Murine leukemia virus transmembrane protein R-peptide is found in small virus core-like complexes in cells

Klaus Bahl Andersen, Huong Ai Diep and Anne Zedeler

Department of Pharmacology and Pharmacotherapy, The Danish University of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark

The core of the retrovirus *Murine leukemia virus* (MLV) consists of the Gag precursor protein and viral RNA. It assembles at the cytoplasmic face of the cell membrane where, by an unclear mechanism, it collects viral envelope proteins embedded in the cell membrane and buds off. The C-terminal half of the short cytoplasmic tail of the envelope transmembrane protein (TM) is cleaved off to yield R-peptide and fusion-active TM. In Moloney MLV particles, R-peptide was found to bind to core particles. In cells, R-peptide and low amounts of uncleaved TM were found to be associated with small core-like complexes, i.e. mild detergent-insoluble, Gag-containing complexes with a density of 1.23 g ml⁻¹ and a size of 150–200 S. Our results suggest that TM associates with the assembling core particle through the R-peptide before budding and that this is the mechanism by which the budding virus acquires the envelope proteins.

During the formation of the retrovirus particle, the pre-proteins Gag and Env have different routes of formation and co-localize at the plasma membrane before virus budding. Gag \([\text{Pr}65^{\text{gag}}]\) in *Murine leukemia virus* (MLV) is translated in the cytoplasm. The assembly of Gag and viral RNA into the core particle differs among retroviruses. Beta- and spumaretroviruses, as exemplified by *Mason–Pfizer monkey virus* (MPMV), assemble their core in the cytoplasm, whereas most other retroviruses, including MLV and human immunodeficiency virus (HIV), utilize the type C pathway, i.e. assemble their core at the inner face of the plasma membrane, although assembly and budding into intracellular vesicles have been reported in some cell types [see, for example, Sherer et al. (2003)]. Fully assembled cores are not observed until just before budding. Env \((\text{gPr80}^{\text{env}})\) in MLV) is translated into the rough endoplasmic reticulum, trafficked to the Golgi, glycosylated and cleaved into the surface protein (SU) and the transmembrane protein (TM) by a cellular protease. SU and TM are linked by S–S bridges and join to form trimer complexes, which are trafficked to the cytoplasmic membrane (Einfeld & Hunter, 1988).

Gag plays a central role in the assembly of the retroviral particle (reviewed by Cimarelli & Darlix, 2002; Demirov & Freed, 2004). MLV Gag is transported rapidly to the membrane (Suomalainen et al., 1996), where it directs particle formation. SU–TM co-localizes with Gag (Hermida-Matsumoto & Resh, 2000) and, during budding, SU–TM becomes concentrated in the viral membrane, whereas cellular membrane proteins are excluded (Kuznetsov et al., 2004). Gag particles can bud in the absence of SU–TM (Kuznetsov et al., 2004), but SU–TM is necessary for efficient budding (Fischer et al., 1998) and high infectivity (Rein et al., 1994).

The mechanism ensuring that budding virions contain SU–TM is unknown. In immature virions of HIV, a specific association between the cytoplasmic TM tail and Gag has been shown (Wyma et al., 2000), but whether this is important for the assembly process is not known. After budding, the core matures by cleavage of Gag into the mature core proteins, the matrix (MA), capsid (CA) and nucleocapsid proteins \((\text{p15C, p30 and p10, and the additional p12 in MLV})\). However, cleavage and budding do not occur synchronously, as illustrated by the existence of both mature core proteins in cells and uncleaved Gag in virions [see, for example, Melamed et al. (2004)]. Analytically, immature viral cores can be distinguished from mature cores by their electron-dense structure and their ability to resist mild detergents (Oshima et al., 2004). In this respect, cellular core material behaves (at least in part) in the same way as the immature core (Hansen et al., 1993). Thus, after treatment with mild detergents, Gag is expected to sediment and CA to be in solution. TM is then expected to be in low-density material or in solution, depending on its raft location.

In some retroviruses (mammalian type C, MPMV and others; Bobkova et al., 2002), TM matures further by cleavage by the viral protease around the time of budding. The C-terminal part of the cytoplasmic tail, called R-peptide \((\text{p2E in MLV})\), is cleaved off. R-peptide *per se* was first observed in MLV
virions by Henderson et al. (1984). In Moloney MLV (MoMLV), the sequence is H-VLTQYHQLKI~YEP-OH. The R-peptide tail apparently prevents premature fusion of the immature TM (pre-TM) by keeping it in a fusion-inactive state (Ragheb & Anderson, 1994; Rein et al., 1994). The events following TM cleavage are intriguing, as the majority of R-peptide is found in cells (Olsen & Andersen, 1999), whereas mature, cleaved TM is seen mainly in virions (Green et al., 1981; Olsen & Andersen, 1999).

The sedimentation coefficient (\(s_{n,20}\)) of the retrovirus particle is reported to be in the range 580–750 S [MoMLV (Sharma et al., 1997); Avian myeloblastosis virus and Rous sarcoma virus (Bellamy et al., 1974)]. Despite the smaller size, the core has almost the same \(s_{n,20}\) [750 S, as calculated for intracellular, fully assembled Gag MPyMV cores from data by Parker & Hunter (2000) and Parker et al. (2001)]. This is due to the higher density of 1·24 g ml\(^{-1}\) of the core in sucrose (Wyma et al., 2000) than of the whole virion (1·16 g ml\(^{-1}\)). The \(s\) value generally follows \(d^2\Delta\rho\), where \(d\) is the particle diameter and \(\Delta\rho\) is the density difference between particle and centrifugation medium. Cellular core material of retroviruses using the type C pathway does not show a uniform size. Hansen et al. (1993) estimated the core material to be larger than 165 S and in vitro assembly studies have shown a large profile for Gag material (Lingappa et al., 1997).

Previously, we found R-peptide as a 7 kDa band in Tricine gels by immunoblotting with an antiserum raised against a synthetic MoMLV R-peptide. Despite the fact that the formula mass of the R-peptide is 1968 Da, we believe that the 7 kDa band is R-peptide for the following reasons (Olsen & Andersen, 1999): (i) the antiserum also recognized Env and pre-TM, but not cleaved TM; (ii) the 7 kDa band was observed only in infected cells; (iii) the band resolved at an isoelectric point of 5·5, which was correct according to the sequence; and (iv) it is known that small peptides can run irregularly in Tricine gels (Schägger & von Jagow, 1987). Furthermore, the band was shown to be associated with palmitic acid, possibly changing its electrophoretic properties. Kubo & Amanuma (2003) observed R-peptide in a haemagglutinin-tagged form, which changed its size.

NIH 3T3 cells infected chronically with a molecular clone of MoMLV derived from pMov3 (Reik et al., 1985) were grown as described previously (Olsen & Andersen, 1999). Cell monolayers were washed twice with PBS, once with NTE [100 mM NaCl, 10 mM Tris/HCl (pH 7·5), 1 mM EDTA] and lysed with 1% Triton X-100 (Rohm and Haas) in ice-cold NTE containing Protease Inhibitor Cocktail tablets (Roche). Nuclei and large cellular debris were removed by centrifugation at 1000 r.p.m. for 5 min.

The Svedberg unit of centrifugation is calculated as:
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S = \frac{10^{13} \text{s}^{-1} \ln \frac{\eta_{r,20}}{\eta_{w,20}}}{\omega^2 r},
\]
where \(\omega\) is the angular speed and \(r\) is the radius from the rotational axis. Values were corrected to the corresponding value in water at 20°C (\(s_{w,20}\)) by the factor \(\eta_{r,20} \Delta \rho_{w,20} / \eta_{w,20} \Delta \rho_{w,20}\), where \(\eta_{r,20}\) and \(\eta_{w,20}\) were, respectively, the viscosities of the centrifugation medium at the temperature used (5°C) and that of water at 20°C [1·004 × 10\(^{-3}\) Pa s (1·004 centipoise)]. \(\Delta \rho_{w,20}\) and \(\Delta \rho_{w,20}\) were density differences between the centrifuged particles and water, respectively, and the centrifugation medium. In sucrose gradients, the \(s_{w,20}\) values were integrated as:
\[
s_{w,20} = \frac{10^{13} \text{s}^{-1}}{V \omega^2} \int_{f_{\text{final}}}^{f_{\text{final}}} \frac{\eta_{r,T} \Delta \rho_{w,20}}{\eta_{w,20} \Delta \rho_{w,20}} dr
\]
The density of 1·23 g ml\(^{-1}\) for the particles was used in calculations unless otherwise noted. Fractions from sucrose gradients were concentrated by fourfold dilution and centrifugation in a microfuge (22 000 r.p.m. for 7 h, yielding 13S material). Material at equilibrium in 1·23 g sucrose ml\(^{-1}\) was thus required to be larger than 37\(s_{w,20}\) to pellet fully. Because low-S material was expected at the top of the gradients, the top fractions were also analysed directly. Proteins were separated by Tricine SDS-PAGE (Schägger & von Jagow, 1987) and blotted on to Hybond-P membranes (Amersham Biosciences) followed by immunostaining using rabbit antiserum raised against synthetic R-peptide (Olsen & Andersen, 1999), TM (from Alan Rein, National Cancer Institute, MD, USA), CA (from Bjørn A. Nexø, Department of Human Genetics, University of Aarhus, Denmark) and actin (Sigma-Aldrich). The secondary antibody was horseradish peroxidase-conjugated swine anti-rabbit immunoglobulin (Dako). Bands were visualized by ECL Plus (Amersham Biosciences).

The location of R-peptide in virions was analysed (Fig. 1). Virions were density-purified, treated with Triton X-100 or left untreated and rerun to equilibrium on new sucrose gradients. When virions were untreated, they ran as expected at 1·16 g ml\(^{-1}\), as seen for the majority of each tested protein. Small amounts of Gag, CA and cleaved TM ran at approximately 1·06 g ml\(^{-1}\), which probably represented fragments of virions. After treatment with Triton X-100, R-peptide shifted to 1·22 g ml\(^{-1}\) together with large amounts of Gag, a hallmark of the immature core. Minor amounts of pre-TM, cleaved TM and CA were also observed in this band. In conclusion, R-peptide appeared to be bound to immature cores, but whether all R-peptide was bound was difficult to judge due to the small amount of R-peptide present in virions.

Previously, in infected cells, we observed R-peptide in membrane preparations (Olsen & Andersen, 1999). Membranes were prepared from cells according to the method of Maeda et al. (1983). Of the total cellular amounts, the majority (63–88%) of R-peptide, Gag and CA and all (97%) pre-TM were found in membranes.

To determine whether R-peptide was attached to cellular core material, we carried out size and density separation of cells lysed in Triton X-100, which dissolves membranes. A post-nuclear lysate was pelleted stepwise to decreasing S values. Gag and R-peptide co-sedimented in two ranges: above 62 000 S and between 16 and 1000 S (results not
The low-S material could have been cores in the process of assembly and the high-S material could have been core material bound to cellular debris/cytoskeletal fragments. Gag is known to bind to actin in the cytoskeleton (Chen et al., 2004; Wilk et al., 1999). Approximately one-third of the total R-peptide was present in each size range, with the last third in a final 5S supernatant, which, as expected, contained the majority of CA and pre-TM.

The density of the low-S complexes was examined by equilibrium centrifugation (Fig. 2). The majority of R-peptide was clearly located in a band of 1.03–2.3 g ml\(^{-1}\) together with a considerable amount of Gag and a small amount of pre-TM, properties similar to those of the viral core seen in Fig. 1. As also observed in the stepwise centrifugation, some R-peptide and most of the CA were present in solution. Some Gag and most of the pre-TM were also present as low-density pelletable material, possibly rafts.

The size of the core-like material was examined by velocity centrifugation (Fig. 3a). R-peptide and Gag co-located, both in the gradient pellet (larger than 460 S) and in a band around 170 S. In similar experiments, R-peptide was not observed in the range between 400 and 1200 S (not shown), where whole cores are expected. Pre-TM and CA were mainly present in solution, as expected; however, low

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**Fig. 1.** Equilibrium centrifugation of MoMLV with or without Triton X-100 treatment. Two 0.5 ml portions of density-purified MoMLV from a total of 250 ml medium were diluted fivefold in NTE buffer with or without 1% Triton X-100 for 1 h on ice and rerun on 10 ml 13–65% sucrose gradients at 36,000 r.p.m. for 20 h in an SW40 rotor. Fractions (~1 ml) are indicated by density (g ml\(^{-1}\); determined by refractometry). Pelleted fractions (600 µl) were analysed together with 20 µl of the loaded virus dilutions (V) and 30 µl of the top fractions (T) by immunoblotting using antisera against R-peptide (α-R), CA (α-CA) and TM (α-TM). Possible Gag cleavage intermediates are indicated by ‘i’.

**Fig. 2.** Equilibrium centrifugation of Triton X-100 lysate from infected NIH3T3 cells. A Triton X-100 lysate from ~6 x 10\(^6\) cells was centrifuged to give a 25,000S supernatant (3 ml), which was run on a 10 ml 13–65% sucrose gradient at 36,000 r.p.m. for 20 h in an SW40 rotor. Fractions (~1 ml) are indicated by density (g ml\(^{-1}\)). T, Top fraction. Fractions were analysed either directly (30 µl) or after pelleting of 300 µl by immunoblotting using antisera against R-peptide, CA or both (α-R + α-CA). In the CA immunoblot of pelleted fractions, fractions were pooled as indicated. MoMLV and MoMLV cores were run in parallel and their densities are indicated as V and C, respectively.
To determine whether the 1·23 g ml⁻¹ and 170S material can explain directly how Env proteins become associated with the assembling Gag core in the cell. The extensive binding of R-peptide indicates that pre-TM is associated by its R-tail. Mutational analysis supports the importance of the TM–Gag association. In R-peptide-containing retroviruses, R-cleavage-negative mutants do produce virions, whereas R-truncated mutants give a low yield; in both cases, the specific virus infectivity is low (Rein et al., 1994; Kubo & Amanuma, 2003). Low infectivity of R truncations is also observed for MPMV (Brody et al., 1994), indicating that the R-peptide–core association is not restricted to retroviruses with the type C assembly pathway. Furthermore, mutations in MPMV MA, to which the binding presumably occurs, have been shown to suppress cleavage of pre-TM (Brody et al., 1992). In HIV, mutations in MA prevent incorporation of envelope proteins into virions (Yu et al., 1992; Freed & Martin 1995, 1996; Cimarelli & Darlix, 2002) and incorporation can be restored by truncations in the TM cytoplasmic tail (Mammano et al., 1994). Furthermore, the TM–core association in HIV has been shown to have implications for fusion ability (Wyma et al., 2004).

Some cleaved TM also appeared to be bound to viral cores (Fig. 1). This association cannot be explained directly by the R-peptide association. It may occur indirectly through the TM–core association in HIV has been shown to prevent incorporation of envelope proteins into virions (Yu et al., 1992; Freed & Martin 1995, 1996; Cimarelli & Darlix, 2002) and incorporation can be restored by truncations in the TM cytoplasmic tail (Mammano et al., 1994). Furthermore, the TM–core association in HIV has been shown to have implications for fusion ability (Wyma et al., 2004).

The location of R-peptide and pre-TM in cellular core-like material can explain directly how Env proteins become associated with the assembling Gag core in the cell. The extensive binding of R-peptide indicates that pre-TM is associated by its R-tail. Mutational analysis supports the importance of the TM–Gag association. In R-peptide-containing retroviruses, R-cleavage-negative mutants do produce virions, whereas R-truncated mutants give a low yield; in both cases, the specific virus infectivity is low (Rein et al., 1994; Kubo & Amanuma, 2003). Low infectivity of R truncations is also observed for MPMV (Brody et al., 1994), indicating that the R-peptide–core association is not restricted to retroviruses with the type C assembly pathway. Furthermore, mutations in MPMV MA, to which the binding presumably occurs, have been shown to suppress cleavage of pre-TM (Brody et al., 1992). In HIV, mutations in MA prevent incorporation of envelope proteins into virions (Yu et al., 1992; Freed & Martin 1995, 1996; Cimarelli & Darlix, 2002) and incorporation can be restored by truncations in the TM cytoplasmic tail (Mammano et al., 1994). Furthermore, the TM–core association in HIV has been shown to have implications for fusion ability (Wyma et al., 2004).

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oligomers are stable in mild detergents (Einfeld & Hunter, 1988).

The cellular core-like complexes had the same density as viral cores (1.23 g ml⁻¹), but were of a smaller size. According to the proportionality between S and d², the core-like complexes had approximately half the diameter of viral cores. We do not know whether the complexes are involved in the formation of virus particles or whether they represent abortive material. The large amount of R-peptide associated with the cellular core-like complexes compared with the amount of R-peptide in virions suggests an accumulation in cells. Further studies are required, therefore, to elucidate the fate of R-peptide and the core-like complexes.

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References


envelope become infectious in the presence of lipofection reagents.  


