 5′-cataacaaactgtgctttgatgacyt-3′.

Cells were transfected two times (48-h interval) according to the manufacturer’s instructions using Lipofectamine RNAiMAX. Knockdown efficiency was confirmed by quantitative PCR and immunoblotting.

Quantitative Real-time PCR—RNA was isolated 72 h after the first siRNA transfection using the RNAeasy Mini kit (Qiagen) according to the manufacturer’s instructions. A 2.5-μg sample of each RNA was transcribed to cDNA using the Super-
### TABLE 1

Primers used

| Gene fragment | Template | Primer sequence 5’-3’ |
|---------------|----------|-----------------------|
| m-p24γ2       | p24γ2 cDNA | F 5′-GGAATTCAGGCGGCCGCAAG-3′; R 5′-GCTCTCGAGGCCGCACGACG-3′ |
| m-p24γ1†     | p24γ1 cDNA | F 5′-GGAATTCAGGCGGCCGCAAG-3′; R 5′-GCTCTCGAGGCCGCACGACG-3′ |
| Epitope       | m-p24γ1, Chimera 1 and 2 | F 5′-GGAATTCAGGCGGCCGCAAG-3′; R 5′-GCTCTCGAGGCCGCACGACG-3′ |

†: Indicated reverse primers were used to clone m-p24γ1, chimera 1, and chimera 2 containing the C-terminus of p24γ1. To allow detection of all chimeric constructs, the C-terminal part was subsequently changed to p24γ1 using the primer pair listed under “Epitope.”

Script Vilo CDNA Synthesis Kit (Invitrogen). Primers used for the quantitative PCR were as follows: p24γ1, 5′-caagtgtgctgctcaatgaggtg-3′ (forward) and 5′-gactgtgctgctcaatgaggtg-3′ (reverse); p24γ2, 5′-aggtgctgctcaatgaggtg-3′ (forward) and 5′-gactgtgctgctcaatgaggtg-3′ (reverse); p24γ3, 5′-aggtgctgctcaatgaggtg-3′ (forward) and 5′-gactgtgctgctcaatgaggtg-3′ (reverse); p24γ4, 5′-cttccaggtgctgctcaatgaggtg-3′ (forward) and 5′-cttccaggtgctgctcaatgaggtg-3′ (reverse).

The quantitative PCR using SYBR Premix Ex TaqII (Tli RNaseH Plus), Bulk (TaKaRa) was performed by Thermal Cycle Dice Real Time System according to the manufacturer’s instructions. The RNA expression level was normalized to HPRT and the relative expression calculated using the formula $\Delta\Delta C_T$. 

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**Role of p24γ2 in GPI-anchored Protein Transport**

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Transport Assay of the Reporter GPI-AP—FCAT5 cells harboring Tet-inducible VSVGts-FLAG-GFP-GPI (VFG-GPI) were incubated at 40 °C in the presence of 1 μg/ml doxycycline. Due to misfolding of the VSVGts domain at 40 °C, the reporter is retained in the ER. After 24 h of incubation, the cultures were harvested with trypsin-EDTA solution (Sigma-Aldrich), and transferred to 32 °C to allow proper folding and transport. Samples collected after different incubation times were stained with anti-FLAG antibody, followed by PE-conjugated goat anti-mouse secondary antibody. The surface amount of VFG-GPI reporter protein was analyzed using FACSCanto II (BD Biosciences) (19).

GPI-AP Immunoprecipitation and Immunoblotting—FCAT5 cells stably transfected with p24_2 or p24_2 shRNA or empty vector-infected cells (V) were probed with specific antibodies against p24_2, p24_1, p24_1, p24_3, p24_4, and p24_1. Blots are representative of at least two experiments.

Role of p24_2 in GPI-anchored Protein Transport

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GPI-AP Immunoprecipitation and Immunoblotting—FCAT5 cells stably transfected with p24_2 siRNA or p24_2 siRNA were harvested and transferred to 32 °C to allow transport. Samples were collected after 0 and 45 min and stained with anti-FLAG antibody followed by PE-conjugated goat anti-mouse secondary antibody. The surface amount of VFG-GPI reporter protein was analyzed by flow cytometry. The geometric mean fluorescence values of surface VFG-GPI in cells transfected with scrambled siRNA or p24_2 siRNA are shown in histograms (C). The quantified data of all cells are shown in D, the graph represents the mean ± S.E. of three independent experiments.

FIGURE 1. Knockdown of p24_2 impairs GPI-AP transport. Cells were transfected with a scrambled siRNA, p24_1 siRNA, or two different siRNA against p24_1, p24_1, p24_1, or p24_1. The transfection using the same oligonucleotides was repeated after 48 h. A, RNA was isolated 72 h after the first transfection and the relative amount of mRNA determined by quantitative PCR. The graph represents the mean ± S.E. (error bars) of three independent experiments. B, 72 h after the first transfection cells were lysed and the protein level analyzed by immunoblotting. C = control (scrambled siRNA), y_1 = p24y_2 siRNA, y_2 = p24y_2 siRNA, y_3 = p24y_3 siRNA, y_4 = p24y_1 siRNA, y_5 = p24y_1 siRNA, y_6 = p24y_2 siRNA. C and D, 72 h after the first transfection the expression of the GPI reporter (VFG-GPI) was induced by the addition of 1 μg/ml doxycycline and the cultures incubated at 40 °C. Another 24 h later the cells were harvested and transferred to 32 °C to allow transport. Samples were collected after 0 and 45 min and stained with anti-FLAG antibody followed by PE-conjugated goat anti-mouse secondary antibody. The surface amount of VFG-GPI reporter protein was analyzed by flow cytometry. The geometric mean fluorescence values of surface VFG-GPI in cells transfected with scrambled siRNA or p24y_2 siRNA are shown in histograms (C). The quantified data of all cells are shown in D, the graph represents the mean ± S.E. of three independent experiments.

FIGURE 2. p24 proteins occur in hetero-oligomeric complexes. Lysate of FCAT5 cells stably expressing p24_6, or p24_2, shRNA or empty vector-infected cells (V) were probed with specific antibodies against p24_2, p24_1, p24_1, p24_3, p24_4, p24_1, p24_1, p24_3, p24_4, and p24_1. Blots are representative of at least two experiments.
After centrifugation (20,000 g, 15 min, 4 °C) the soluble lysate was incubated with anti-FLAG M2-agarose beads (Sigma-Aldrich) for 2 h at 4 °C, followed by four washing steps (20 mM MES/HEPES (pH 7.4), 100 mM NaCl, 0.5% digitonin). Finally, the beads were boiled in SDS-PAGE sample buffer and analyzed by immunoblotting.

RESULTS

Transient Knockdown of p24γ but Not of Other p24γs Impairs GPI-AP Transport—To investigate the role of p24γ proteins in the transport of GPI-AP, we used FCAT5 cells, which express a Tet-inducible, temperature-sensitive GPI reporter protein, VFG-GPI. After expression and accumulation of VFG-GPI in the ER at 40 °C, cultures were transferred to 32 °C to allow protein folding and transport. The kinetics of GPI-AP transport to the cell surface can be determined by measuring the amount of surface-associated VFG-GPI at different time points by flow cytometry using anti-FLAG antibody.

We transiently knocked down the p24γ subfamily members in FCAT5 cells using two different siRNAs. After transfecting the cells two times at a 48-h interval, the mRNA levels of

![FIGURE 3. Alignment of p24γ with other p24γ proteins. Alignment of mouse p24γ proteins was performed using the BLAST tool of the National Center for Biotechnology Information (NCBI). Similar amino acids are indicated by . Matching amino acids are indicated by the amino acid letter code. Sequence information: p24γ1 (NP_034874), p24γ2 (NP_083152), p24γ3 (NP_079974), p24γ4 (NP_079636), and p24γ5 (NP_079734).](image-url)

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Role of p24γ₂ in GPI-anchored Protein Transport

p24γ₁–₄ were decreased to 3–16% of those in control cells (Fig. 1A). Substantial decreases in protein levels were also detected (Fig. 1B). Impaired GPI-AP transport could be observed only upon knockdown of p24γ₂ (Fig. 1, C and D).

The basic mRNA expression level of p24γ₂ was substantially lower compared with the other p24γ subfamily members. This might explain why in p24γ knockdown cells a relative decrease to only 36 and 45%, respectively, could be observed (Fig. 1A). Under the conditions of the reduction of the mRNA to <50%, we could not observe any transport delay in our assay (Fig. 1D). Although a role of p24γ₆ in GPI-AP transport cannot certainly be excluded under these circumstances, it is rather unlikely. The restricted tissue expression of p24γ₆ (6) further indicates a very specific function of this protein and hence questions a role in the ubiquitous GPI-AP transport. These data provide evidence that p24γ₂ may be a key subunit in the cargo receptor for the specific recognition of GPI in transport of GPI-APs. This finding is also supported by our previous result, where in a mass spectrometry analysis p24γ₂ was the only member of the p24γ family found to be associated with VFG-GPI (10).

Various Hetero-oligomeric p24 Complexes, Differing in Their p24γ Subunit, Can Be Built—To study further the interaction among p24 proteins we established a stable p24γ₂ knockdown FCAT5 cell line and compared it with the stable p24δ knockdown cell line used previously (10). p24α₁, p24β₁, and p24γ₂ proteins were destabilized upon p24δ knockdown (Fig. 2, left), confirming the occurrence of a hetero-oligomeric p24 receptor complex consisting of p24α₁, p24β₁, and p24γ₂ and mutual stabilization among these subunits (10). The protein levels of p24γ₁, p24γ₃, and p24γ₄ were also reduced significantly upon p24δ knockdown (Fig. 2, left), suggesting that other members of the p24γ subfamily can form a complex with p24α₁, p24β₁, and p24δ, in a way similar to p24γ₂. However, upon stable knockdown of p24γ₂, levels of p24α₁, p24β₁, and p24δ were unchanged, and the levels of p24γ₁, p24γ₃, and p24γ₄ proteins were slightly increased (Fig. 2, right). We therefore assume that p24γ proteins compete for the common p24α₁, p24β₁, and p24δ subunits leading to the occurrence of various hetero-oligomeric receptor complexes.

Localization of Regions in p24γ₂ for Recognition of GPI Anchor—Among the p24γ proteins, p24γ₁ is most closely related to p24γ₄, revealing 53.6% sequence identity (Fig. 3). Despite this similarity, only the expression of p24γ₂, but not p24γ₄, was able to restore the transport phenotype in stable p24γ₂ knockdown cells (Fig. 4). This finding further points toward specific roles of p24γ₁ and γ₂ proteins with no or only limited redundancy.

Because the p24γ₁ did not influence GPI-AP transport kinetics (Figs. 1C and 4), we next tested p24γ₁/p24γ₂ chimeric constructs for their ability to restore the transport phenotype in stable p24γ₂ knockdown cell line to determine which region in p24γ₂ is functionally important (Fig. 5A). To assure the detection by the p24γ₂ antibody that recognizes the cytoplasmic domain (C-term), each construct was designed to contain the C-term of p24γ₂. All chimeric constructs could be detected by immunoblotting (Fig. 5B).

Using chimeras 1–4, we first studied the role of the GOLD domain, the α-helical region, and the transmembrane domain in GPI-AP transport. Chimera 1 consisted of a p24γ₂ GOLD domain and α-helical region, but a p24γ₁ transmembrane region. Chimera 2 contained a p24γ₂ GOLD domain but the p24γ₁ α-helical region and transmembrane domain. Chimeras 3 and 4 were the opposite of chimeras 2 and chimera 1, respectively (Fig. 5A). Chimeras 1 and 3 were able to restore the transport delay. At time point 40 min, their level of VFG-GPI on the surface reached 79.1% and 97.0% of vector-transfected FCAT5 cells, respectively (Fig. 5C). However, expression of chimeras 2 and 4 was not sufficient to restore the transport delay (56.5% and 50.1%, respectively). This implies that the α-helical region rather than the GOLD domain is involved in GPI-AP recognition and transport.

The α-helical regions of p24γ₁ and p24γ₂ have only 27.6% sequence identity in amino acids 127–153, whereas the remaining α-helical region (amino acids 154–196) shows a sequence identity of 65.1% (Fig. 5D). Four chimeric constructs (chimeras 5–8) were designed to test the role of these two parts of the α-helical region in GPI-AP transport (Fig. 5A). Chimeras 5 and 8 were able to restore the transport phenotype (92.2% and 83.1% respectively), whereas chimeras 6 and 7 were not (42.3% and 41.6%, respectively) (Fig. 5C). The result indicates that the critical region for GPI-AP transport is not localized in amino acids 127–153 but in amino acids 154–196. The α-helical region spanning amino acids 154–196 was further divided into two parts (154–174 and 175–196), and their influence was tested with the expression of chimeras 9–12 (Fig. 5A). All cell lines showed an intermediate phenotype (67.4%, 66.5%, 70.2%, and 61.1%, respectively), neither reaching the level of p24γ₂ knockdown cells restored by wild-type p24γ₂ nor showing a reduction comparable with nonrestored p24γ₂ knockdown cells. This suggests that motifs from both regions are required to restore the transport delay fully.
Role of p24γ2 in GPI-anchored Protein Transport

When the p24γ2 part spanning amino acids 175–196 was extended to 2 or 4 more amino acids to amino acids 173–196 (chimera 13) or 171–196 (chimera 14), transport was fully recovered (158.5% and 118.3%, respectively) (Fig. 5C). We therefore conclude that the IQ motif at positions 173/174 together with a motif present within amino acids 175–196 are involved in GPI-AP binding. Although this combination was shown to be very effective, chimera 15, full-length p24γ2, was still able to restore the knocked-down induced phenotype (135.5%) (Fig. 5C). Hence, although the IQ motif at 173/174 together with amino acid 175–196 of p24γ2 is sufficient to allow normal GPI-AP transport, the motif seems to be negligible in other sequence combinations.

Transport Phenotype of Chimeric Constructs Correlates with GPI-AP Binding—To correlate the result of the transport assay with binding ability of various cargo receptor complex, we next studied the interaction of chimeric constructs 7, 8, 12, and 13 with the reporter GPI-AP by immunoprecipitation. Chimeric constructs 8 and 13, which were very efficient in restoring the knockdown-induced phenotype, were tested in combination with their counterpart constructs (chimeras 7 and 12), which showed delayed transport.

FIGURE 5. The α-helical region of p24γ2 is relevant for GPI-AP transport. A, schematic overview shows used p24γ2/p24γ1, chimeric constructs. 8, stable p24γ2 knockdown FCAT5 cells expressing chimeric constructs were lysed and the protein level analyzed by immunoblotting. Note that a 25-kDa band seen in vector lane (left end) is endogenous hamster p24γ2, which was significantly reduced with p24γ2 shRNA and was more greatly reduced with p24γ2 shRNA. C, stable p24γ2 knockdown FCAT5 cells expressing chimeric constructs were prepared as described under “Experimental Procedures.” The expression of the GPI reporter (VFG-GPI) was induced by the addition of 1 μg/ml doxycycline, and the cultures were incubated at 40 °C. After 24 h the cells were harvested and transferred to 32 °C to allow transport. Samples were collected after 0 and 40 min and stained with anti-FLAG antibody, followed by PE-conjugated goat anti-mouse secondary antibody. The surface amount of VFG-GPI reporter protein was analyzed by flow cytometry. The graph represents the quantified data. Error bars, S.E. D, α-helical region of p24γ2 and p24γ2 were aligned using the BLAST tool of NCBI. Similar amino acids are indicated by +. Matching amino acids are indicated by the amino acid letter code. Amino acid positions mentioned in the text are indicated. They correspond to the position in mouse p24γ2 (NP_083152). The IQ motif of p24γ2 (chimera 13) as well as the IQML motif of p24γ1 (chimera 15) are framed.

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Role of p24γ₂ in GPI-anchored Protein Transport

After accumulating VFP-GPI in the ER at 40 °C, the temperature was shifted to 32 °C for 20 min to allow protein folding, receptor binding, and transport initiation, and then lysed in buffer containing 1% digitonin. VFG-GPI was collected with anti-FLAG beads and the amount of bound chimeric constructs analyzed by immunoblotting. Blots are representative of at least three experiments.

DISCUSSION

Ten different p24 proteins, involved in the specific transport between the ER and the Golgi, are expressed in vertebrates. They can be divided into four subfamilies, p24α, β, γ, and δ. Whether p24 proteins exist mainly as monomers, dimeric, tetrameric, or larger complexes is still debated. Jenne et al. showed that the state of oligomerization and concentration of p24 proteins vary, depending on the cellular localization (7). Several studies confirmed the occurrence of heterotetrameric complexes involving one member of each subfamily (8–11). In contrast to the limited diversity of the p24α, β, and δ subfamilies, five different p24γ proteins are expressed in vertebrates. It is therefore likely that in heterotetrameric receptor complexes, the respective γ subunit determines the cargo specificity. In this study we also showed that levels of p24 are interdependent. The inverse correlation among the levels of p24γ proteins supports the competition for the common p24α, β, and δ interaction partners. Consistent with that, we additionally detected elevated kinetics of GPI-AP transport upon knockdown of p24γ₁, γ₃, γ₄, and γ₅, which further strengthens the competition among these proteins (Fig. 1D). Redundancy among p24α and p24γ proteins had been described recently in yeast (11). Because the GPI-AP transport was impaired in p24γ₂ knockdown cells and could not be compensated by the slight overexpression of other p24γ proteins (Fig. 1D), p24γ proteins seem, however, to have a specific function in the GPI-AP transport in mammalian cells.

Until now, impaired transport of GPI-APs had only been reported upon knockdown of p24β₁ and p24δ₁ (12, 16, 17). Because these knockdowns lead to the instability and reduction of all p24 proteins, no conclusions about the specificity could be drawn. In the present study we could, however, show that knockdown of p24γ₂ specifically affects the GPI-AP transport without impairment of basic p24 complex formation.

A major conclusion of this study is that the α-helical region of p24γ₂ is involved in the binding of GPI. The α-helical region had previously been implicated in oligomerization and stabilization of p24 receptor complexes and the more prominent GOLD domain was suggested as a cargo binding site. The GOLD domains are not only implicated in protein-protein interactions (20), but are also often observed in sugar- and lipid-binding proteins (21). It therefore seemed feasible that the GOLD domain is capable of interacting with a variety of cargos including sugar-lipid motifs like GPI. We however found that the GOLD domains of p24γ₁ and p24γ₃ are interchangeable, as chimera 3, although containing the GOLD domain of p24γ₁, was still able to restore the p24γ₂ knockdown-induced GPI-AP transport delay (Fig. 5A). The α-helical regions of p24 proteins in complexes interact and form coiled-coil structures. It is therefore possible that in this region protein-overlapping binding motifs arise. Changes in amino acids directly involved in cargo binding as well as changes in residues important for proper p24 protein interaction could thereby affect efficient cargo binding and transport. As all of our chimeric constructs were stably expressed, we assume that the basic complex assembly was efficient. Small conformational changes, however, might have led to masking or unfavorable positioning of the binding motif.

We were able to narrow down the binding site to the IQ motif at positions 173/174 together with a motif present within amino acids 175–196. We did not, however, conclude that the IQ motif is involved directly in interaction with GPI-AP. Chimera 15, full-length p24γ₂ with the GHIQ motif at positions 171–174 changed to the respective amino acid in p24γ₁ (IQML), was still able to restore the knockdown-induced phenotype. We therefore propose a model that amino acids 175–196 of p24γ₂ are indispensable for proper GPI-AP transport and presumably involved directly in the binding process. The IQ motif at 173/174, however, does not seem to be involved directly in the interaction but rather has a role in increasing the affinity of binding or favoring proper folding or binding motif accessibility. Also, amino acids 154–170 seem to be able to undertake such a supportive function, and it appears that either amino acids 154–170 or IQ motif at 173/174 in combination with amino acids 175–196 is sufficient to allow efficient GPI-AP transport.

Two remodeling reactions in GPI occur in the ER after its attachment to proteins. First, the acyl chain linked to inositol is removed by PGAP1 followed by the removal of a side chain ethanolamine phosphate attached to the second mannose by PGAP5 (22, 23). We showed previously that these reactions are crucial for association with p24 proteins and the recruitment of GPI-APs into ER exit sites (23). As in our chimeric constructs the transmembrane regions of p24γ₁ and p24γ₂ were interchangeable (Fig. 5), we conclude that the lipid part of GPI is not...
involved directly in p24 binding. The removal of the acyl chain from inositol ring is nevertheless a crucial step for the interaction with p24 proteins. We expect that cleavage by PGAP1 changes the orientation of inositol to the membrane and structure of the glycan plus inositol and could consequently contribute to the formation of the correct binding site for the p24 complex. The same might be the case for the removal of the ethanolamine phosphate. It is also likely that a combination of both remodeling reactions is required to generate the final binding site. Our findings provide evidence that p24γ proteins show only a limited redundancy and that p24γ2 is specifically important for GPI-AP transport in mammalian cells.

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