Association of glycosylphosphatidylinositol-anchored protein with retroviral particles

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ABSTRACT **We describe for the first time the association of glycosylphosphatidylinositol (GPI) -anchored proteins with retroviral and lentiviral particles, similar to a process well established for cells, termed "painting." The aim of the study was to assess the feasibility of modification of retroviral vectors by exogenous addition of recombinant protein, removing the need for genetic engineering of virus producer cell lines. The recombinant GPI protein CD59his was purified *via* fast protein liquid chromatography and associated with concentrated virus stock in a controlled incubation procedure. Reaction mixtures were purified in order to remove nonassociated GPI protein and endogenous protein. Analysis of samples by immunoblotting revealed that CD59his was only detectable in the presence of viral particles. From this, we conclude that CD59his could be stably associated with retroviral particles. In addition, we demonstrated by flow cytometry that virus particles remain infectious after these procedures. As well as suggesting a novel possibility for interaction between enveloped virus and host, we believe that the stable association of recombinant GPI proteins to retroviral particles can be developed into an important tool for both research and clinical applications, especially in the fields of gene therapy and vaccine development.—Metzner, C., Mostegl, M. M., Günzburg, W. H., Salmons, B., Dangerfield, J. A. Association of glycosylphosphatidylinositol-anchored protein with retroviral particles. *FASEB J.* 22, 000–000 (2008)**

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Glycosylphosphatidylinositol (GPI) anchors are attached to protein precursors at the ER membrane by the transamidase enzyme complex and delivered to the outer leaflet of the plasma membrane. GPI-linked proteins serve a number of different functions, for example, in the regulation of complement activity (CD59, CD55) or as hydrolytic enzymes (alkaline phosphatase, renal dipeptidase). GPI anchors consist of a hydrophilic oligosaccharide and a lipophilic fatty acid part. The lipophilic component can mediate the reinsertion of purified GPI proteins into lipid membranes, for example, the membrane of other cells, in a process termed "painting" (1). Transfer of GPI-anchored proteins from cell to cell has been reported in a variety of *in vivo* and *in vitro* systems (2, 3). In addition, it has been demonstrated that GPI anchors can be added to previously non-GPI-linked proteins [*e.g.*, green fluorescent protein (GFP)] by addition of a GPI signaling sequence (GSS) to their C-terminal end and that these proteins retain their biological functions (4–6). Reinsertion of purified GPI-linked proteins has been demonstrated on cells (1, 4–6) and artificial lipid membranes (7, 8). For this "painting" process, purified GPI extracts are mixed with lipid membranes and incubated at 37°C for 2 to 24 h with occasional shaking. In this work, we describe procedures to modify the lipid envelope of retroviral (RV) and lentiviral (LV) vectors by exogenous addition of recombinant GPI protein directly onto the viral particle. Virus remains infectious after these procedures. Uptake of GPI-anchored proteins from the surrounding fluid phase might constitute a novel form of virus-host interactions. In addition, exogenous modification of RV particles can be a valuable tool for molecular manipulation of virions. Potential applications include novel targeting strategies for gene therapy, immune modulation (9, 10) (*e.g.*, enhancement of vaccine efficacy by immune stimulation, protection of RV gene therapy vectors by immune inhibition), or implementation of pharmacogenetics in RV gene therapy by quick adaptation to different therapy requirements in patient subgroups. In addition to potential clinical uses, virus painting provides a quick way to specifically tag and modify viral envelopes for research purposes, for example, using GPI-linked GFP (4).

Our model GPI-linked protein CD59 (protectin, MACIF, SwissProt accession no. P13987) has been widely studied and was also shown to confer partial

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resistance against human complement to modified murine leukemia virus (MLV) particles after transfection of producer cells with CD59 (11), implying that RV particles can accommodate GPI-linked proteins. To facilitate purification and analysis of CD59, six consecutive histidine residues were introduced directly at the N terminus of the mature protein (*i.e.*, after signal peptide cleavage). These histidine tags are also potentially useful in downstream applications; after painting of virions with CD59his, they can be associated with magnetic nanoparticles to allow for easy purification and concentration. In addition, magnetic virus could be targeted by magnetic fields or used for magnetothermic therapy (12, 13), thus creating a new link between protein engineering and nanobiotechnology. Earlier studies demonstrated the usefulness of magnetic particles in antitumor strategies (14, 15).

MATERIALS AND METHODS

Plasmids and cell lines

CrFKCD59hisneo cells expressing the recombinant CD59his were derived from parental CrFK cells by lipofection using Lipofectin reagent (Invitrogen, Carlsbad, CA, USA) with pCD59hisneo. For generation of pCD59hisneo, a polymerase chain reaction (PCR) fragment derived from cDNA [using primers CD59 (2)FK*Hind*III 5′cacgacaagcttaccatgggaatccaaggagggtctgtcctgtt3′ and CD59 (2)R*Apa*I 5′atgacgggcccttagggatgaaggctccaggctgctgccagaa3′] from human embryonic kidney (HEK) 293 cells was cloned into the expression vector pcDNA3. The histidine tag was introduced by a two-step mutagenesis PCR protocol, using first two primer pairs [CD59 (2)FK*Hind*III and CD59RHis 5′gtgatggtgatggtgatggctatgacctgaatggcagaag3′; CD59FHis 5′catcaccatcaccatcacctgcagtgctacaactgtccta3′ and CD59 (2)R*Apa*I] in two different PCR reactions. Subsequently, a mix of both primary fragments was hybridized and amplified using primers CD59 (2)FK*Hind*III and CD59 (2)RApaI. The fragment was recloned into pCDNA3 using the *Hind*III and *Apa*I sites. 293gpalfpLXSNeGFP cells are derived from HEK293 cells and were generated in house (16–18). STAR-A-HV cells (19) are derived from HEK293T cells and were used with kind permission from Dr. Mary Collins.

Purification of protein

Four to six confluent T175 flasks of CrFKCD59hisneo were harvested by scraping after washing cells with 10 ml PBS. Cells were scraped into a total of 25 ml sample application buffer (50 mM Tris HCl, 50 mM NaCl, 35 mM imidazole, 0.5% sodium deoxycholate, and 1% Nonidet P-40, pH 7.4). Eighty microliters of protease inhibitor complex (Sigma-Aldrich, St. Louis, MO, USA) was added before sonification of samples for 30 s. Samples were incubated for 30 min on ice before centrifugation for 30 min at 3000 *g*. Samples were filtered through 0.45-μm filters (Sarstedt, Nümbrecht-Rommelsdorf, Germany) before application to an ÄktaPrime plus fast protein liquid chromatography (FPLC) device (GE HealthCare, Chalfont St. Giles, UK). Prepacked 5-ml HisTrap FF Crude columns (GE HealthCare) were used. Samples were washed using washing buffer (50 mM Tris HCl, 50 mM NaCl, and 35 mM imidazole, pH 7.4) and eluted from columns by elution buffer (50 mM Tris HCl, 50 mM NaCl, and 600 mM imidazole, pH 7.4). Fractions were collected during elution. Presence of CD59his in fractions was determined by immunoblotting. Positive fractions were pooled and concentrated by ultrafiltration using Amicon Ultra filter devices (Millipore, Billerica, MA, USA; 5 kDa molecular mass cutoff). Samples were washed twice with 5 ml painting buffer (50 mM Tris HCl and 50 mM NaCl, pH 7.4). Concentrations were measured using the DC protein assay (Bio-Rad, Hercules, CA, USA).

Concentration of virions

Culture supernatants were filtered through 0.45-μm filters (Sarstedt) before ultracentrifugation for 2 h at 20,000 rpm at 4°C in a Beckman XL-70 ultracentrifuge using an SW28 rotor (Beckman Coulter, Fullerton, CA, USA). Pellets were resuspended in Dulbecco modified eagle medium (DMEM; Invitrogen).

Painting procedure

For painting, concentrated supernatants derived from of 2–6 T175 culture flasks were incubated with purified protein at final concentrations between 20 and 100 ng/μl or painting buffer alone. Incubation was carried out at 37°C, in 5% CO_2 under constant shaking. Incubation times were 3 h (infection experiments) to ~21 h (standard experiments).

Postpainting treatment

Painting samples were diluted by addition of 34 ml of DMEM and ultracentrifuged as described above. Samples for determination of CD59his content were pretreated using the MagneHis kit (Promega, Madison, WI, USA).

Product-enhanced reverse transcriptase (PERT) assay

PERT assay was carried out as described previously (20). In brief: 20 μl of concentrated supernatants from virus producer cells was mixed with an equal amount of disruption buffer [40 mM Tris Cl, pH 8.1; 50 mM KCl; 20 mM dithiothreitol (DTT); and 0.2% Triton X-100] and incubated at room temperature for 2 min to lyse the virions. Starting from this dilution, 1:100 and 1:1000 dilutions were made from each sample. As a standard, a serial dilution of purified MoMLV RT (Promega) in RT dilution buffer (20 mM Tris Cl, pH 7.5; 50 mM KCl; 0.25 mM EDTA, pH 8.0; 0.025% Triton X-100; 50% glycerol; and 0.2 mM DTT) was used. In the RT step of the assay, 10 μl of either standard, sample, or negative control was incubated with 20 ng of brome mosaic virus (BMV) RNA for 1 h at 37°C. BMV DNA, generated during the RT step, was quantified by real-time PCR. PCR was performed with a 7500 PCR Detection System (Applied Biosystems, Foster City, CA, USA).

Immunoblotting

Samples were electrophoretically separated on precast 4–12% gradient gels (Invitrogen) before electroblotting (1.1 mA/cm^2) onto polyvinylidene difluoride (PVDF) membranes (GE HealthCare). Monoclonal antiCD59 was purchased from AbD Serotec (Oxford, UK). Mouse anti-human HIV-1 p24 was a kind gift from Polymun Scientific (Vienna, Austria). Rat anti-MLV capsid antibody was purified by Biomedica (Vienna, Austria). Horseradish peroxidase (HRP) -conjugated anti-rat and anti-mouse secondary antibodies were purchased from Dako (Glostrup, Denmark). Signal detection was carried out using the ECLplus kit (GE HealthCare).

Silver staining

Silver staining of protein extracts was carried out as described previously (21). In brief: after fixing and washing, the polyacrylamide gels were sensitized in a 0.02% sodium thiosulfate solution for 1 min. An aqueous 0.1% silver solution was used for the incubation before development in a sodium carbonate/formaldehyde solution. Color development was stopped by washing in 5% acetic acid in water.

Infection and flow cytometry

For infection, 8–9 $\times$ 10^5 HeLa target cells were seeded 6 h prior to infection in 6-well plates. Virus supernatants after postpainting ultracentrifugation were diluted to 1 ml with DMEM supplemented with 10% fetal calf serum (FCS; Invitrogen) and 10 μl/ml polybrene (0.8 μg/μl). After 36 h, supernatants were saved for analysis of CD59his content. Cells were trypsinized, fixed, washed 2 times in PBS, and analyzed for expression of GFP in a FACsCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA) using CellQuest software.

RESULTS

Association of CD59his with RV and LV vectors

In a first set of experiments, supernatants from the stable LV producer cell line STAR-A-HV (19) and the MLV-based RV producer cell line 293gpalfpLXSNeGFP (16–18) were harvested, filtrated, and concentrated by ultracentrifugation before incubation with CD59his at final concentrations between 20 and 100 ng/μl for 21–24 h at 37°C and 5% CO_2. To separate potentially painted virus from free GPI-linked proteins, samples were ultracentrifuged after painting. To allow for the differentiation between recombinant CD59his and endogenous CD59 present on virus producer cells, samples were subjected to purification with Ni magnetic particles after painting and ultracentrifugation to remove endogenous CD59 derived from producer cells (for an overview of procedure see **Fig. 1*A***). CD59his was detected only in samples containing virus and purified CD59his, suggesting that both constituents are necessary (Fig. 1*B*, lane VP+). No influence on painting was observed as a result of the media used (Fig. 1*B*, lane ME−) or the parental (non-virus-producing) cells (Fig. 1*B*, lane PC+) used. However, if cells undergo considerable stress, *i.e.*, overgrowing in culture, prominent amounts of nonviral membrane vesicles can be shed (22), leading to the potential for painting of these exosomal bodies as well (data not shown). In addition, postpainting procedures were sufficient to remove unpainted CD59his (seen by the absence of a signal for CD59his in the ME+ sample, Fig. 1*B*), as well as endogenous CD59 (seen by the absence of a signal for CD59 in the PC- and VP-samples, Fig. 1*B*).

Specificity of virus painting

Proteins may stick to viral envelopes regardless of GPI anchoring in a nonspecific manner. To exclude this possibility, we analyzed the protein content of the samples directly after painting and after the postpainting ultracentrifugation. A high amount of protein carryover would suggest low specificity of the painting process. However, silver staining of painted samples before and after purification *via* ultracentrifugation showed that the majority of proteins are removed in the purification step (Fig. 1*C*). In addition, we added rat IgG at the same levels as CD59his to the painting reaction. IgG was not retained by the virus as the CD59his was (Fig. 1*C*). This indicates that the process is at least semispecific, being restricted to proteins with highly hydrophobic residues.

Infectivity of viral particles postpainting

Optimization was carried out to determine the minimal incubation time necessary for the interaction of virions and CD59his. Preliminary results suggested that an incubation time of 3 h is sufficient for maximal viral painting (data not shown). Using the minimal incubation time, we repeated the painting experiments to assess infectivity of painted virus. HeLa cells were infected with painted virions and analyzed by flow cytometry 36 h postinfection. After infection, supernatant was collected and analyzed for CD59his to confirm painting (**Fig. 2**). Painted virus remains infectious, however, at reduced levels. Differences in infectivity between samples that received CD59his and mock-painted samples that did not receive GPI proteins are small (Fig. 2, compare samples VP− and VP+). The difference of infectivity to samples before painting was comparatively large (~15-fold, Fig. 2, compare samples VP, VP−, and VP+). This indicates that the reduction in infectivity is rather a result of the duration of the process than the process itself and suggests that further optimization could improve the infection efficiency.

Quantification of virion painting

In addition, we were interested in the stoichiometry of the process, especially the numbers of GPI proteins incorporated per virus particle, a parameter termed the density *D* (**Fig. 3**). Results for the experiments depicted in Fig. 1*B* suggested that between 2 and 150 molecules (LV and RV, respectively) can be found per virion (**Table 1**). Calculations are based on viral titers determined by PERT and by determination of painting efficacy from immunoblots. The density values, however, reflect only rough estimates of the numbers of GPI molecules per virion.

DISCUSSION

Our results clearly demonstrate that exogenously added recombinant CD59his associates with concentrated RV and LV particles. The association of virions and GPI-linked protein is carried out at near-physiological conditions (*i.e.*, at 37°C, 5% CO_2, and in a buffered

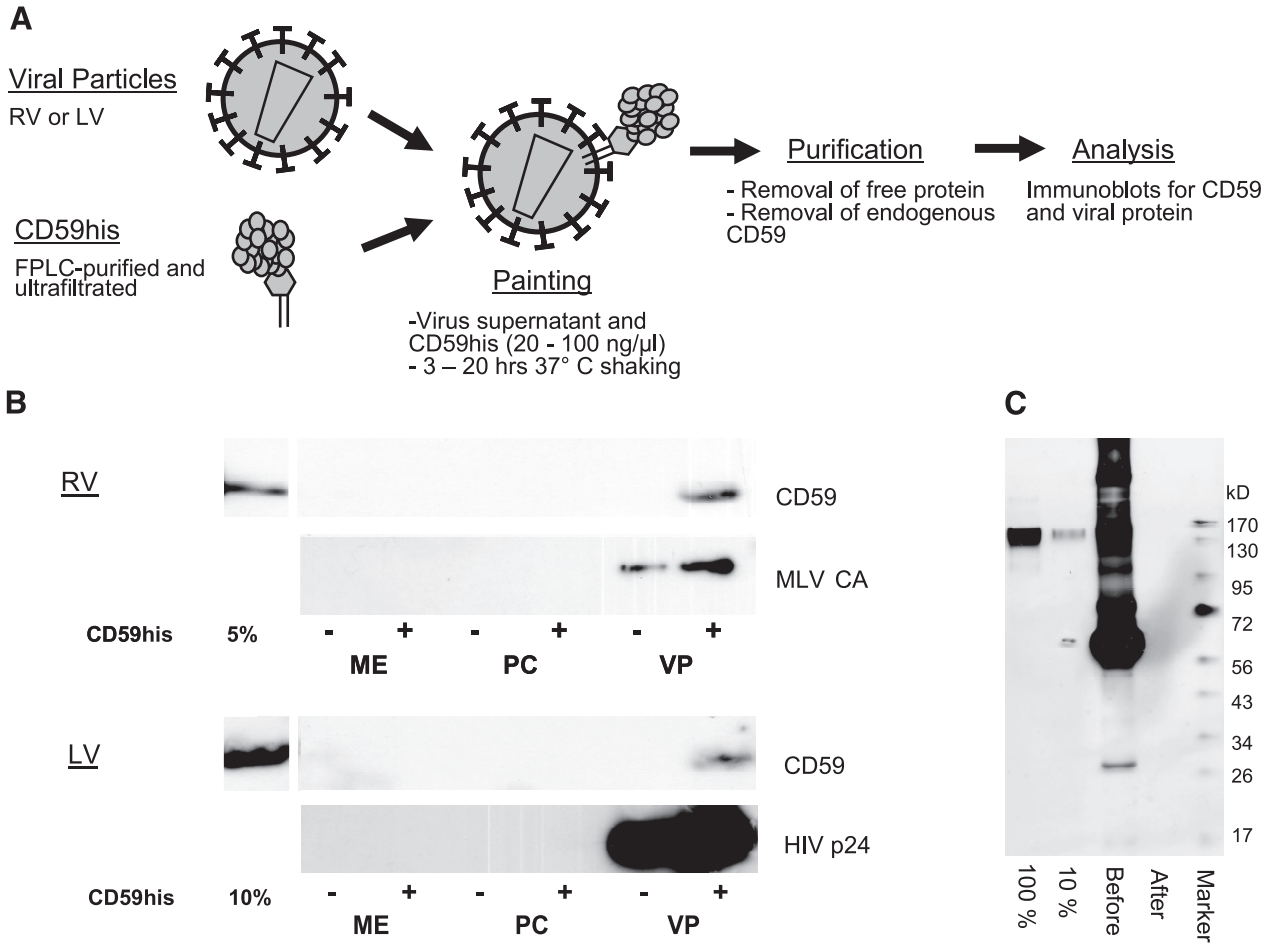

Figure 1. Painting of RV and LV vector particles. *A*) Overview: concentrated supernatants from RV or LV producer cell lines (293gpalfpLXSNeGFP and STAR-A-HV, respectively) are incubated with purified and concentrated CD59his for 3–20 h at 37°C under constant shaking. After incubation, samples are purified by ultracentrifugation (2 h, 20,000 rpm, 4°C) to remove non-virus-associated proteins. Before analysis of CD59his, endogenous CD59 is removed by using magnetic nickel beads. Samples were analyzed by immunoblotting using antibodies directed specifically against CD59, MLV capsid (CA), and HIV-1 p24. *B*) Analysis of painted retrovirus: concentrated supernatants from parental cells (PC) and virus-producing cells (VP) were incubated in the presence or absence of CD59his for 21 h at 37°C under constant shaking. In addition, cell culture medium (ME) was also incubated under the same conditions. After purification as detailed above, cells were analyzed by immunoblotting. Results show that CD59his is only retained during purification in the presence of virus and CD59his (RV and LV, top panels, respectively; compare lanes VP− and VP+), indicating association of the protein with viral particles. On the same gels, either 5 or 10% of the amount of CD59his used for painting was loaded to assess efficiency of painting. Levels of viral gag proteins are shown *via* immunoblots using MLV capsid and HIV-1 p24 antibodies. Viral proteins are only present in supernatants derived from viral producers. (RV and LV, bottom panels, respectively; compare lanes VP− and VP+). *C*) Specificity of painting: concentrated viral supernatant were mixed with CD59his and the same amount of a non-GPI protein (rat IgG; molecular mass 150 kDa). The sample was incubated for ~21 h. Aliquots were taken before and after ultracentrifugation and silver stained to assess protein content; 100 and 10% of the used amount of IgG was loaded for comparison. Ultracentrifugation removes the majority of total protein, as well as the IgG contaminant.

nutrient solution). This suggests that virions might be capable of uptake of GPI-linked proteins from the surrounding medium *in vivo*, similar to the transfer of GPI-linked proteins between cells (2, 3). Enveloped virus might exploit this feature as a means of immune evasion, especially in cross-species infection. The uptake of host GPI proteins, *e.g.*, complement regulatory factors such as CD55 [decay accelerating factor (DAF)] and CD59 might efficiently protect viral particles from the host's immune system after zoonotic infection.

Data from immunoblotting suggest that the efficacy of the incorporation of GPI-linked proteins by viral particles allows for the association of 1–5% of the total amount of CD59his (Fig. 1*B*, compare 5 and 10% lanes, respectively, with corresponding VP+ lanes), leading to estimates of 2–150 molecules of CD59his per virion (Table 1). These numbers are approximately in the same range as estimates for the number of viral surface envelope glycoprotein molecules (23–25). Therefore, we believe that the observed levels will be sufficient to elicit biologically relevant effects. The vast majority of proteins are removed by postpainting ultracentrifugation; this is strong evidence that the process is specific. Our control protein (rat IgG) is a soluble protein of

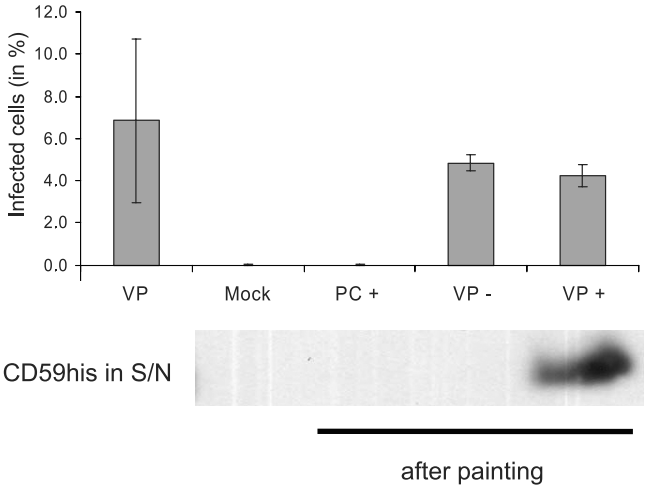

Figure 2. Infectivity after painting. Virus supernatants postpainting are purified by ultracentrifugation and used to infect target HeLa cells. After 36 h, supernatant is removed and analyzed by immunoblotting for presence of CD59his to confirm painting. Cells were fixed and washed before analysis by flow cytometry. No infection was observed in HeLa cells treated with medium (mock) or supernatant from parental cells that was incubated with CD59his (PC+). Virus supernatant in the absence or presence of CD59his (VP−; VP+) shows infection rates of ~4%. Supernatants treated with CD59his performed slightly worse in infection experiments. As an infection control, viral supernatant saved before painting (VP; 0.1 of the volume used for infection after painting) was used. Infectivity was reduced significantly after painting. Error bars represent means ± SD.

hydrophilic character with consequentially low affinity for hydrophobic surfaces. The possibility remains that proteins with substantial hydrophobic sequence motifs, *e.g.*, transmembrane proteins, could show comparable behavior to GPI proteins. However, when GPI-anchored proteins are purified prior to painting, this should not pose any problems. Virions remain infectious after painting and postpainting treatments (Fig. 2). Infectivity was reduced when compared to infection levels before painting. However, when comparing infection levels for GPI-associated virions to nonassociated virus particles, only a small difference was observed (Fig. 2, compare lanes VP− and VP+). This suggests that the duration of the process (5–6 h in total) rather than the painting process itself is responsible for the decrease in infectivity. Using shorter incubation times for painting and alternative purification procedures postpainting, *e.g.*, ultrafiltration or magnetic purification, we could decrease handling times, leading to higher infection rates.

$$D = k \times (M \times E_A)/N_V$$

$$\text{For } k = (E_P \times N_a)/(M_W \times 10E9)$$

D	Density (molecules per virion)
k	Constant factor k
M	Total amount of protein used (in ng)
E_A	Efficiency of association (ratio associated/total GPI molecules)
N_V	Number of virus particles
E_P	Efficiency of protein purification (ratio desired molecules/total molecules)
N_a	Loschmidt or Avogadro constant (6.022x10E23)
M_W	Molecular weight of GPI protein (in Dalton)

Figure 3. Calculation of painting density. The density of CD59his molecules per virion is dependent on the amount of CD59his M, the efficacy of the association process E_A, and the number of virions N_V, determined by PERT assay. The constant factor k contains the parameters supposed not to change between experiments, such as the molecular mass M_W of the GPI protein, the efficacy of purification E_P, and Avogadro's number N_a. The given calculations yield a rough approximate of the densities. See Table 1 for an example.

TABLE 1. *Painting density values and results of calculation*

Parameter	RV	LV
M (ng)	36,696	18,348
N_V	2.90E + 08	2.43E + 09
E_A	0.04	0.01
D	~150	~2

Calculated for $k = 3.01 \times 10E7$. Experimental parameters from Fig. 1; see Fig. 3 for description of parameters.

Another advantage of using GPI anchors for modification of proteins is that the amino acid sequence of the mature protein is not altered, increasing the possibility for functionally intact proteins. The addition of the hydrophobic moiety might, however, influence folding of the recombinant GPI protein, thereby destroying conformational epitopes or the active centers of enzymes. Moreover, preliminary experiments suggest that levels of painting increase with an increase in the numbers of virions and GPI protein amount. This indicates that the amount of GPI proteins after painting on virions is controllable.

There are a number of potential uses for this technique. The addition of immune-stimulatory GPI-linked factors could enhance the efficacy of vaccination (10) or provide an important added benefit for viral suicide gene therapy. Immune inhibitory molecules might help to protect viral gene transfer vectors from unwanted or premature immune responses. Quite recently, it has been shown that viruses outfitted with GPI anchor-fused proteins can be used to manipulate cells in such a way that they undergo differentiation (9), demonstrating that viral particles containing recombinant GPI-linked proteins may elicit specific responses from target cells. The inherent flexibility of a modular system (*i.e.*, adding different types of GPI-linked molecules to viruses directly prior to use) would allow for quick and adaptive responses to therapy needs, for example, in response to genetic heterogeneity in patients. Viral painting also allows for the direct labeling of viral envelope membranes, thus facilitating visualization of virions. In addition, we believe that our results may be easily transferable to other enveloped viruses (*e.g.*, influenza or hepatitis B virus) thereby further increasing the range of potential applications.

Although the histidine tags are engineered into CD59his mainly for the purpose of easy purification, the tags can also be used for linkage of magnetic particles to CD59his and subsequently to virus particles, thus providing a platform that combines retrovirology

and nanotechnology. Magnetic retrovirus could be easily concentrated and purified *via* exposure to magnetic fields (12). In addition, magnetic fields could be used for targeting particles and inducing hyperthermia (13–15). Taken together, the direct transfer of GPI-linked his-tagged proteins onto enveloped viral particles is a potentially valuable tool for a variety of both research and clinical applications. FJ

STAR-A-HV cells were used with kind permission from Dr. Mary Collins. The p24 antibodies were a kind gift from Polymun Scientific (Vienna, Austria). PERT assays were performed by Reinhard Ertl.

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