Minimal exclusion of plasma membrane proteins during retroviral envelope formation

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The retrovirus forms its envelope by budding at the plasma membrane (PM). This process is primarily driven by its cytoplasmic core-precursor protein, Gag, as shown by the efficient formation of virus-like Gag particles in the absence of its envelope protein, Env. Most interestingly, several studies have demonstrated incorporation of various PM proteins into retrovirus, but the underlying mechanism of this phenomenon has remained elusive. We have purified Moloney murine leukemia virus Gag particles by sedimentation in an iodixanol gradient and donor PMs by flotation in a sucrose gradient and compared their protein compositions at equal lipid basis. We found that most PM proteins are present at similar density in both membranes. The inclusion of PM proteins was unaffected by incorporation of Env protein into the envelope of the Gag particles and whether these were produced at high or low level in the cells. These findings indicate that most PM proteins become incorporated into the retrovirus envelope without significant sorting. This feature of retrovirus assembly should be considered when studying retrovirus functions and developing retrovirus vectors.

According to a prevailing model, virus-specific membrane proteins are incorporated into the viral envelope by means of specific interactions with the viral core, whereas host membrane proteins, lacking possibilities to undergo such interactions, will be excluded (1). Although, experimental results support the validity of this model for some viruses—e.g., the alphaviruses (2)—it is not applicable to others—e.g., the retroviruses. In particular, several studies with HIV-1 suggest that many plasma membrane (PM) proteins of the host become incorporated into the viral envelope. These proteins include cell adhesion molecules such as CD44, LFA-1, and ICAM-1 and the antigen presenters HLA-I and II (reviewed in ref. 3). Similarly, studies with several different retroviruses show that they can be pseudotyped with envelope proteins of nonrelated viruses if the latter are expressed at the PM of the host cell (reviewed in refs. 4 and 5). These phenomenon may be related to the fact that retrovirus budding is not, like that of alphavirus, dependent on core–envelope protein (Env) interactions but depend on interactions of core proteins alone (reviewed in ref. 5). Thus, expression of the *gag* gene—i.e., the gene encoding the internal core protein (the Gag precursor)—in the absence of other viral genes results in formation of retrovirus-like Gag particles (reviewed in ref. 6). This Env-independent budding might favor host protein incorporation into the retrovirus envelope. However, the exact mechanism for the incorporation is still unclear. In particular, it is not known whether only certain or most PM proteins are incorporated into the retrovirus envelope and whether that incorporation occurs passively. To characterize this process it is necessary to compare the densities of PM proteins in the donor PM of the host cell and in the envelope of the retrovirus. Here we present such a study with Moloney murine leukemia virus (Mo-MuLV) Gag particles.

Materials and Methods

Cell Culture. BHK-21 baby hamster kidney cells were grown as described (7). MOV-3 mouse fibroblast cells (NIH 3T3 cells transformed with wild-type Mo-MuLV genome) were obtained from G. Schmidt (GSF-National Research Center for Environment and Health, Neuherberg, Germany) and grown as NIH 3T3 cells as described (8).

Virus, Vectors, and Infection. Semliki Forest virus (SFV) stocks were produced in BHK-21 cells transfected with RNA transcribed *in vitro* from plasmid pSP6-SFV4 (9). The SFV vector RNAs were transcribed *in vitro* from plasmids: pSFV-C/Pr65^{gag}, which contains a SFV *capsid*–Mo-MuLV *gag* fusion gene; pSFV-1/Pr65^{gag}, which contains the Mo-MuLV *gag* gene; pSFV-1/Pr65^{gag}+Pr80^{env}, which contains the Mo-MuLV *gag* and *env* genes in two separate transcription units; and pSFV-C/NP, which contains a SFV *capsid*–influenza virus A/PR/8 *nucleoprotein* (NP) fusion gene (10, 11). Infectious SFV vectors were produced by cotransfection of cells with vector RNA and helper 1 RNA as described (12). The titers of vector stocks were determined by indirect immunofluorescence using anti-Pr65^{gag} or -NP antibodies (7, 11). For infection, nearly confluent BHK-21 cells were incubated with SFV or SFV vectors (multiplicity of infection = 5–10) for 1 h at 37°C as described (7).

Metabolic Labeling. *Labeling with [^{35}S]methionine.* Cells were seeded in phosphate-free Dulbecco's modified Eagle's medium supplemented with L-arginine, sodium cystine, D-glucose, L-glutamine, *i*-inositol, L-leucine, and L-methionine, as recommended by the manufacturer (GIBCO/BRL/Life Technologies), and further with 5% FCS, 20 mM Hepes, and 1/10 of the regular concentration of sodium phosphate (low-phosphate medium). After 24 h, the medium was changed to a similar one, but with only 1/10 of the regular concentration of L-methionine (low-phosphate, low-methionine medium) and supplemented with 100 μCi/ml [^{35}S]methionine (Amersham; 1 μCi = 37 kBq) ([^{35}S]methionine labeling medium). The cells were labeled for 15 h and then infected with SFV or SFV vectors. After infection, labeling was continued for 3.5 or 5.5 h in fresh [^{35}S]methionine labeling medium. Particles were collected for 30 or 60 min by incubation in new [^{35}S]methionine labeling medium, the last 15 min in excess of unlabeled methionine (300 μg/ml).

Labeling with [^{32}P]orthophosphate. Cells were seeded in low-phosphate medium supplemented with 25 μCi/ml [^{32}P]orthophosphate (Amersham). After 24 h, the medium was changed to low-phosphate, low-methionine medium supplemented with 25 μCi/ml [^{32}P]orthophosphate ([^{32}P]orthophosphate labeling medium) and labeling was continued for 15 h. The

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Abbreviations: EM, electron microscopy; Mo-MuLV, Moloney murine leukemia virus; PM, plasma membrane; PL, phospholipids; SFV, Semliki Forest virus.

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cells were infected with SFV vectors for 1 h, and the labeling was continued in fresh [^{32}P]orthophosphate labeling medium for 3.5 or 5.5 h. Particles were collected for different periods in low-phosphate, low-methionine medium without further labeling.

Labeling with [^{3}H]uridine. Cells were grown in low-phosphate medium for 40 h, infected with SFV-C/Pr65^{gag} vectors for 1 h, and incubated in low-phosphate, low-methionine medium supplemented with 12.5 μCi/ml [^{3}H]uridine for 5 h. Finally, particles were collected in low-phosphate, low-methionine medium containing 10 μCi/ml [^{3}H]uridine for 1 h.

Isolation of Gag Particles and SFV. Media from [^{32}P]orthophosphate- and [^{35}S]methionine-labeled cells, respectively, were mixed with medium containing unlabeled carrier Gag particles (approximately 3–5 μg) and clarified by low-speed centrifugation. The particles were isolated from the supernatant by either of two methods. (*i*) The medium was applied on top of 3 ml of 10% (wt/wt) sucrose in 50 mM Tris·HCl, pH 7.4/100 mM NaCl/0.5 mM EDTA (TNE) in a Beckman SW41 tube, and particles were pelleted by centrifugation for 1.5 h at 35,000 rpm and 4°C. (*ii*) Medium was applied on top of a 5–20% (5–30% for SFV) iodixanol gradient (wt/vol) (Optiprep, Nycomed Pharma, Oslo) in a Beckman SW41 rotor, and particles were sedimented for 1.5 h at 36,000 rpm and 4°C. The fractions (700 μl) were diluted 2-fold with TNE, and particles were pelleted by centrifugation in a Beckman JA18.1 rotor for 1.5 h at 17,000 rpm and 4°C. Alternatively, particle-containing fractions were identified by scintillation counting, pooled, and diluted 5-fold with TNE, and particles were pelleted by centrifugation in a Beckman SW41 rotor for 1.5 h at 35,000 rpm and 4°C.

Electron Microscopy (EM). EM analyses of negatively stained (2% uranyl acetate) particles and of ultrathin sections of pelleted Pr65^{gag}-enriched PMs were done as described (7). Cryosections of MOV-3 cells and infected BHK-21 cells and subsequent labeling with biotinylated concanavalin A (Con A)/mouse anti-biotin monoclonal antibody/rabbit anti-mouse IgG/10-nm protein A-gold conjugate was performed as described (13). The biotinylated Con A and the corresponding antibody were from Sigma. The number of gold particles labeling the PM and the viral membrane was systematically sampled. The length of the membrane profile was estimated by intersection counting (14). The formula used was Q/Id, where Q is the number of gold particles, I is the number of intersections, and d is the distance between the test lines (1.13 μm).

Other Methods. Homogenization of cells and isolation of Pr65^{gag}-containing PMs by flotation in a sucrose step gradient was done as described (7). Peak fractions, identified by scintillation counting, were pooled, diluted in 10 mM Tris·HCl, pH 7.4, and pelleted by centrifugation in a Beckman SW41 rotor for 1 h at 35,000 rpm at 4°C. Chloroform/methanol extraction, protein analyses by 6–15% gradient SDS/PAGE, quantification of radioactivity in protein bands, and Triton X-114 extraction of membrane proteins were done as described (7, 15, 16).

Results

Purification of Gag Particles. We used the SFV-C/Pr65^{gag} vector for expression of the *gag* gene of Mo-MuLV. This is an efficient RNA vector that takes over most of the translational activity of the cell. Therefore, to follow host proteins during budding of Gag particles we labeled cells with [^{35}S]methionine for 15 h before vector infection and continued labeling until particles were collected. This protocol ensured steady-state labeling of both host- and vector-specific proteins. Fig. 1 *Top* shows a protein analysis of all particles released from SFV-C/Pr65^{gag}-vector infected cells, separated in an iodixanol gradient. The most intensively labeled protein in the gradient is Pr65^{gag}. Its highest concentration is found in fraction 14. Of the total Pr65^{gag}, 71% is found in fractions 13–15. Most interestingly, there are several additional proteins accumulating in the same fractions. The second-most-abundant protein in the gradient is a 46-kDa protein. It is seen almost across the entire gradient with a predominance in its upper and middle parts. Furthermore, there are abundant 63- to 52-kDa proteins, which are seen in the top fractions of the gradient. This analysis suggests that several different kinds of particles have been released from the cells. Fractions 13–15, with the bulk of Pr65^{gag}, seem to contain one major class of Gag particles. Fractions 8–10, where the ratio of the 46-kDa protein to Pr65^{gag} is increased, might contain another class of Gag particles. Finally, the particles in the top fractions with the 63- to 52-kDa proteins seem to represent host-derived material. This interpretation was supported by morphological analyses using EM. The total extracellular particle preparation showed many particles that were heterogenous in size (diameter 40–370 nm) (Fig. 2 *Left*). In contrast, the particles in the pooled fractions 13–15 showed mostly spherical particles in the size range of the Mo-MuLV virion (diameter 80–130 nm) (Fig. 2 *Right*). These results suggest that the iodixanol gradient can be used for separation of retrovirus-like Gag particles from many other particles that are also released from the SFV-C/Pr65^{gag}-infected cells.

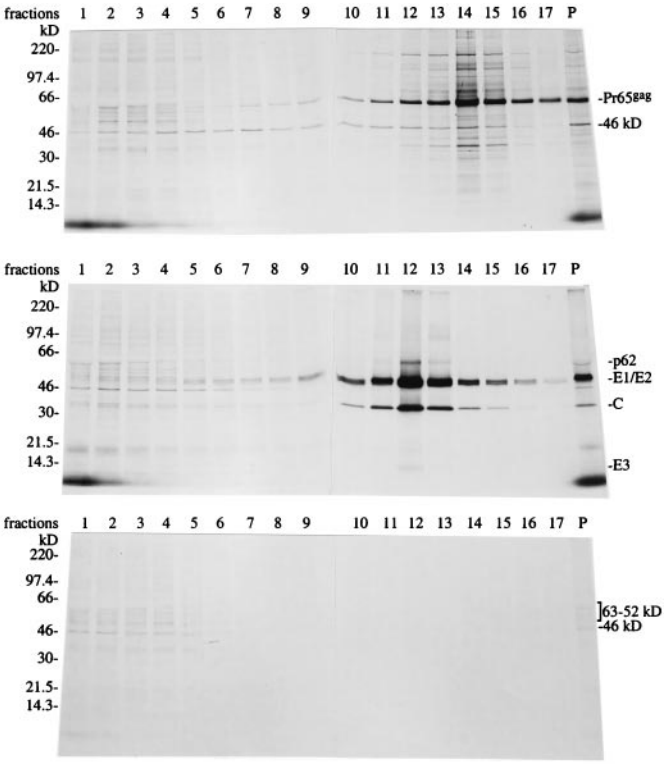

Fig. 1. Purification of Gag particles. (*Top*) Cells (4×10^6) were infected with SFV-C/Pr65^{gag} vectors and labeled with [^{35}S]methionine both before and after infection. Released particles in medium were collected between 5.5 and 6.0 h after infection and analyzed by sedimentation on a 5–20% iodixanol gradient. Particles were recovered from each fraction by pelleting and analyzed by SDS/PAGE. Autoradiographies of the gels are shown. Major proteins are indicated. P, pellet in gradient. (*Middle*) Cells were infected with SFV. Labeling of cells and particle analysis were as described above. Note that the iodixanol gradient was in this case 5–30%. (*Bottom*) Cells were infected with SFV-C/NP vectors. Labeling of cells and analysis of medium were as described for *Top*.

Host-Specific Proteins Are Incorporated into Gag Particles. The additional proteins that cofractionated with the Pr65^{gag} in retro-

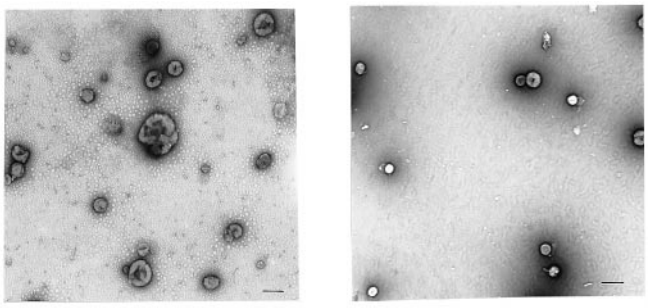

Fig. 2. EM analyses of released particles. Particles were produced as described in the legend to Fig. 1. (*Left*) Particles recovered from medium by direct pelleting through a sucrose cushion. (*Right*) Particles from fractions 13–15 of the iodixanol gradient in Fig. 1. (Bar = 200 nm.)

virus-like Gag particles could correspond to Pr65^{gag}-related oligomerization/degradation products, host-specific proteins of a contaminating particle population, or host-specific proteins incorporated into the Gag particles. The first possibility was tested in an experiment where we compared the protein profile of Gag particles produced in cells labeled with [^{35}S]methionine, before and after vector infection, to that of particles obtained from cells labeled only before infection. In the former case both SFV-C/Pr65^{gag}-vector and host-specific proteins should be labeled and in the latter case only host-specific ones. The results are shown in Fig. 3. The Pr65^{gag} was a prominent band in particles isolated from cells labeled both before and after vector infection (lane 1), whereas it was absent from the particles isolated from cells labeled only before infection (lane 2). Two other bands were also missing from the latter particles, namely one 33-kDa and one 38-kDa protein. The 33-kDa protein fits the size of the SFV C protein which was also produced by the vector. The 38-kDa protein could be Pr65^{gag}-derived or, alternatively, a host protein induced by vector infection. We conclude that other proteins in the Gag particles are host-specific.

The question whether the host proteins are constituents of a contaminating particle population or incorporated into the Gag particles was studied by analyzing particles released from cells that were infected with either wild-type SFV or another SFV vector, SFV-C/NP, which carries the NP gene of influenza virus A. The SFV particles are known to be virtually free from host-specific proteins, and influenza NP protein (56 kDa) cannot bud when expressed alone, but accumulates in the cell nucleus (2, 11). A sedimentation analysis of particles released from SFV-infected cells is shown in Fig. 1 *Middle*. The SFV particles, which contain the C protein, the almost comigrating spike subunits E1 and E2, and the small E3 protein as well as a small amount of the E2 and E3 precursor protein p62, peak in fractions 11–13. It is evident that there are no host-specific proteins that follow the SFV particles in the gradient. A similar analysis of the medium of SFV-C/NP-infected cells shows complete absence of protein-containing particles in the lower part of the gradient. However, both SFV- and SFV-C/NP-infected cells released slowly migrating particles with protein profiles similar to the corresponding particles from SFV-C/Pr65^{gag}-infected cells. These results show that SFV or SFV-vector-infected cells do not produce particles that sediment like Gag (or SFV) particles. Consequently, the host proteins cofractionating with the Gag particles must be constituents of these rather than contaminating particles.

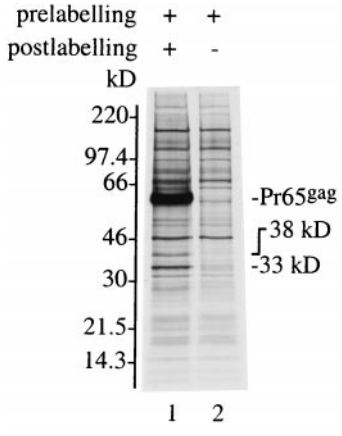

Fig. 3. Host-specific proteins cofractionate with Gag particles. Two cultures were infected with SFV-C/Pr65^{gag} vectors in parallel. One was labeled both before and after infection and the other one only before infection. The retrovirus-like Gag particles, collected 5.5–6.0 h after infection, were purified as described in the legend of Fig. 1 and analyzed by SDS/PAGE. Vector-specific proteins are indicated.

Isolation of Donor PMs for Gag Particles. We have earlier described a microsome flotation procedure for isolation of a Pr65^{gag}-enriched (sub)fraction of the PM, which might be used for Gag-particle formation (7). This possibility was confirmed by a morphological characterization of these membranes by EM. This technique showed vesicular structures, many of which contained budding profiles of Gag particles (Fig. 4). These budding profiles were easy to identify by their size (100–150 nm) and characteristic multilayered surface structure.

Quantification of Phospholipids (PLs). We chose to measure surface areas of Gag-particle envelopes and PMs on the basis of their PL content. For this purpose the PLs of BHK-21 cells were steady-state labeled with [^{32}P]orthophosphate, and the cells were then used for production of ^{32}P-labeled Gag particles and isolation of ^{32}P-labeled PMs. The preparations were solubilized in an excess of hot SDS, and labeled material was separated by SDS/PAGE (20%). Both PM and Gag-particle preparations (Fig. 5, lanes 1 and 2) give one broad heavily labeled band in the separating gel, a faint band in the gel front, two or three very faint bands migrating slower than the broad band, and bands at the top of the separating and stacking gels. Control analysis using radioactively labeled orthophosphate (lane 3), PLs (lane 4), and RNA (lanes 5 and 6) showed that the material in the front was free orthophosphate, the broad band was PLs in SDS micelles (18 kDa) (17), and the material at the top of the separating and

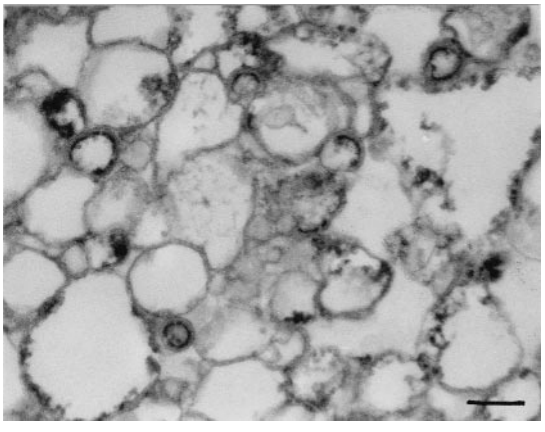

Fig. 4. EM analysis of Pr65^{gag}-enriched PMs. Cells were infected with SFV-C/Pr65^{gag} vectors and homogenized, and microsomes were separated by flotation in a sucrose step gradient. The figure shows a section of Pr65^{gag}-enriched PMs. (Bar = 200 nm.)

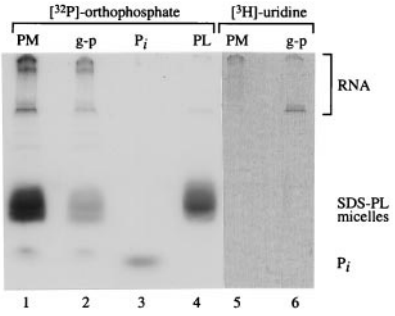

Fig. 5. Separation of PLs in PM microsomes and Gag particles by SDS/PAGE. Shown are SDS/PAGE analyses of ^{32}P-labeled PMs (lane 1) and Gag particles (g-p) (lane 2) isolated 5.0–6.0 h after infection, [^{32}P]orthophosphate (P_i) (lane 3), and ^{32}P-labeled PLs, extracted by chloroform/methanol from ^{32}P-labeled PMs (lane 4). Also shown are PM and Gag particles produced in [^{3}H]uridine-labeled cells (lanes 5 and 6).

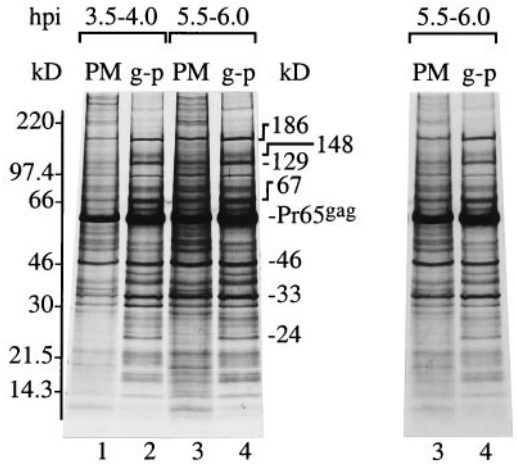

Fig. 6. Sorting of host proteins during budding of Gag particles. ^{35}S-labeled Gag particles (g-p) and PMs were adjusted to contain an equal amount of membranes and then analyzed by SDS/PAGE. Samples in lanes 1 and 2 are from a particle production between 3.5 and 4.0 h after infection and those in lanes 3 and 4 are from a production between 5.5 and 6.0 h after infection. Host proteins in Gag particles are indicated. (*Right*) A shorter exposure of the gel analysis of the samples from the later collection period.

stacking gels was RNA. Any ^{32}P-labeled proteins were expected to migrate slower than the PLs and might correspond to the two or three very faint bands in the upper part of the separating gel. Thus, this simple procedure allowed accurate quantitation of PL content in PM and Gag-particle preparations.

Sorting of PM Proteins During Budding of Gag Particles. The behavior of the PM proteins during budding of Gag particles was studied as follows: Gag particles were produced in two cultures under identical conditions. One was labeled with [^{35}S]methionine (both before and after infection with SFV-C/Pr65^{gag} vectors) for isolation of Gag particles and PMs with labeled proteins, and the other one with [^{32}P]orthophosphate for isolation of Gag particles and PMs with labeled PLs. The ^{32}P-labeled PM and Gag-particle preparations were subjected to SDS/PAGE for quantification of total PLs, and the values obtained were used to normalize the membrane contents of the ^{35}S-labeled PM and Gag-particle preparations to each other. The labeled proteins were then analyzed by SDS/PAGE. As we were concerned about the increased accumulation of Pr65^{gag} that occurs with time in vector-infected cells, we performed all our experiments before 6 h after infection—i.e., 6–8 h before appearance of visible cytopathic effects in vector-infected cells. Furthermore, we used two different collection times for particles: one between 3.5 and 4.0 h after infection, when the Pr65^{gag} synthesis was still increasing in the cell, and another between 5.5 and 6.0 h after infection, when full *gag* gene expression had been achieved. Single, rather than double, labeling of cells with [^{32}P]orthophosphate and [^{35}S]methionine was used in these experiments because the intensively labeled PLs interfered with the weaker ^{35}S label in the proteins. A drawback of the separate labeling protocol was that the membrane equalization became dependent on the reproducibility of yields of PMs and Gag particles in the parallel purifications. Separate tests showed that the variability of yields was within 10% for both preparations when these were isolated at the later time point and within 25% when prepared at the early time point.

An examination of the protein profiles of Gag particles collected at the early time period and of particles collected at the later time showed that the protein compositions are virtually identical (Fig. 6, lanes 2 and 4). This result indicated that incorporation of host proteins into Gag particles was not significantly influenced by the increased accumulation of Pr65^{gag} in vector-infected cells. As steady-state labeling conditions were used in the experiment, it was possible to compare the amounts of host proteins in the particles to the amount of Pr65^{gag} by measuring their ^{35}S radioactivities. Quantification showed that the radioactivity of the most intensively labeled host proteins each constituted 3–5% of the Pr65^{gag} radioactivity. As Pr65^{gag} contains only about 1/5 of the average frequency of methionine residues in proteins (18), this result suggests that they each constitute 0.6–1.0% of Pr65^{gag} by mass. Most surprisingly, the protein composition of the PM preparations was found to be strikingly similar to that of the Gag particles (compare lanes 1 and 2, and lanes 3 and 4). This finding suggested that the majority of the PM proteins were included in the Gag particles. A closer examination revealed that all proteins of the Gag particles were present in the PM and hence must be considered as PM proteins. The PM preparations contained, in addition, some proteins that were absent from Gag particles, or alternatively, present in very low amounts. Examples of such were the proteins migrating at the top of the gel, between the 186-kDa and the 148-kDa proteins, and also between the Pr65^{gag} and the 46-kDa protein. As samples in lanes 1 and 2 and in lanes 3 and 4, respectively, had been equalized on the basis of their PL content, we could roughly assess the sorting of individual PM proteins into the envelope of the Gag particle. At the later time point (lanes 3 and 4) the majority of the shared bands appeared with equal intensities in the two samples, suggesting no sorting but passive inclusion in a nondiluting and nonconcentrating manner into the budding particles. A few, most notably the 24-kDa protein, but also Pr65^{gag} (as expected) and the 67-kDa and 148-kDa proteins, were more concentrated in the Gag particle than in the PM, suggesting that these were actively sorted into the envelope of the Gag particle. Still other proteins, already mentioned above, were excluded from the envelope of the Gag particle. At the earlier time point (lanes 1 and 2) more proteins appeared to be concentrated in the Gag particle during budding. However, this was not a constant finding. In some experiments sorting conditions similar to those found at the later time point were observed. These differences were probably due to variation in the yields of ^{35}S- and ^{32}P-labeled Gag particles. As already noted, the variation was larger for particle preparations collected at the early time than for those collected at the later time.

Most Host Proteins in Gag Particles Are Integral Membrane Proteins. To identify integral membrane proteins in Gag particles, we subjected particles to Triton X-114 extraction and subsequent phase separation. The result is shown in Fig. 7 *Left*. Except for

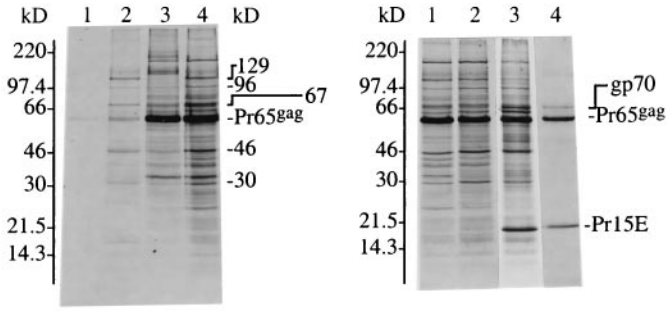

Fig. 7. Host proteins in Gag particle: Detergent binding and effect of Env coassembly. (*Left*) ^{35}S-labeled Gag particles were solubilized with Triton X-114, and protein partitioning was followed during detergent and aqueous phase separation. Unsolubilized material (lane 1) and solubilized material in the aqueous (lane 2) and detergent (lane 3) phases were analyzed by SDS/PAGE together with a sample of starting material (lane 4). Host proteins are indicated. (*Right*) Gag particles were produced in cells infected with SFV-1/Pr65^{gag}+Pr80^{env} and labeled both before and after infection (lane 3) or only after infection (lane 4). The particles were purified by sedimentation in an iodixanol gradient and analyzed by SDS/PAGE. Gag particles produced in cells infected with SFV-C/Pr65^{gag} (lane 1) and SFV-1/Pr65^{gag} (lane 2), respectively, were analyzed as controls.

a small amount of Pr65^{gag} (lane 1), all proteins of Gag particles were solubilized by Triton X-114. The majority of the PM-derived proteins partitioned preferentially into the detergent phase and hence were considered to be integral membrane proteins (lane 3). This was also the case with Pr65^{gag}. A few proteins partitioned preferentially into the aqueous phase (e.g., the 129-, 46-, and 30-kDa proteins) (lane 2), and some proteins distributed approximately equally between the two phases (e.g., the 96- and 67-kDa proteins). In the presence of high salt (0.5 M NaCl) the Pr65^{gag} distributed equally between the detergent and aqueous phase, whereas the other proteins partitioned as before (data not shown).

Incorporation of Env into Gag Particles Does Not Affect Host Protein Inclusion. To test the effect of Env on host protein inclusion into Gag particles we coexpressed the *gag* and *env* genes of Mo-MuLV by using an SFV-1 vector that carried separate transcription units for *gag* and *env*. As this SFV-1/Pr65^{gag}+Pr80^{env} vector drives 8-fold lower gene expression than does the SFV-C vector, we collected particles for a longer time—i.e., for 1 h between 5 and 6 h after infection (19). The SFV-1 vector was used, because high expression with the SFV-C vector caused most Pr80^{env} to form disulfide-linked aggregates (H. Andersson and H.G., unpublished results). Fig. 7 *Right*, lane 4, shows the ^{35}S-labeled proteins of Gag particles from SFV-1/Pr65^{gag}+Pr80^{env}-infected cells, labeled only after infection. Under these conditions only the Mo-MuLV-specific Pr65^{gag} and the Env subunits gp70 and Pr15E were seen. Quantification indicated that there was about one Env complex per five Pr65^{gag} molecules. When particles were produced in cells labeled both before and after vector infection, several additional proteins were seen (lane 3). The pattern of this was very similar to those of the host proteins in particles produced in cells infected with the high-level *gag*-expression vector, SFV-C/Pr65^{gag} (lane 1), and in particles produced in cells infected with a low-level *gag*-expression vector, SFV-1/Pr65^{gag} (lane 2). Note the 33-kDa protein in particles from SFV-C/Pr65^{gag}-infected cells. This is most likely vector-specific SFV C protein. We conclude that host proteins are not significantly excluded from the Gag particle by the simultaneous incorporation of the homologous Env.

Table 1. Quantitation of Con A labeling of PM with budding structures

Virus	Density, Au particles/μm: Viral membrane	PM
Mo-MuLV Gag particles	5.4 ± 0.9	3.3 ± 0.4
Mo-MuLV wild type	8.5 ± 0.8	2.3 ± 0.3
SFV	31 ± 10.1	3.8 ± 1.2

BHK-21 cells infected with SFV-C/Pr65^{gag}, MOV-3 cells, and BHK-21 cells infected with SFV were sectioned and labeled with Con A, and Con A was detected in EM using a combination of antibodies and protein A-gold conjugate. The number of gold particles per boundary length of the viral membrane (column 2) and PM (column 3) was estimated (n = 3; ±SD).

Cryo-Immuno-EM. To extend our biochemical data to the ultrastructural level we used cryo-immuno-EM. BHK-21 cells infected with SFV or SFV-C/Pr65^{gag} vectors and NIH 3T3 cells infected with wild-type Mo-MuLV (MOV-3 cells) were cut into ultrathin sections and labeled with Con A to detect glycoproteins. The labeling density was then calculated in parts of the PM that were, or were not, involved in viral budding. This was done by quantitative estimation of the number of gold particles per boundary length (μm). The results are presented in Table 1. The labeling density in budding-free regions of the PM is approximately the same in all infected cells (Table 1, column 3). A very similar labeling density is also found in membranes of budding Gag particles, whereas it is somewhat increased in those of budding Mo-MuLV and much higher in those of budding SFV (Table 1, column 2). Fig. 8 shows representative EM pictures of a budding Gag particle (*A*), an apparently released wild-type Mo-MuLV (*B*), and a budding SFV (*C*). We conclude that these results are consistent with our biochemical finding that PM proteins in general are included in the Gag particle during budding. The clear increase in Con A binding to SFV buds is most likely due to the high concentration of viral spike proteins. The less significant increase of label in budding Mo-MuLV might be due to recruitment of the glycosylated Env complexes to the particle.

Discussion

Our results show that most PM-associated proteins become passively incorporated into the envelope of Mo-MuLV Gag particles during budding. Surprisingly, coassembly of Env into the particles did not reduce their content of PM proteins. Insufficient Env incorporation appeared to be a simple explanation, but our analyses showed that the particles contained about one Env complex per five Pr65^{gag} molecules—i.e., about as much as previously reported for wild-type MuLV (20). Furthermore, we have recently demonstrated that Env becomes about 3-fold concentrated when sorted from the PM into the envelope of the Gag particle (M.H. and H.G., unpublished results). This finding suggests that passive incorporation of most PM proteins

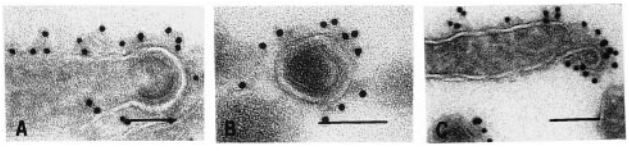

Fig. 8. Con A labeling of budding Gag particles. Ultrathin cryosections of cells infected with SFV-C/Pr65^{gag} vectors, wild-type Mo-MuLV, and SFV were labeled with Con A, and the Con A was detected in EM using a combination of antibodies and a protein A-gold (10 nm) conjugate. (*A*) Con A labeling of a budding Gag particle and the adjacent PM. (*B*) Labeling of wild-type Mo-MuLV. (*C*) Labeling of budding SFV and adjacent PM. (Bar = 100 nm.)

could also occur during the formation of the wild-type Mo-MuLV. Unfortunately, this could not be studied directly because of the low production rate of wild-type particles in Mo-MuLV-infected cells.

One might argue that the host protein incorporation into Gag particles is, at least in part, a consequence of the high gene expression obtained with the SFV-C/Pr65^{gag} vector. However, we did not observe any significant effect on host protein incorporation when Gag particles were produced at early times after vector infection—i.e., when comparatively little Pr65^{gag} had been produced in the cells, or when using SFV-1/Pr65^{gag} or SFV-1/Pr65^{gag}+Pr80^{env} vectors, which produced 8- and 12-fold less Pr65^{gag}, respectively, than the SFV-C/Pr65^{gag}-vector (19) (M.H. and H.G., unpublished results). Although, this still represents about 10-fold higher Pr65^{gag} production than in wild-type Mo-MuLV-transformed cells, our results do show that the host protein incorporation is not critically dependent on the intracellular Pr65^{gag} concentration.

It should be stressed that not all PM proteins were passively incorporated into the Gag particles. A few PM proteins were found to be excluded from the particle. The reason for this exclusion remains unclear. These proteins might share some physical and topological features that excludes them from the submembranous Pr65^{gag} lattice, or they might not be part of the PM regions where Gag-particle formation takes place. A most intriguing finding was that a few host proteins were concentrated into the Gag particles. The most notable one was the 24-kDa protein. These host proteins might have functional roles in the assembly or entry process of Mo-MuLV and clearly deserve to be studied further.

The validity of our interpretations is primarily based on the purity of our Gag-particle and PM preparations and the accuracy of our PL quantifications, all of which can be criticized. We found that BHK-21 cells infected with various SFV vectors released, in general, significant amounts of host-derived vesicles, but these could be separated from the retrovirus-like Gag particles by sedimentation in an iodixanol gradient. This problem and its solution have been noted before (21–23). The PM preparation isolated by the sucrose step gradient was most likely to some extent contaminated by other membranes. However, the facts that our PM preparation carries a PM marker protein (7), is separated from endoplasmic reticulum and trans-Golgi membranes (7), and is significantly enriched in Pr65^{gag} and Gag-particle budding profiles (this study) make us confident about its suitability as a donor membrane preparation for the Gag particles in our experiments. Our estimation of the PM and Gag-particle membrane ratio was based on PL quantification. The results were interpreted assuming similar lipid composition in the two preparations. This assumption might, however, be somewhat erroneous. The lipid composition of retroviruses has been studied before, and the results suggest that they contain relatively more sphingolipids and cholesterol than the PM of the host cell (24, 25). If this is also the case with Gag particles produced in BHK-21 cells, it means that the envelope of the Gag particles contain less labeled PLs per unit area than the host PM and that we hence overestimate the protein densities in the envelope of the Gag particles. However, it is unclear how much the intercalation of additional cholesterol molecules in between the PL molecules of a membrane actually increases the surface area of the latter (26).

Our biochemical results were corroborated by our immunocytochemical analyses at ultrastructural level. The Con A labeling resulted in similar densities of label in budding and budding-free regions of the PM. Assuming that Con A detects glycoproteins rather than glycolipids, we have interpreted our results as the presence of similar concentrations of glycoproteins in the two regions. The validity of our assumption is supported by the facts that (*i*) Con A binds preferentially to mannose, which is a frequent component of the sugar units of membrane glycoproteins but not glycolipids (14, 27) and (*ii*) we observe a much increased Con A labeling in SFV buds, structures which are known to involve spike protein clustering.

Bulk incorporation of PM proteins into the retrovirus envelope gives a natural explanation for the frequent observations of various host PM proteins (and functions) in retroviruses as well as for the pseudotyping phenomenon (3, 4). Moreover, it can explain the significant unspecific binding of retrovirus to cells (28). The realization of this process opens up new possibilities for modulation of the targeting and fusion functions of retrovirus vectors. However, it also raises concerns about vector purity and PM protein transport from producer cells to target cell surfaces.

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