Tissue-inherent fate of GPI revealed by GPI-anchored GFP transgenesis

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Abstract To clarify the fate of glycosylphosphatidylinositol (GPI) in mammals, we developed GPI-anchored enhanced green fluorescent protein (EGFP-GPI) and transgenic mice carrying this fusion construct. When it was introduced to culture cells, the EGFP-GPI protein was correctly sorted to plasma membranes and microsomes depending on GPI biosynthesis. Transgenic mice carrying EGFP-GPI were found to show a broad transgene expression. Histologically, a prominent polarized localization of EGFP-GPI protein was observed in various epithelia, the nervous system and liver and secreted from some exocrine glands, as well as non-polarized presence in non-epithelial tissues, demonstrating a tissue-inherent manner of GPI sorting.

Key words: Glycosylphosphatidylinositol-anchor; Enhanced green fluorescent protein; Cell surface; Transgenic mouse; Polarization

1. Introduction

Numerous membrane proteins in eukaryotes are transported and attached to the plasma membrane via a glycosylphosphatidylinositol (GPI)-anchor [1–3]. Deficient GPI biosynthesis in hematopoietic cells causes paroxymal nocturnal hemoglobinuria, demonstrating dysfunction of GPI associated with human disease [4]. Previously, to elucidate the roles of GPI in mammals, knockout experiments were performed and found that the GPI system plays crucial roles in embryogenesis and keratinocyte differentiation [5,6]. Recently, the mosaic mice for disrupting GPI biosynthesis were produced and the extreme differences in GPI requirements among tissues were shown [7]. These results prompted us to investigate the fate of GPI in global tissue and here, we developed transgenic mice carrying GPI-anchored green fluorescent protein (GFP) as an indicator [8,9].

Generally, GPI-anchored proteins are synthesized as precursors which contain two signal sequences: the amino-terminal signal sequence for membrane translocation into the endoplasmic reticulum (ER) and the carboxy-terminal GPI-anchor signal polypeptide [2,3]. Both signal peptides are cleaved soon after translocation into the ER lumen and then, the carboxy-terminal of the protein is covalently attached to GPI which has been pre-synthesized en bloc in the ER. For our purpose, the amino-terminal signal sequence of the sperm secretory protein acrosin and the carboxy-terminal GPI-anchor signal sequence of Thy-1 were fused to either end of enhanced GFP (EGFP). Here, we report the development of EGFP-GPI and characterization of EGFP-GPI transgenic mice.

2. Materials and methods

2.1. Generation of EGFP-GPI fusion gene

The EGFP was first fused with the membrane translocation signal sequence of acrosin at the amino-terminal as described previously [10]. The acrosin-EGFP fusion DNA fragment was further ligated to the 5′-end of the mouse Thy-1 GPI-anchoring sequence. The amino acid residue for GPI attachment (ω site) was changed from cysteine to asparagine for a more efficient attachment (K. Ohishi, personal communication). According to both gene linkages, additional 14 amino acid sequences (KDNTTLQEFAT and LEN) would be resided in the mature EGFP-GPI protein. Then, this fusion gene was ligated down stream of the CAAG promoter, an established strong and broad driving element containing the cytomegalovirus immediate-early enhancer and chicken β-actin promoter [11].

2.2. Cell culture and transfection

F9 and COS7 cells were cultured in DMEM supplemented with 10% FCS. For DNA transfection, lipofectamine reagents (Life Technologies, Rockville, MD, USA) were used according to the manufacturer's protocol.

2.3. Phosphatidylinositol-specific phospholipase C (PI-PLC) treatment

COS7 cells transfected with EGFP-GPI were treated with 2 IU/ml of PI-PLC (Boehringer Mannheim, Mannheim, Germany) in DMEM for 1 h at 37°C. The incubated supernatant from each dishes was collected, the fluorescent activity was measured at 488 nm excitation and 518 nm emission wave lengths by a fluorometer and the total protein was precipitated with 10% trichloroacetate, washed with cold acetone and subjected to Western blotting.

2.4. Western blotting

Cells and tissues were first homogenized in TNE solution (10 mM Tris-HCl pH 7.8, 1 mM EDTA, 150 mM NaCl) with Complete protease inhibitor (Boehringer Mannheim, Mannheim, Germany) at an ice cold temperature. The homogenates were centrifuged at $100\,000 \times g$. The supernatants were collected and stocked (the water-soluble fraction). The precipitates were washed with TNE buffer and then homogenized in 1% Triton X-114 (Nacalai tesque, Kyoto, Japan)-TNE solution with Complete protease inhibitor and centrifuged at $100\,000 \times g$. The supernatants were collected and stocked (the detergent-soluble fraction). Both fractions of each tissue were subjected to SDS-PAGE and electrophoretically transferred to a nitrocellulose membrane. The membrane was probed with rabbit polyclonal antibody against GFP (MBL, Nagoya, Japan) and staining was detected with ECL system-conjugated anti-rabbit IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK).

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Abbreviations: GPI, glycosylphosphatidylinositol; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; PI-PLC, phosphatidylinositol-specific phospholipase C

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2.5. Subcellular fractionation

COS7 cells transfected with EGFP-GPI were mechanically scraped off the culture dish. To prepare the membrane fraction, we followed the methods of Koizumi et al. [12]. Briefly, cells were suspended in 0.5 mM $CaCl_2$ in 1.0 mM $NaHCO_3$ buffer to prepare the cell ghost and applied to a serial sucrose gradient centrifuge (42-68% sedimentation and 42-48-68% sedimentation). To prepare cytoplasmic and microsome fractions, we followed the methods of Hogeboom et al. [13]. Briefly, cells were suspended in 0.25 M sucrose-TKM (20 mM Tris, pH 7.6, 50 mM KCl, 2 mM $MgCl_2$) solution and homogenized. Lysates were serially centrifuged at 1000, 12 000 and 105 000 × *g*. The supernatants (cytoplasmic fraction) and precipitates (microsome fraction) of the final centrifuge were collected and stocked. The method for preparing the nuclear fraction was described before [14]. Briefly, cells were homogenized in 0.25 M sucrose-TKM solution. The homogenate was overlaid on 2.4 M sucrose-TKM solution and centrifuged for 30 min at 14 000 × *g*. The precipitates (nuclear fraction) were collected and sonicated. All fractions were finally dissolved in 1% Triton X-114-TNE solution and subjected to Western blotting.

2.6. Transgenic mouse production

The CAAG-EGFP-GPI transgene was injected into one-cell embryos of B6C3F1 mice. By screening 42 newborn mice, we obtained six transgenic founders and established three transgenic lineages (lines 1–3), all of which transmitted the transgene in a Mendelian fashion.

2.7. Histological analyses

Mice were deeply anesthetized with phenobarbital and fixed by perfusing with 4% (w/v) paraformaldehyde-PBS via the left ventricle of the heart. Excised tissues were fixed again in 4% paraformaldehyde-PBS and then incubated in 20% sucrose-PBS for 48 h at 4°C. Pieces of tissues were then embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA, USA), quickly frozen with dry ice and sectioned on a cryostat in 5–10 μm thicknesses. Preparations were examined by fluorescence microscopy with GFP-specified filters (Olympus, Tokyo, Japan).

3. Results and discussion

3.1. Characterization of EGFP-GPI in culture cells

When the EGFP-GPI fusion construct was stably expressed in F9 cells, a marked green fluorescence was detected on the plasma membrane and in intracellular structures, which was quite different from the original EGFP transfectants (Fig. 1A). The intracellular structure was identified as a Golgi complex by co-staining with wheat germ agglutinin (data not shown). To compare both EGFP molecules further, Western blotting was performed and showed that EGFP-GPI was about 4 kDa larger than EGFP (31 versus 27 kDa), which almost corresponds to the sum of GPI plus the additional 14 amino acid molecular weights (> 3800 Da). Moreover, the EGFP-GPI molecule was effectively solubilized when detergent was added to the lysis buffer, while the original EGFP was effectively extracted without detergent. These findings suggest that EGFP-GPI contains a highly hydrophobic moiety.

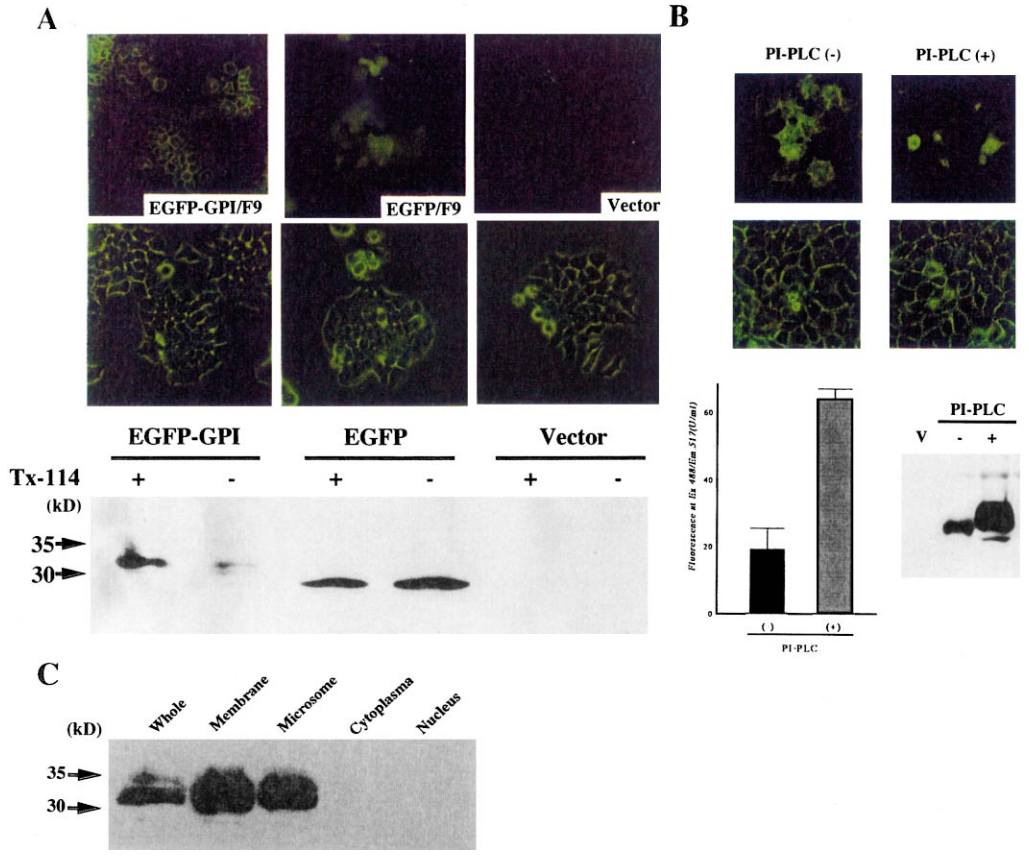


Fig. 1. Expression and characterization of EGFP-GPI in culture cells. (A) Stable expression of EGFP-GPI in F9 cells. Upper panels, fluorescence microscopic views. Middle panels, phase-contrast views. Lower panel, Western blotting. Tx-114 +, detergent-plus lysis buffer; Tx-114 −, detergent-minus lysis buffer. (B) Release of EGFP-GPI by PI-PLC cleavage. Upper panels, fluorescence microscopic views. Middle panels, phase-contrast views. Lower panel left, fluorescence of culture supernatants. Means after background subtraction with an S.D. bar are indicated. Lower panel right, Western blotting. Spontaneous release of EGFP-GPI was observed in both assays. PI-PLC (+), PI-PLC treated; PI-PLC (−), PI-PLC non-treated; v, vector-transfected cells. (C) Subcellular fractionation of EGFP-GPI transfectants. Western blotting. Whole, cell lysate without fractionation. Membrane, plasma membrane.

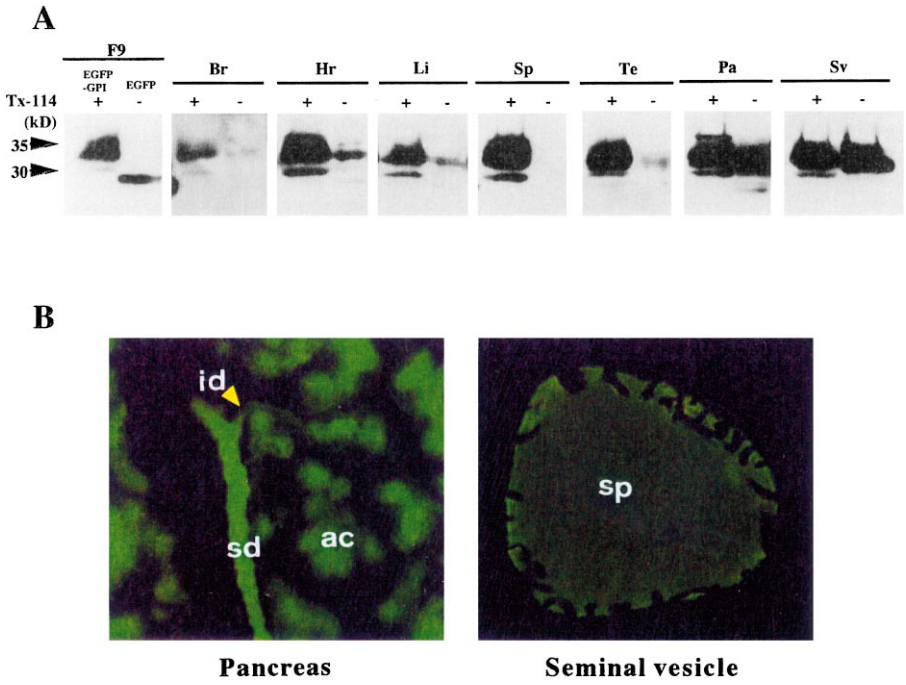


Fig. 2. Expression of EGFP-GPI in various tissues of EGFP-GPI transgenic mice. (A) Expression of EGFP-GPI protein in multiple organs. Western blotting. Tx-114 +, detergent-plus lysis buffer; Tx-114 −, detergent-minus lysis buffer. (B) The fluorescent histology of pancreas and seminal vesicle. Magnification: pancreas, ×200; seminal vesicle, ×100. F9, F9 transfectants; Br, brain; Hr, heart; Li, liver; Sp, spleen; Te, testis; Pa, pancreas; Sv, seminal vesicle; id, intercalated duct; sd, secretory duct; ac, acinus; sp, seminal plasma. The presenting results were obtained from a line 3 transgenic mouse.

To confirm that EGFP-GPI was anchored via GPI, sensitivity to PI-PLC was tested. It is well-known that PI-PLC cleaves the GPI phosphate-glycerol bond and releases GPI-anchored protein from the membrane to the water-soluble phase, which is a useful diagnostic tool for confirming GPI-anchorage of the protein [15]. Bacterial PI-PLC was added to the culture medium of EGFP-GPI-transfected COS7 cells. In the presence of PI-PLC, membranous fluorescence was diminished. In proportion to this, total fluorescence of culture supernatants was increased and elevation of EGFP-GPI in the supernatant was detected by Western blotting (Fig. 1B). We also observed membrane attachment of EGFP-GPI in GPI-anchor positive cells, but not in GPI-anchor negative counterparts (data not shown). These findings demonstrate that EGFP-GPI is attached to the outer surface of plasma membranes by GPI.

To elucidate the effects of fused peptides and over-expression, which would cause mis-localization of EGFP-based fusion protein, subcellular fractionation was performed. The lysates of COS7 cells transfected with EGFP-GPI were fractionated and subjected to Western blotting. Even in extreme over-expression conditions using the SV40 replicative system in COS cells, EGFP-GPI was solely fractionated to the plasma membrane and microsomes where GPI-anchored proteins are harbored, suggesting that peptide fusion and over-expression have no effects on the sorting of EGFP-GPI (Fig. 1C).

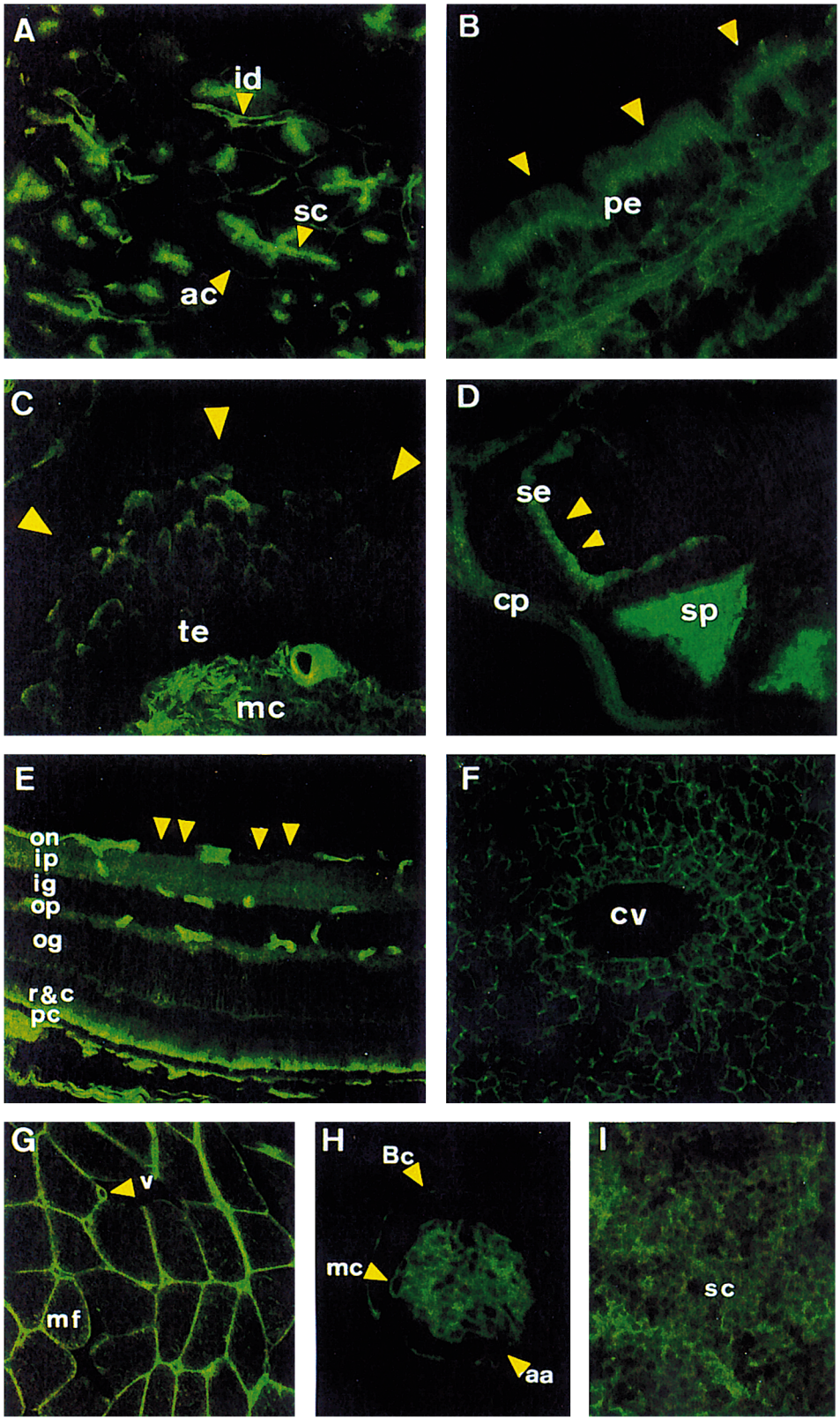
3.2. Development of EGFP-GPI transgenic mouse

To clarify the fate of GPI in vivo, transgenic mice carrying an EGFP-GPI fusion construct were raised. All transgenic mice were born normal size, had no morphological abnormalities and were fertile. The expression of transgene mRNA was first examined and widely detected in tissues (data not shown).

Expression of EGFP-GPI protein was then examined in various tissues. Both water-soluble and detergent-soluble fractions of each tissue were subjected to Western blotting. As shown in Fig. 2A, EGFP-GPI protein was solubilized in both fractions, but to different degrees in each tissue. In tissues like brain, heart, liver, spleen and testes, most of the EGFP-GPI was solubilized in detergent-plus buffer. In contrast, large amounts of EGFP-GPI were solubilized in water-soluble as well as detergent-soluble fractions in pancreas and seminal vesicles. Histologically, strong homogeneous fluorescence in the secretory duct of pancreas and the plasma of seminal vesicles was observed (Fig. 2B), suggesting that EGFP-GPI was abundantly released here. It was previously reported that secretion of GP-2 and CDw52, kinds of the GPI-anchored proteins, were noted in rat pancreatic acinar cells and human seminal plasma, respectively [16,17]. Our findings may support these observations.

3.3. Histological survey of EGFP-GPI transgenic mice

Fixed tissues were sectioned frozen and immediately examined by fluorescence microscopy. Localization of EGFP-GPI protein could be classified as polarized/localized or non-polarized. All kinds of epithelial cells showed polarized localization of EGFP-GPI. The presence in representative tissues, such as pancreatic acinar cells, intestinal pinoepithelium, transitional epithelium of the urinary bladder and seminal epithelium, is shown (Fig. 3A–D). These appearances were similar to those of some endogenous GPI-anchored proteins, such as N-CAM in intestinal pinoepithelium and GP-2 in pancreatic acinus [16,18]. We also observed EGFP-GPI apical localization in the transitional and squamous epithelia (X.-H. Gao, unpublished data), suggesting that the apical polarization of GPI-anchored proteins is a more general phenotype among epithe-



lia despite of their morphology and distribution. In the nervous system, EGFP-GPI was specifically localized in nerve fibers (Fig. 3E). For instance, fine fluorescence could be seen on nerve fibers, but not on perikaryons, in the local neural networks of retina, suggesting that the mature GPI molecules were constitutively produced here, sorting and anchoring GPI-anchored proteins to nerve fibers. In the liver, EGFP-GPI seemed to localize along the network of bile canaliculi (Fig. 3F). This pattern of expression is similar to alkaline phosphatase and 5′-nucleotidase, a well-known GPI-anchored

Fig. 3. Fluorescent histology of EGFP-GPI transgenic mice. (A) Pancreas. Bright fluorescence can be seen on the apical side of acinar cells, followed by intercellular secretory canaliculi (sc). ac, acinus; id, intercalated duct. Magnification, ×200. (B) Duodenum. Bright fluorescence was markedly localized to the apical side of pinoepithelial cells (pe). Magnification, ×400. (C) Urinary bladder. Apparent apical polarization of fluorescence can be seen on transitional epithelium (te). mc, tunica propria mucosae. Magnification, ×400. (D) Seminal vesicle. Apical but not basal localization of fluorescence can be seen on seminal epithelium (se). sp, seminal plasma; cp, capsular smooth muscle cells. Magnification, ×400. (E) Retina. Bright fluorescence can be seen on nerve fiber-rich layers and, reciprocally, fluorescence-lucent areas correspond to the perikaryon-rich layers. Worm-like structures are blood vessels. on, optic ganglion cell layer; ip, inner plexiform layer; ig, inner granular layer; op, outer plexiform layer; og, outer granular layer; r and c, rod and cone layer; pc, pigment cell layer. Magnification, ×200. (F) Liver. Bright fluorescence can be seen along the bile canaliculi network. cv, central vein. Magnification, ×200. (G) Skeletal muscle. Muscle fibers (mf) are surrounded by strong homogeneous fluorescence. v, blood vessel. Magnification, ×200. (H) Renal corpuscle. Strong homogeneous fluorescence on mesangium cells (mc) of gromerulus can be seen. Bc, Bowman's capsule; aa, arteriola afferense. Magnification, ×400. (I) Spleen. Strong homogeneous fluorescence can be seen on the membrane of splenocytes (sc). Magnification, ×200. Arrowheads in B–D indicate the apical side of each organ and in E, they indicate perikaryons of optic ganglion cells. The presenting results were obtained from a line 3 transgenic mouse. ←

protein specifically localized to bile canaliculi [19,20], demonstrating that the expression of EGFP-GPI faithfully represents endogenous GPI-anchored protein. However, the expression of alkaline phosphatase in EGFP-GPI transgenic mice was reduced in comparison with wild-type mice (data not shown), suggesting that the amount of GPI is quite limited here. As bile canaliculi are composed of the plasma membrane of liver parenchymal cells, they might correspond to apical surfaces of the epithelium and GPI may specifically act as an intracellular sorting apparatus in the liver parenchymal cells. In contrast to these observations, non-epithelial cells, such as muscle cells, mesenchymal cells, hematopoietic cells and vascular endothelial cells, showed non-polarized localization, comparable with EGFP-GPI transfectants (Fig. 3G–I).

For labelling cell surfaces, EGFP-GPI might be applicable to many eukaryote systems, including primitive protozoa and yeast, because it is processed and targeted to the plasma membrane via the ubiquitous GPI-anchoring system. Another benefit of EGFP-GPI is that it is fully exposed to the cell surface. This means that EGFP-GPI could be used as an immuno target for antibody-mediated collection or elimination of target cells. Moreover, as fine fluorescence was detected along networks of nerve fibers and bile canaliculi, it might be a simple way of visualizing these structures under physiological and pathological conditions.

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References

[1] Low, M.G. and Saltiel, A.R. (1988) Science 239, 268–275.
[2] Takeda, J. and Kinoshita, T. (1995) Trends Biochem. Sci. 20, 367–371.
[3] Stevens, V.L. (1995) Biochem. J. 310, 361–370.
[4] Kinoshita, T., Inoue, M. and Takeda, J. (1995) Adv. Immunol. 60, 57–103.
[5] Nozaki, M., Ohishi, K., Yamada, N., Kinoshita, T., Nagy, A. and Takeda, J. (1999) Lab. Invest. 79, 293–299.
[6] Tarutani, M., Itami, S., Okabe, M., Ikawa, M., Tezuka, T., Yoshikawa, K., Kinoshita, T. and Takeda, J. (1997) Proc. Natl. Acad. Sci. USA 94, 7400–7405.
[7] Keller, P., Tremml, G., Rosti, V. and Bessler, M. (1999) Proc. Natl. Acad. Sci. USA 96, 7479–7483.
[8] Stauber, R.H., Horie, K., Carney, P., Hudson, E.A., Tarasova, N.I., Gaitanaris, G.A. and Pavlakis, G.N. (1998) BioTechniques 24, 462–471.
[9] Chalfie, M. and Kain, S. (1998) Green Fluoresscent Protein, Wiley-Liss, New York.
[10] Nakanishi, T., Ikawa, M., Yamada, S., Parvinen, M., Baba, T., Nishimune, Y. and Okabe, M. (1999) FEBS lett. 449, 277–283.
[11] Niwa, T., Yamamura, K. and Miyazaki, J. (1991) Gene 108, 193–199.
[12] Koizumi, K., Ito, Y., Kojima, K. and Fujii, T. (1976) J. Biochem. 79, 739–748.
[13] Hogeboom, G.H., Schneider, W.C. and Palade, G.E. (1948) J. Biol. Chem. 172, 619.
[14] Li, Q., Yoshioka, N., Yutsudo, M., Inafuku, S., Aozasa, K., Aizawa, S., Kitamura, Y., Nishimune, Y., Hakura, A. and Kondoh, G. (1998) Virology 252, 28–33.
[15] Davitz, M.A., Low, M.G. and Nussenzuweig, V. (1986) J. Exp. Med. 163, 1150–1161.
[16] Freedman, S.D., Kern, H.F. and Scheele, G.A. (1998) Eur. J. Cell. Biol. 75, 163–173.
[17] Hale, G., Rye, P.D., Warford, A., Launder, I. and Brito-Babapulle, A. (1993) J. Reprod. Immunol. 23, 189–205.
[18] Koller, E. and Ranscht, B. (1996) J. Biol. Chem. 271, 30061–30067.
[19] De Wolf-Peeters, C., De Vos, R. and Desmet, V. (1972) Tissue Cell 4, 379–388.
[20] De Valck, V., Geerts, A., Schellinck, P. and Wisse, E. (1988) Histochemistry 89, 357–363.