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The retrovirus MA and PreTM proteins follow immature MLV cores

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ABSTRACT

We have used mild detergent to analyze the core of Moloney Murine Leukemia Virus (MoMLV) and core-like complexes in infected cells. The immature core consists of the Gag polyprotein (PrGag) and viral RNA (vRNA). It is known to be detergent-resistant, in contrast to the mature Gag core. The core matures by cleavage of PrGag into MA (matrix), p12, CA (capsid) and NC (nucleocapsid) protein. We found that mature Gag proteins were bound to the PrGag cores. The degree of binding differed widely. No (<0.1%) p12 bound, low amount of CA (3-5%), and higher amount of MA (13-20%) bound. Varying NC was bound (5-15%). NC could be released by RNase A in agreement with its binding to viral RNA. The TM (transmembrane) protein was also examined. A low amount of TM was bound to the PrGag core (approximately 5%), whereas a very high amount (65%) of the PreTM (TM with the cytoplasmic R peptide tail) bound. The binding in the PrGag core appears to occur by direct protein–protein interactions as only minute amounts of lipids including raft lipids were observed after detergent treatment.

1. Introduction

Type C retroviruses assemble at the cytoplasmic membrane. The MA•p12•CA•NC polyprotein PrGag binds viral RNA and drives the assembly at the inner face of the membrane, followed by attachment of the virus Envelope (Env) proteins TM and SU and budding of the virus particle. It is however immature and non-infectious. The virion matures when PrGag is cleaved by the viral protease. MA then locates just inside the virus membrane forming the matrix. CA forms the capsid which surrounds the nucleocapsid consisting of NC and vRNA. In MLV the protease also cleaves the R peptide off PreTM to form the mature TM, a necessity for virus infectivity (Rein et al., 1994; Löving et al., 2011).

p12 is unique for MLV and a few other mammalian gammaretrovirus. The p12 protein has both early and late functions in the infection cycle (Yuan et al., 1999). The late function involves the p12 domain in PrGag where its PPPY motif is important for virus assembly (see review by Freed, 2002). The early function involves mature p12 (Lee et al., 2005; Rulli et al., 2006; Wight et al., 2012). It is important for reverse transcription, and nuclear integration of viral RNA as part of the pre-integration complex PIC (Kyere et al., 2008; Prizan-Ravid et al., 2010). Elis et al. (2012) have shown that p12 binds PIC to the chromosomes only during mitosis, explaining why only growing cells can be infected by MLV. The location of p12 in the virion is not known.

Retroviruses are assembled at the membrane by tight PrGag–PrGag attachments. *In vitro*, PrGag can assemble into almost icosahedral 60 nm or 60S particles (Klikova et al., 1995; Campbell & Vogt, 1995; Morikawa et al., 2004), whereas the virion has a size of approximately 80-100 nm. A glycosylated transmembrane form of PrGag (gGag) appears to be important for the budding and formation of spherical particles rather than tubular particles (Low et al., 2007; Nitta et al., 2010). gGag is known to remain in the cell (Fujisawa et al., 2001).

CA has structured N- and C-terminal domains (NTD and CTD) joined by a flexible region (Wright et al., 2007). CA and especially CA•NC can together with RNA under certain conditions self-assemble *in vitro* into icosahedrons consisting of pentamers (Hyun et al., 2010), or tubes consisting of hexamers (Gross et al., 1997, 2000; Zuber et al., 2000; Mayo et al., 2003; Li et al., 2000; Noviello et al., 2011, Bharat et al., 2012). The mature cone-shaped HIV retrovirus capsid consists of 1056 CA units in a lattice of hexamers and 12 pentamers (Pornillos et al., 2011; Ganser-Pornillos et al., 2008) alike a skewed fullerene molecule. The gammaretrovirus capsid is more spherical, possibly determined by a more even distribution of the pentamers (Briggs et al., 2009).

PrGag and Env proteins collect at rafts from where the virions bud (reviewed by Ono, 2010; Li et al., 2002; Pickl et al., 2001; Chan et al., 2008; Briggs et al., 2003; Waheed and Freed, 2010). The virus membrane is highly enriched in raft lipids, e.g. sphingolipids and cholesterol (Quigley et al., 1972; Pessin and Glaser, 1980; Chan et al., 2008). The N-terminal end of MA, respectively the MA domain in PrGag, is myristoylated, which is important for directing PrGag to the membrane (see e.g. Dalton et al., 2005; Mayo et al., 2002). Phosphatidylinositol-4,5-bisphosphate (PI[4,5]P_2) has furthermore

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been shown to aid HIV MA binding to the membrane (Alfadhli et al., 2009; Monde et al., 2011; Hamard-Peron and Muriaux, 2011). This is presumably also true for MLV, as it is enriched in phosphoinositoles and as its budding is dependent on PI[4,5]P_2 (Chan et al., 2008).

Approximately 50 trimeric Env spikes (consisting of TM and the surface protein SU) are observed on the MoMLV surface (Förster et al., 2005; Sjöberg et al., 2007). The mechanism of Env incorporation to the virus particle is not totally clear. It might involve various factors (Johnson, 2011). MA–TM interaction is of some importance in HIV and HTLV (Manrique et al., 2003; Cosson, 1996, reviewed by Göttlinger, 2001; Rayne et al., 2004). A direct TM–MA interaction is not obvious in MLV as the cytoplasmic domain of TM is short. The TM to MA stoichiometry is low (approximately 1:7, as calculated from the number of Env spikes and CA molecules in the mature capsid). TM is palmitoylated which is important for its location to rafts (Yang and Compans, 1996; Li et al., 2002).

Mild, nonionic detergents, such as NP40 and Triton X100, have been used to examine the internal virion bonds (Khorchid et al., 2002; Oshima et al., 2004; Andersen et al., 2006). After detergent treatment, the immature core, consisting of PrGag is stable, in contrast to the mature core consisting of the Gag proteins. The immature core has a buoyant density in sucrose of approximately 1.22 g/ml, in comparison to the 1.16 g/ml of the whole virion, showing that the low-density virus membrane has been removed (Andersen et al., 2006). Low amounts of CA followed the PrGag core. We have therefore set out to investigate the binding of the Gag proteins to the PrGag core. We have earlier found core-like PrGag complexes in infected cells, to which low amounts of CA likewise was bound (Andersen et al., 2006). Whole virions have an S value of 700 (Sharma et al., 1997). The cellular core-like PrGag complexes were smaller, approximately 100 S.

Below, we show that no p12, low amounts of CA, NC and TM, but higher amounts of MA and PreTM followed the detergent-resistant PrGag cores and cellular core-like PrGag complexes.

2. Materials and methods

MoMLV and chronically infected NIH3T3 cells have been described earlier (Andersen et al., 2006). Virus purification and Triton X100 cell lysate isolation have likewise been described (Andersen et al., 2006). Roche "Complete Mini, EDTA free" protease inhibitor was added to the buffers. Plasma membrane vesicles (PMV) were prepared according to Puri et al. (1992) and were isolated by centrifugation through 10% sucrose onto a 42% sucrose cushion in a SW60 rotor for 30 min at 45,000 rpm. The PMV preparation was diluted and collected by centrifugation in a microfuge.

Equilibrium centrifugation: 13-65% sucrose gradients were made in NTE (100 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5) and samples were loaded on top. The gradients were then centrifuged at 36,000 rpm for 18-20 h in a SW40 rotor. Fractions were collected from the bottom. Centrifuged material was concentrated by four-fold dilution in NTE followed by pelleting at 45,000 rpm in a Ti70 rotor for 6 h, thus material larger than 30 S was collected. The pellets were dissolved in gel sample buffer.

The MLV preparation was also pelleted directly after treatment without or with Triton X100 treatment in order to analyze the virions and immature cores. The centrifugation was through 500 μl 20% sucrose at 30,000 × *g* for 4 h (giving 60 S), followed by two washes of the pellets with NTE. The pellets were dissolved in sample buffer and run on gels.

Samples were run on tricine gels and blotted onto Hybond-P membranes (GE healthcare) followed by sequential immunostaining with: rabbit anti-NC, anti-MA (kindly from David Ott, NCI-Frederick, USA), rabbit anti-TM, rabbit or goat anti-CA (Andersen et al., 2006), mouse anti-p12 (kindly from Finn Skou Pedersen, University of Aarhus, Denmark), and rabbit anti-R peptide (Olsen and Andersen, 1999). Each was followed by peroxidase conjugated secondary antisera and was visualized with ECL-Advance or ECL-Prime (GE healthcare). The immunoblots were recorded (2-400 s) with a Leica DC500 camera (sensitive to 14 bit). In general, the light emission had a half-time of approximately 2 min. The NC antiserum also detected MA as a secondary band.

For measurements, the samples were run in serial dilutions, and serial exposures were recorded. The following band measure was used: the sum of each pixel times its density ranging from 0 to 1 (i.e. from no to full exposure), minus the density of unexposed neighbor areas. Fully exposed bands generally had a measure of 400 pixel-density-units. The protein amount was determined relative to a control within the same immunoblot. This was done as follows. The density units of the bands was plotted in a Hill diagram where $log(measurement/(Max - measurement))$ was plotted against $log(sample\ volume)$. The maximal density unit (*Max*) was chosen in order to linearize the control curve (i.e. that of untreated MLV). The relative protein amount between different samples is then the horizontal distance on the plot.

^{3}H-Cholesterol labeled virus was obtained from 150 cm^2 infected cultures grown 24 h with 50 μCi ^{3}H-Cholesterol (PerkinElmer 50 μCi/mmol) in DMEM media supplemented with 2% lipid-depleted serum (Biosera). Media was filtered (0.45 μm) and virus was pelleted to 100 S and re-suspended in NTE.

3. Results

3.1. Virion cores

The virus preparation appeared to be a mixture of immature and mature virions as it contained both CA and PrGag (Fig. 1A). However, another explanation is that partly matured virions are present (see later). When the virions were centrifuged to equilibrium on a 13-65% sucrose gradient, they located as expected at 1.16 g/ml, see Fig. 1A. When the virions were treated with Triton X100, a large part of the PrGag centrifuged down to a higher density of approximately 1.22 g/ml, whereas almost all CA remained on top. PrGag at 1.22 g/ml is a hallmark of the immature core, and CA in the top is a hallmark of the dissolved mature virions. As only low volumes of sucrose fractions could be loaded directly on the gel (Frames A and B) the peaks were concentrated by pelleting.

PrGag and Gag proteins in the heavy (TH) and the light (TL) peaks of the Triton treated virus was compared to the heavy (nH) and light (nL) peaks of the not treated virus. As expected, all virus proteins of the intact virions were observed in the light fraction nL. After the detergent treatment, the heavy fraction TH mainly contained PrGag, but also low amounts of MA, CA, and NC, though no p12 were present. Only minute amounts of proteins were observed in nH relative to nL, and in TL relative to TH. This shows the purity of the 1.16 g/ml virus (nL) and the 1.22 g/ml PrGag core fraction (TH).

The protein amounts were determined by densitometry (Table 1A), and were related to that of PrGag in the untreated 1.16 g/ml virions. Thus 12% of the MA followed the PrGag core after the detergent treatment. Low but significant amounts of CA and NC (2-4%) followed the PrGag core. p12 was not found in the PrGag core (at least not down to the detection limit of 0.06%). The binding of mature Gag proteins to the immature PrGag cores is in contrast to earlier findings (Oshima et al., 2004). The gradient was repeated with similar results (however NC values differed, see also below).

In order to verify the results, virions were treated with Triton X100 or left untreated and pelleted through 20% sucrose. See Fig. 3 and Table 1C and D. Without Triton treatment, the whole virions are expected to pellet. And with Triton treatment only the PrGag