Mammalian GPI-anchored proteins require p24 proteins for their efficient transport from the ER to the plasma membrane

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The GPI (glycosylphosphatidylinositol) moiety is attached to newly synthesized proteins in the lumen of the ER (endoplasmic reticulum). The modified proteins are then directed to the PM (plasma membrane). Less well understood is how nascent mammalian GPI-anchored proteins are targeted from the ER to the PM. In the present study, we investigated mechanisms underlying membrane trafficking of the GPI-anchored proteins, focusing on the early secretory pathway. We first established a cell line that stably expresses inducible temperature-sensitive GPI-fused proteins as a reporter and examined roles of transport-vesicle constituents called p24 proteins in the traffic of the GPI-anchored proteins. We selectively suppressed one of the p24 proteins, namely p23, employing RNAi (RNA interference) techniques. The suppression resulted in pronounced delays of PM expression of the GPI-fused reporter proteins. Furthermore, maturation of DAF (decay-accelerating factor), one of the GPI-anchored proteins in mammals, was slowed by the suppression of p23, indicating delayed trafficking of DAF from the ER to the Golgi. Trafficking of non-GPI-linked cargo proteins was barely affected by p23 knockdown. This is the first to demonstrate direct evidence for the transport of mammalian GPI-anchored proteins being mediated by p24 proteins.

Key words: glycosylphosphatidylinositol-anchored protein (GPI-anchored protein), p24, post-translational modification, trafficking.

INTRODUCTION

Hundreds of eukaryotic proteins are modified with a GPI (glycosylphosphatidylinositol) anchor. Among all species known to have these modified proteins, GPI anchors share a common core glycolipid structure that is composed of ethanolamine phosphates, three mannose residues, glucosamine and inositol-phospholipid [1]. Molecules that are implicated in GPI biosynthesis and anchor attachment have been identified and characterized [2]. The GPI moiety is sequentially assembled and transferred *en bloc* to a nascent protein in the lumen of the ER (endoplasmic reticulum). One critical role for the GPI attachment is to target the modified proteins to the cell surface where they primarily function. It is known that proteins with GPI modification are transported from the ER to their final destination along the classical secretory pathway. Yet relatively little is known about how the modified proteins are targeted to the proper subcellular location. Upon ER exit, cargoes must be packaged into COPII (coatamer protein II)-coated vesicles. In yeast, one of the GPI-anchored proteins, Gas1p, has been shown to exit out of the ER via vesicles distinct from those carrying other non-GPI linked proteins [3–5]. Furthermore, in ER-derived vesicles prepared *in vitro,* Gas1p was shown to be cross-linked with Emp24p and Erv25p, members of transport-vesicle constituents called p24 family proteins [3]. Much less well understood is how mammalian GPI-anchored proteins transit along the secretory pathway. In the present study, we investigated the molecular mechanisms underlying the trafficking of a newly synthesized GPI-anchored protein, focusing on the early secretory pathway.

The p24 family of proteins are small type I integral membrane proteins found in eukaryotes from yeast to mammals [6]. The proteins are commonly divided into four subgroups: α, β, γ and δ. In mammals, there are at least seven members identified to date: gp25l (p24α1), GMP25 (p24α2), p24 (p24β1), p26 (p24γ1), Tp24 (p24γ2), gp27 (p24γ3) and p23 (p24δ1). Increasing evidence suggests that p24 proteins form a functional heteromeric complex and mutually stabilize each other [7–10]. It has been generally agreed that the proteins play crucial roles in the fidelity of vesicular transport between the ER and the Golgi. However, currently available data from mutational analysis have provided a somewhat murky picture regarding how p24 proteins work. A yeast mutant with deletion of all eight genes encoding p24 family members was viable and displayed no marked defects in vesicle trafficking [11], whereas strains with a single deletion exhibited delayed transport of selected proteins [12]. No direct evidence has been reported for transport of mammalian cargoes being mediated by p24 proteins. However, homozygous disruption of p23 in mice resulted in embryonic lethality [13], underscoring the physiological significance of p24 proteins in mammals.

Previous work has used ectopic overexpression systems to examine p24 proteins in mammalian cells. Yet no previous study has investigated how the proteins regulate cargo transport by interfering with their corresponding mRNA. Using a cell line that we developed to track movement of inducible thermoreversible GPI-anchored reporter proteins from the ER to the PM (plasma membrane), we suppressed p24 proteins employing RNAi (RNA interference) techniques and examined the effects on transit of mammalian GPI-anchored cargoes. To date, this is the first study

Abbreviations used: CHO, Chinese-hamster ovary; COPII, coatamer protein II; DAF, decay-accelerating factor; EGFP, enhanced green fluorescent protein; mEGFP, modified EGFP; ER, endoplasmic reticulum; GFP, green fluorescent protein; GPI, glycosylphosphatidylinositol; MDCK, Madin–Darby canine kidney; PI-PLC, phosphatidylinositol-specific phospholipase C; PM, plasma membrane; RNAi, RNA interference; siRNA, small interfering RNA; VSVG, vesicular stomatitis virus G protein; VSVGts, temperature-sensitive VSVG.

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to demonstrate directly the transport of mammalian proteins being mediated by p24 proteins.

EXPERIMENTAL

Cells

3B2A cells, CHO (Chinese-hamster ovary) cells stably expressing DAF (decay-accelerating factor) and CD59, were described previously [14]. FF8 and #36 cells were established by stably transfecting 3B2A cells with pTRE2puro-VSVGts-FF-mEGFP-GPI and pTRE2puro-FLAG-VSVGts-EGFP respectively, in conjunction with pUHrT62-1, an expression plasmid for reverse tetracycline-controlled transactivators (a gift from Dr Hermann Bujard, Center for Molecular Biology, University of Heidelberg, Heidelberg, Germany, and Dr Wolfgang Hillen, Department of Microbiology, University of Erlangen, Erlangen, Germany) [15]. pTRE2puro-VSVGts-FF-mEGFP-GPI encodes a reporter protein consisting of the extracellular domain of the VSVGts [temperature-sensitive VSVG (vesicular stomatitis virus G protein)], a furin cleavage site, a FLAG tag, mEGFP [modified EGFP (enhanced green fluorescent protein)] and a GPI-attachment signal. pTRE2puro-FLAG-VSVGts-EGFP contains a sequence for a non-GPI reporter protein consisting of FLAG, VSVGts and EGFP. Transfectants were selected with puromycin, and surviving cells were isolated by limited dilution. The cells were grown in Ham's F-12 medium supplemented with 10 % (v/v) fetal bovine serum, 600 μg/ml G418 and, if necessary, 6 μg/ml puromycin, and maintained at 37 °C in 95 % air, 5 % CO_2-humidified atmosphere.

siRNA (small interfering RNA) constructs

pSINsi-hU6, an expression plasmid for siRNA, was purchased from TaKaRa Bio. Sequences of siRNA targeting p23 were selected using BLOCK-iT RNAi Designer (Invitrogen) and cloned into pSINsi-hU6. pSINsi-hU6-242, -256, -345 and -465 contain the siRNA sequences of GCCATATTCTGTATGCCAA, GCCAAAGAGGATGCAACTA, GCGGATACCTGACCAACTA and GGACCTTTCAGAGTCTATT respectively. These sequences are unique to p23 and should not interfere with mRNA levels of other p24 family members.

Time-course experiments and flow-cytometric analysis

In the presence of doxycycline cells were incubated at 40 °C overnight and transferred to 32 °C. After incubation at 32 °C for the indicated times, the cells were stained with an anti-FLAG M2 monoclonal antibody (Sigma) and then a phycoerythrin-conjugated anti-mouse antibody (BD Biosciences). Stained cells were analysed using a FACSCaliber (BD Biosciences).

Metabolic labelling and pulse–chase analysis of DAF

3B2A cells were transfected with siRNA vectors by electroporation. Cells were electroporated by using a Gene Pulser (Bio-Rad). Pulse–chase experiments were performed as described previously [16]. Briefly, at 60 h after transfection, cells were incubated with [^{35}S]methionine and [^{35}S]cysteine. After 10 min of pulse labelling and chase with unlabelled methionine and cysteine for up to 60 min, DAF was precipitated using an anti-DAF monoclonal antibody (IA10) and Protein A/G PLUS–agarose (Santa Cruz Biotechnology). Labelled DAF was separated by SDS/10 % (w/v) PAGE under reducing conditions. Gels were dried and exposed to a BAS IP film for autoradiography. Resulting images were analysed using a BAS PhosphoImager (Fujifilm).

Materials

Doxycycline and puromycin were purchased from Sigma. G418 was from Nacalai Tesque. Anti-p23 and anti-p24 rabbit polyclonal antibodies were kindly provided by Dr Felix Wieland (Center for Biochemistry, University of Heidelberg, Heidelberg, Germany) and Dr Irene Schulz (Physiology Institute, University of Saarland, Saarland, Germany). PI-PLC (phosphatidylinositol-specific phospholipase C) was purchased from Molecular Probes.

RESULTS

Characterization of trafficking of GPI-anchored VSVG in FF8 cells

In order to monitor export of GPI-anchored proteins from the ER to the PM as a synchronous population we established a cell line, FF8, stably expressing a tetracycline-inducible thermoreversible GPI-anchored VSVGts–FLAG–mEGFP construct (Figure 1A). The detailed structure of this fusion protein was described previously [17]. Briefly, this reporter protein is composed of the extracellular domain of VSVG protein, a furin cleavage site, a FLAG tag and a GPI-attachment signal. A mutant viral glycoprotein, VSVGts, misfolds and is retained in the ER at 40 °C, but, upon temperature shift to 32 °C, moves to the PM via the Golgi complex [18]. This property is preserved after fusing with an epitope tag or GFP (green fluorescent protein). Since the FLAG epitope is in its extracellular domain, surface expression of the fusion protein can be tracked by probing FLAG, and an inducible system enables us to follow the movement of newly synthesized proteins to the PM. We first characterized this stable cell line. Doxycycline treatment of cells induced expression of VSVGts–FLAG–mEGFP–GPI as assessed by Western blotting using an anti-GFP or an anti-FLAG monoclonal antibody (results not shown). Images from immunofluorescence staining showed that the GPI-anchored fusion protein was predominantly localized to the ER at 40 °C (Figure 1B, a and c), whereas temperature shift to 32 °C promoted strong PM localization of the GPI-anchored protein (Figure 1B, e and g). More quantitatively, cell-surface expression of the GPI-anchored reporter protein was examined using flow cytometry (Figures 1C and 1D). When grown at 40 °C in the presence of doxycycline, cells displayed little or virtually no detectable PM expression of the GPI-anchored fusion protein. Temperature shift from 40 °C to 32 °C resulted in the release of the GPI-anchored fusion protein from the ER to the cell surface. In time-course experiments, the cells exhibited PM expression of the GPI-anchored reporter protein 20 min after the temperature shift. The reporter in the PM gradually increased with time, and a majority of them reached the PM approx. 60 min after the 40 °C to 32 °C shift. To ensure that the GPI anchor was attached to the fusion protein, we performed a PI-PLC assay. PI-PLC is known to specifically cleave within the GPI anchor of modified proteins and release them from cell membranes [19]. Treatment of cells with PI-PLC reduced the GPI-anchored reporter proteins in the PM (Figure 1E), indicating that the fusion protein was modified with the GPI moiety. Taken together, this cell line provides experimentally traceable systems to monitor secretory traffic from the ER to the PM of a GPI-anchored protein.

siRNA for p23 selectively suppressed the targeted protein

We designed several siRNAs targeting to p23 and first tested their ability to suppress the expression level of p23. The siRNA plasmids were introduced into cells by electroporation, and, 60 h after transfection, cells were lysed, separated by SDS/PAGE, and proteins were analysed by Western blotting using an anti-p23 polyclonal antibody. The whole-cell lysate from CHO cells

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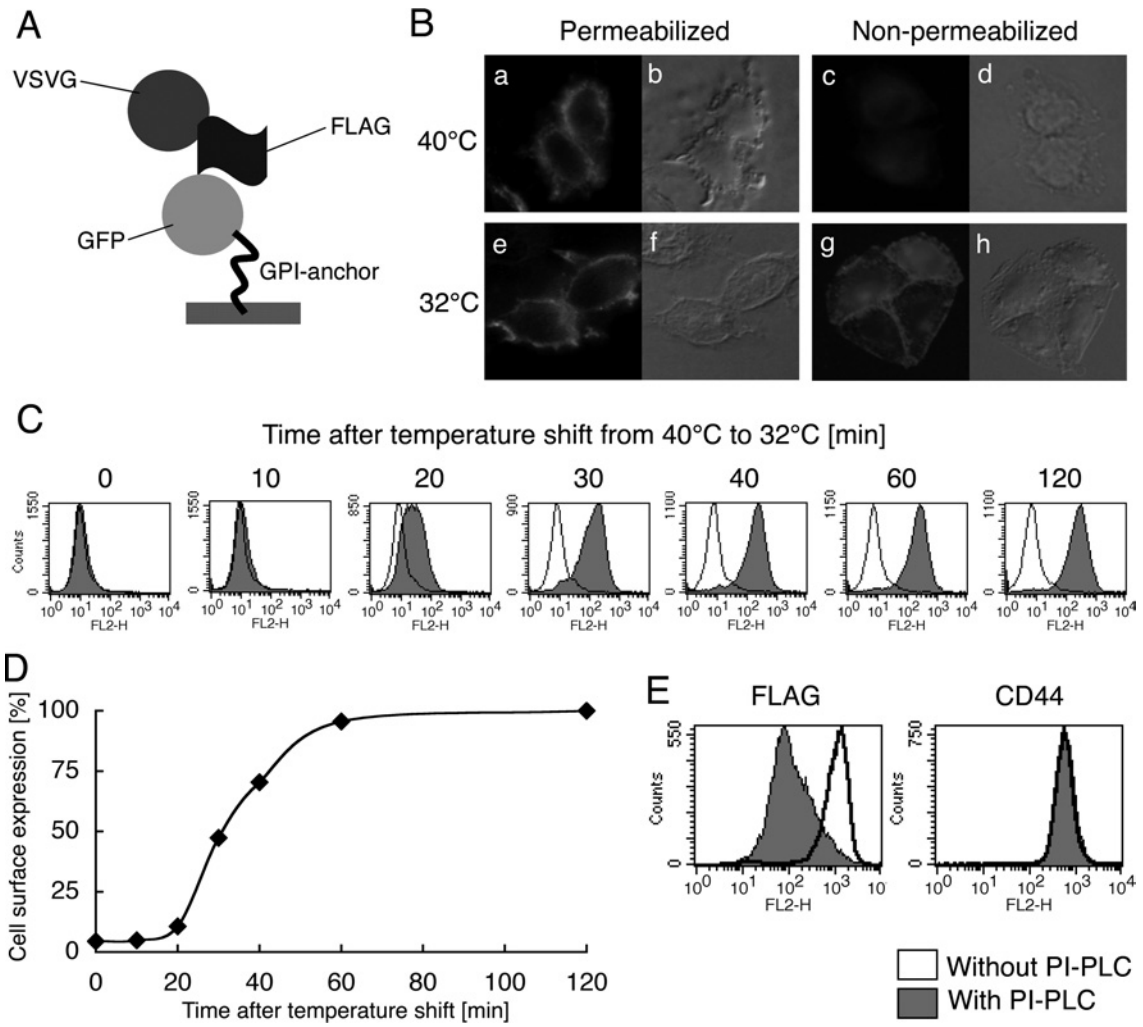

Figure 1 FF8 cells provide a system to monitor a GPI-anchored protein along the secretory pathway

(**A**) A schematic view of the GPI-anchored, FLAG- and GFP-fused VSVG. (**B**) Subcellular localization of the GPI-anchored reporter protein was visualized by immunofluorescence staining under permeabilized (0.1% saponin) (a, b, e and f) and non-permeabilized (c, d, g and h) conditions using a mouse anti-FLAG monoclonal antibody followed by Alexa Fluor® 594 anti-mouse antibody. The right-hand panel (b, d, f and h) shows Nomarski images corresponding to the fluorescent images on the left-hand panel (a, c, e and g). At 40°C, cells exhibited characteristic ER localization, while strong PM staining was observed at 32°C. (**C**) Cell-surface expression of the GPI-anchored reporter protein (filled histogram) was traced using flow-cytometric analysis as described in the Experimental section. An isotype (IgG_1)-matched mouse monoclonal antibody was used for control staining (solid line). (**D**) The geometric mean fluorescence value in cell population at each time was plotted. The geometric mean of control cells at 120 min was plotted as 100%. (**E**) Cells were treated with 1 unit/ml PI-PLC for 1 h at 37°C. The level of the GPI-anchored reporter protein (left) or an endogenous non-GPI-linked protein, CD44 (right) at the PM was measured by flow cytometry as described above. The solid line and shaded histogram indicate surface expression of the GPI-anchored reporter or non-GPI-linked CD44 in the absence and presence of PI-PLC respectively.

transiently transfected with p23 cDNA was utilized as a positive control (Figure 2A, lane 6). Transfection of a control pSINsi-hU6 empty vector had no effect on the level of endogenous p23 (Figure 2A, lane 1). A substantial decrease in p23 was observed in cells transfected with pSINsi-hU6-242 or pSINsi-hU6-345 (Figure 2A, lanes 2 and 4). On the other hand, cells with pSINsi-hU6-256 or pSINsi-hU6-465 exhibited virtually no difference in the level of p23 (Figure 2A, lanes 3 and 5). Therefore siRNA constructs pSINsi-hU6-242 and pSINsi-hU6-345, but not pSINsi-hU6-256 and pSINsi-hU6-465, specifically suppressed p23 in FF8 cells, interfering with its corresponding mRNA. It has been shown that p24 family proteins may form a complex and mutually stabilize each other [7–10]. We next examined the level of p24 protein in p23 siRNA-transfected cells. Cells expressing pSINsi-hU6-242 or pSINsi-hU6-345 (Figure 2A, lanes 2 and 4) had decreased p24 proteins as assessed by Western blotting using an anti-p24 polyclonal antibody, whereas p24 was unchanged in cells transfected with pSINsi-hU6-256 and pSINsi-hU6-465 (Figure 2A, lanes 3 and 5) compared with control cells (Figure 2A, lane 1).

Suppression of p23 protein level results in delayed transport of a GPI-linked protein

We next examined the effects of p23 siRNA on PM targeting of the GPI-anchored reporter protein. Cells were transfected with the siRNA vector and maintained at 37°C. At 48 h after transfection, doxycycline was added to the cells. The cells were grown at 40°C overnight and then transferred to 32°C (zero time). Expression of the GPI-anchored reporter protein at the PM was tracked at time intervals over 120 min, as described in the Experimental section. Transfection itself did not affect the

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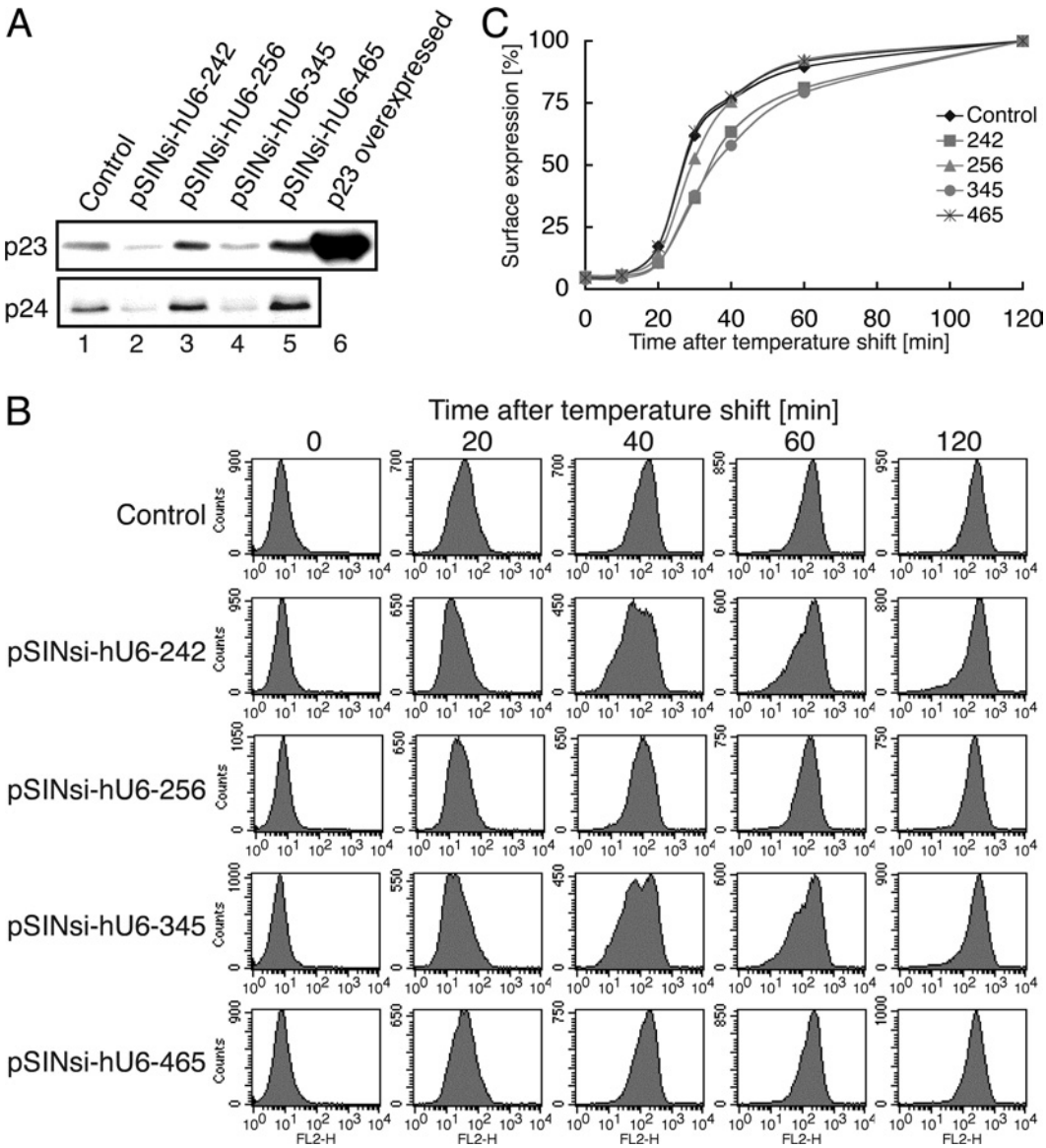

Figure 2 Suppression of p23 leads to delayed PM trafficking of the GPI-anchored protein

(**A**) FF8 cells were transfected with the indicated siRNA vector or an expression vector encoding p23. Cells were lysed and sample proteins were resolved by SDS/PAGE, followed by immunoblotting using a rabbit anti-p23 (top) or anti-p24 (bottom) polyclonal antibody. (**B**) Cell-surface expression of the GPI-anchored reporter protein in cells transfected with the indicated vector was tracked using flow cytometry as described in the Experimental section. (**C**) The geometric mean fluorescence value at each time point was plotted with the geometric mean at 120 min as 100 %.

trafficking of the GPI-anchored reporter protein. Cells transiently expressing an empty pSINsi-hU6 plasmid showed virtually the same time-course pattern of PM expression of the GPI-anchored protein as non-transfected cells shown in Figure 1. In cells transfected with pSINsi-hU6-242 or pSINsi-hU6-345, PM localization of the GPI-anchored reporter was apparently slowed as indicated by much less GPI-anchored reporter protein on the PM at 40 or 60 min after the temperature shift (Figures 2B and 2C). On the other hand, transfection of cells with pSINsi-hU6-256 or pSINsi-hU6-465 had little or virtually no effect on the delivery of the GPI-anchored reporter proteins to the PM. These findings indicate that selective suppression of p23 by siRNA led to delayed transport of the GPI-anchored reporter protein to the PM. Limited efficiency of transient transfection resulted in a mixed population of siRNA-expressing and non-expressing cells. In order to examine the effect of p23 siRNA more precisely, we introduced the gene encoding HcRed into the siRNA expression plasmid as a reporter. The presence of HcRed fluorescence indicated that cells expressed the siRNA vector and thus we could distinguish cells containing siRNA from those lacking it. An empty pSINsi-hU6 vector with HcRed did not affect PM targeting of the GPI-anchored fusion protein. When transfected with pSINsi-hU6-242-HcRed or pSINsi-hU6-345-HcRed, a cell population with low HcRed displayed an almost identical pattern of reporter-protein movement to control cells (Figures 3B and 3D), whereas a population of cells presenting high HcRed fluorescence exhibited striking delays in trafficking of the GPI-anchored protein (Figures 3A and 3C). The mean $\pm$ S.D. surface expression at 40 min after the temperature shift was determined as 79 ± 2.6 ($n = 5$), 49 ± 3.7 ($n = 4$; Student's t test,

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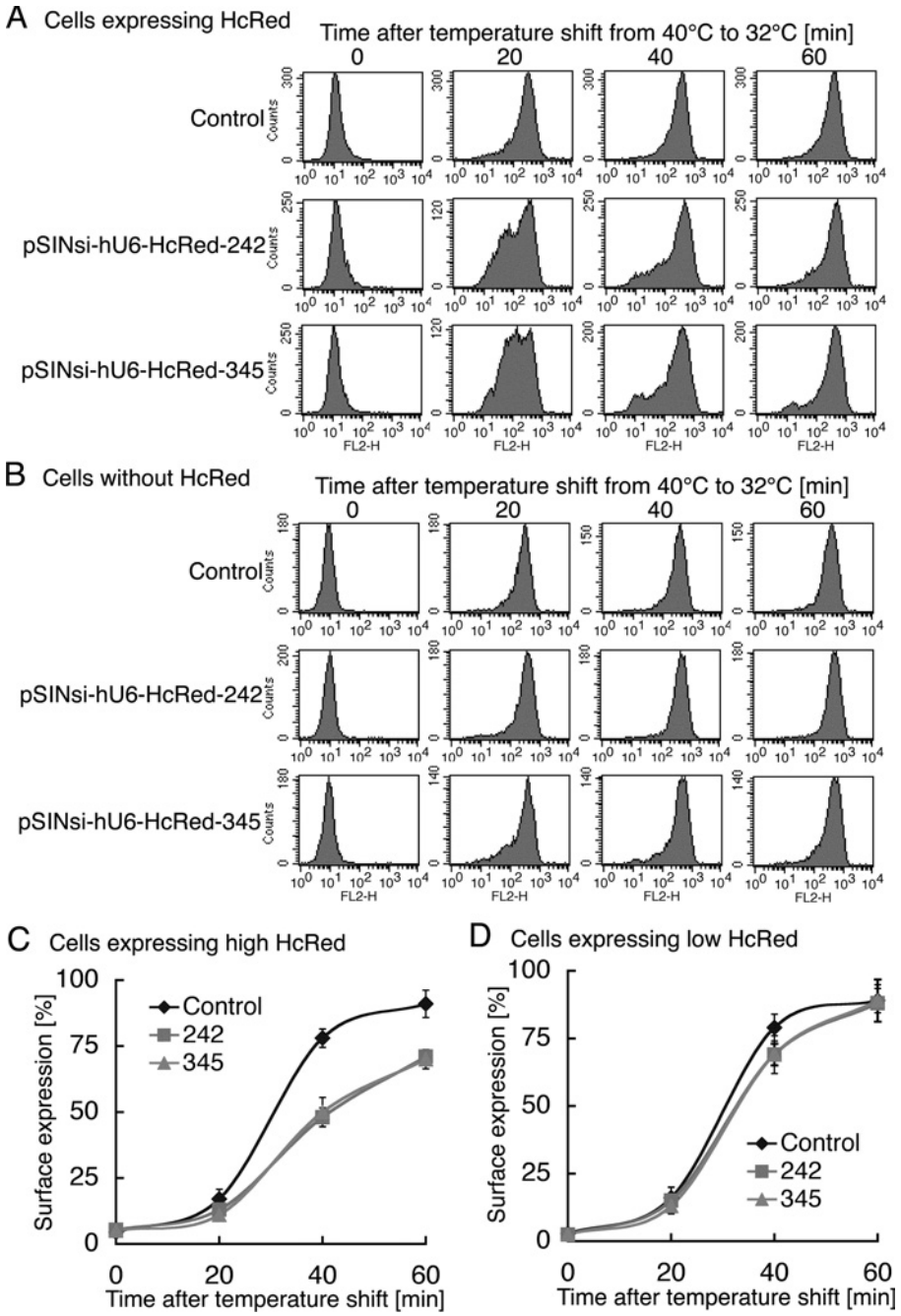

Figure 3 Cells expressing siRNA specifically exhibited the delay in targeting of the GPI-anchored protein to the PM

The gene encoding HcRed was inserted into the pSINsi-hU6 vector as a reporter in order to distinguish cells expressing siRNA from those lacking it. Cells were transiently transfected with pSINsi-hU6-HcRed-242 or pSINsi-hU6-HcRed-345. A population of cells presenting high HcRed fluorescence exhibited substantial delays in trafficking of VSVG–FLAG–GFP–GPI. The geometric mean fluorescence value was plotted in a cell population expressing high (**A**, **C**) or low (**B**, **D**) HcRed fluorescence. The percentage of surface expression at 120 min was plotted as 100 %. An empty pSINsi-hU6 vector with HcRed did not affect PM targeting of the GPI-anchored fusion protein.

$P = 0.001$ relative to control) and 50 ± 5.6 % ($n = 3$; $P = 0.014$ relative to control) for cells expressing control, pSINsi-hU6-242-HcRed and pSINsi-hU6-345-HcRed vector respectively. At 60 min, 91 ± 5.2 % ($n = 5$) of the reporter reached the PM in the control cells, whereas pSINsi-hU6-242-HcRed- and pSINsi-hU6-345-HcRed-expressing cells showed 71 ± 2.0 % ($n = 4$; $P = 0.001$) and 70 ± 2.6 % ($n = 3$; $P = 0.001$) surface expression respectively. Collectively, these results clearly demonstrate that specific suppression of p23 by siRNA resulted in pronounced delay of PM targeting of the GPI-anchored cargo.

Maturation of DAF was slowed by siRNA for p23

We next examined the effect of p23 siRNA on transit of a GPI-anchored protein from the ER to the Golgi. To test this, we monitored the maturation of DAF, a well-studied GPI-anchored

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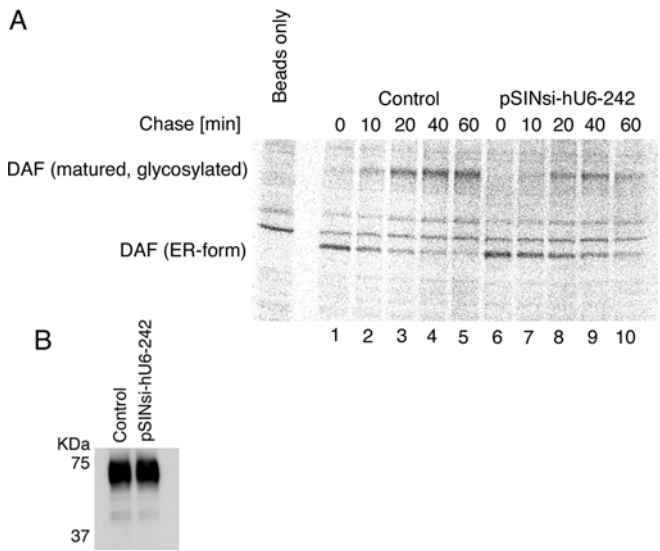

Figure 4 p23-knockdown results in slower maturation of DAF

(**A**) Cells were transfected with pSINsi-hU6-242. At 60 h after transfection, cells were incubated for 10 min in medium containing [^{35}S]methionine and [^{35}S]cysteine labelling mixture (pulse). Cells were washed and incubated in regular medium for the indicated times (chase). DAF was immunoprecipitated using a mouse anti-DAF monoclonal antibody and separated by SDS/PAGE, and radiolabelled proteins were visualized by autoradiography. Note that two non-specific bands between the ER- and Golgi-form DAF were also seen in the sample incubating lysate from labelled control cells with no anti-DAF antibody (Beads only lane). (**B**) Whole-cell lysates of control- and pSINsi-hU6-242-transfected cells were analysed by SDS/PAGE and Western blotting with a monoclonal anti-DAF antibody under non-reducing conditions.

protein in human cells. A newly synthesized DAF traverses from the ER to the Golgi and undergoes O- and N-glycosylation to become the mature form of DAF. The movement from the ER to the Golgi can be traced as an alteration in their apparent molecular mass from the ER form (~45 kDa) to the highly glycosylated form (~75 kDa). We experienced less efficient metabolic labelling, probably because cells were electroporated before pulse. Yet, in cells treated with the control siRNA expression vector, the mature form of DAF was clearly seen after 20 min chase, while the ER form decreases (Figure 4A, lanes 1–5). In contrast, cells transfected with pSINsi-hU6-242 exhibited delayed maturation of DAF (Figure 4A, lanes 6–10). After 20 min of chase, the band for the Golgi form was weaker, and the decrease in the ER form and increase in the Golgi form of DAF was slower than those in control cells. The total amount of DAF was virtually the same in both conditions as assessed by Western blotting (Figure 4B).

p23 siRNA barely affected trafficking of non-GPI-linked proteins

Subsequently, we looked at whether p23 is required for transport of non-GPI-linked proteins. VSVG is the only temperature-sensitive cargo in mammalian cells currently available to us, and we generated a cell line stably expressing a tetracycline-inducible FLAG–VSVGts–EGFP as a reporter protein (Figure 5A). Incubation of cells at 40 °C in the presence of doxycycline resulted in little or no PM localization of the non-GPI-linked fusion protein. Upon shift from 40 °C to 32 °C, surface expression of FLAG–VSVG–GFP increased with time (results not shown). When p23 was suppressed by pSINsi-hU6-242 containing the HcRed gene, PM targeting of the non-GPI-linked reporter protein was barely affected in cells expressing high HcRed (Figure 5B). No delay was seen in cells with low HcRed signals (Figure 5C).

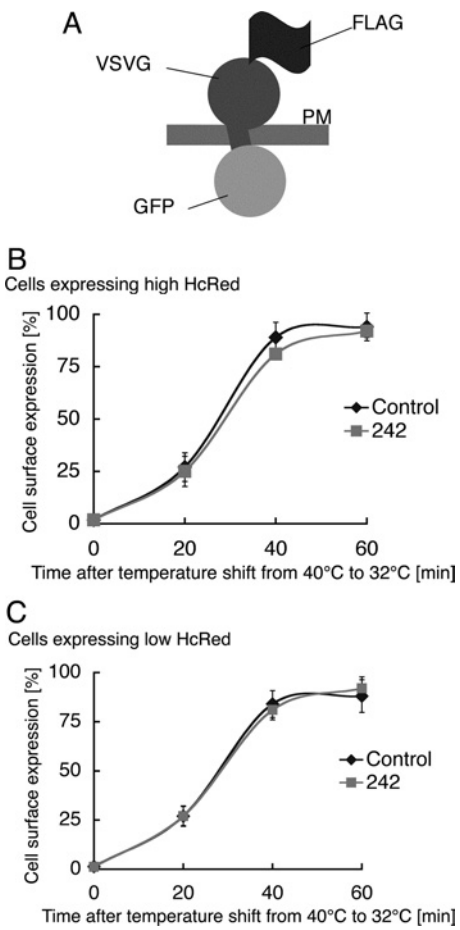

Figure 5 Only slightly delayed transport of the non-GPI-anchored VSVG protein was seen in cells with p23 siRNA

(**A**) A schematic representation of the FLAG- and EGFP-fused VSVG at the PM. (**B**, **C**) The siRNA vector with the HcRed-encoding gene was transfected into cells stably expressing the FLAG- and GFP-fused VSVG protein. Cell-surface expression of the VSVG reporter protein was monitored using flow cytometry as described in the Experimental section. The geometric mean fluorescence values were plotted, with the geometric mean 120 min after temperature shift plotted as 100 %.

DISCUSSION

Transport from the ER to the Golgi apparatus is the initial step in the secretory pathway. Upon ER exit, cargo proteins must be selectively packaged into COPII-coated transport vesicles. Proteins containing sorting signals in their cytoplasmic domain can interact with the coat machinery, whereas cargoes without cytoplasmic signals require alternative mechanisms to be recognized by the transport vesicles. Attachment of the GPI anchor to a newly synthesized protein occurs in the luminal side of the ER membrane, and the modified protein possesses no transmembrane domains, tethering to the ER solely by the GPI moiety.

Although their precise mechanisms remain to be resolved, the family of p24 proteins has been thought to play key roles in maintaining the fidelity of vesicle transport between the ER and the Golgi. Deletion studies in yeast have revealed that disruption of Emp24p caused delayed transport of a GPI-anchored protein, Gas1p [12]. A mutant lacking p24 proteins was found to secrete ER-resident proteins [20]. In mammalian cells, a difficulty in examining molecular machineries of p24 family proteins has been the inability of ectopically introduced p24 proteins to be localized to proper subcellular locations. In the present study, we employed

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an RNAi technique and selectively suppressed p23 expression to study their roles in transit of the GPI-anchored protein in mammalian cells.

We found that cells that suppressed p23 by siRNA had a reduced level of p24 (Figure 2A). This is consistent with previous results showing that the p24 family proteins form a functional hetero-oligomeric complex and mutually stabilize each other [8,9]. In yeast, deletion of one or more of the p24 family proteins affected steady-state levels of other member proteins [7,10]. p23-knockout mice displayed a reduced level of other p24 proteins, namely GMP25 and p26 [13]. The nature of heteromer formation between p24 proteins remains to be investigated.

The temperature-sensitive variant of VSVG has been widely utilized to study secretory transport [18]. We fused the GPI anchor to VSVG and stably expressed the fusion protein into a CHO-derived cell line. Expression of this protein was regulated by doxycycline, which allowed us to track movement of the newly synthesized protein from the ER to the PM. We found that suppression of p23 by siRNA resulted in pronounced delays in PM trafficking of the GPI-anchored reporter protein (Figures 2B, 2C and 3). Moreover, p23 siRNA slowed the maturation of DAF, a well-studied mammalian GPI-anchored protein (Figure 4), suggesting that p23 suppression led to delayed transit of DAF from the ER to the Golgi. Collectively, these results indicate that p23, probably in concert with p24, regulates the transport of GPI-anchored cargoes in mammalian cells.

In yeast, it appears that different cargoes can be sorted into different populations of transport vesicles [3–5]. The observation that Gas1p was packaged into vesicles that were distinct from those that carry α-factor and amino acid permeases has led to a commonly accepted notion of GPI-anchored protein-specific transport machinery. p24 proteins were found to be necessary for Gas1p transport, whereas a non-GPI-linked protein, Gap1p, did not require p24 proteins for its cell-surface delivery [3]. In HeLa cells, it has been reported that GFP fused with GPI exhibited different kinetics of the accumulation into transport vesicles from non-GPI-linked cargoes in the presence of a GTP-restricted mutant of Sar1 [21]. In the present study, using cells stably expressing inducible non-GPI-linked VSVG reporter proteins, we found that suppression of p23 barely affected transit of the reporter to the PM (Figures 5B and 5C). This suggests that, in mammalian cells, p24 proteins play limited roles, if any, in efficient ER exit of non-GPI-linked cargoes. A temperature-sensitive system is probably the most advantageous system to precisely track synchronized release of cargo from the ER. VSVG is the only thermoreversible cargo in mammalian cells currently available to us. Taken together, results of the present study suggest that mammalian GPI-anchored cargoes, but not non-GPI-linked proteins, require p24 proteins for their transport between the ER and the Golgi. Like GPI-anchored proteins in yeast, mammalian counterparts may be packaged into different transport vesicles from non-GPI-linked proteins. Alternatively, proteins with or without GPI may utilize the same vesicle, whereas p24 proteins are implicated in certain steps exclusive to GPI-anchored proteins. Interestingly, a recent study demonstrated that a protein with GPI was packaged into the same carrier as non-GPI-linked proteins in non-polarized HeLa, COS7 or MDCK (Madin–Darby canine kidney) cells and, albeit controversially, in polarized MDCK cells [22–24].

How do p24 proteins work in cargo delivery? Specific association of any p24 proteins with cargoes has not been shown, except in yeast. Using budding vesicles prepared *in vitro,* it was reported that Gas1p can be cross-linked to p24 proteins, Emp24p and Erv25p [3]. Although it remains to be seen whether the association occurs *in vivo*, results imply that yeast p24 proteins can function as a cargo receptor for Gas1p. Whether all yeast GPI-anchored proteins interact with p24 proteins remains to be fully tested. Also unknown is whether it is because of the GPI modification that yeast p24 proteins interact with Gas1p. In our hands, it has been chronically unsuccessful to detect interaction of p23 with mammalian cargoes employing co-immunoprecipitation or chemical cross-linking techniques. At the concentrations of cross-linking reagents reported in yeast, all epitopes we tested in mammalian cells seemed to be masked by covalent modifications. No association between p23 and the GPI-anchored cargo protein was observed at lower concentrations. Yet our results do not rule out the possibility that the interaction of GPI-linked proteins with p24 proteins is extremely weak or transient, or that the cross-linkers that we used were unsuitable for conjugating cargoes with p24 proteins. More studies are needed to clarify the precise nature of p24 functions in cell-surface targeting of the GPI-anchored cargoes.

In conclusion, the present study demonstrates that, in mammals, GPI-anchored proteins specifically require p24 proteins for their transit from ER to the PM. To our knowledge, this is the first report to show direct evidence for trafficking of mammalian cargoes regulated by p24 proteins. The results suggest that RNAi may prove valuable in future studies of functions of mammalian p24 proteins.

We gratefully thank Dr Felix Wieland and Dr Irene Schulz for generously providing the antibodies, Dr Satoshi Tanaka for assistance in the initial stage of the work, and Keiko Kinoshita and Fumiko Mori for technical expertise. This work was supported by grants from Core Research for Evolutional Science and Technology, Japan Science and Technology Agency, and from the Ministry of Education, Science, Sports, Culture, and Technology of Japan.

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Received 13 February 2007/28 September 2007; accepted 11 October 2007
Published as BJ Immediate Publication 11 October 2007, doi:10.1042/BJ20070234

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