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Short communication

In vitro assembly of the feline immunodeficiency virus Gag polyprotein

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ABSTRACT

The retroviral Gag protein is the only viral product that is necessary for the assembly of virions in mammalian cells. We have established an in vitro assembly system to study the assembly properties of purified feline immunodeficiency virus (FIV) Gag protein expressed in bacteria. Under fully defined conditions, the FIV Gag protein assembles into spherical particles of 33 nm in diameter which are morphologically similar to authentic immature particles, albeit smaller than virions. The in vitro assembly of FIV Gag into particles was found to be resistant to the addition of Triton X-100 and required the presence of RNA. Notably, we found that an amino acid substitution in the nucleocapsid domain of Gag that impairs RNA binding and blocks virion production in vivo, also abrogates Gag assembly in vitro. The development of an in vitro assembly system for FIV Gag protein will facilitate the study of the mechanisms by which this protein assembles into immature particles.

In retroviruses, the Gag polyprotein has all the necessary information to direct the assembly and release of virions from the plasma membrane of the infected cells (Hunter, 1994). Indeed, the expression of Gag in eukaryotic cells, in the absence of the other viral proteins, is sufficient for the production of extracellular virus-like particles (see references in Hunter, 1994). Initially, retrovirus particles that bud from the plasma membrane exhibit an immature morphology. However, cleavage of Gag by the viral protease into the matrix (MA), capsid (CA), and nucleocapsid (NC) proteins produces profound morphological changes and generates the mature infectious particles, in which the MA remains associated with the inner side of the viral envelope and the CA forms a shell that surrounds the complex between the NC protein and two molecules of the viral genomic RNA (Briggs et al., 2003; Kingston et al., 2001).

The retroviral MA, CA and NC proteins not only differ in their structural role in the mature particle but also fulfill different functions as domains of Gag during the process of particle assembly in retrovirus-infected cells. In most retroviruses, the MA protein, through its N-terminal myristic moiety and polybasic region, mediates Gag transport to and interaction with the plasma membrane (González et al., 1993; Göttlinger et al., 1989; Manrique et al., 2001; Ono and Freed, 1999; Zhou et al., 1994). Moreover, we and others have demonstrated that in human and simian immunodeficiency viruses (HIV and SIV, respectively) the MA is responsible for the incorporation of the envelope glycoprotein into virions (Dorfman et al., 1994; Freed and Martin, 1996; González et al., 1996; Manrique et al., 2003, 2008; Murakami and Freed, 2000). Regarding the functions of the other domains of Gag, while the CA promotes the Gag self-interactions that result in particle assembly (Accola et al., 2000; von Schwedler et al., 2003), the NC exhibits one or two zinc-finger motifs that mediate its association with the viral genomic RNA thereby ensuring the packaging of the retroviral genome during virus morphogenesis (Jewell and Mansky, 2000). Finally, the Gag sequences responsible for particle budding are variably located when comparing different retroviruses. Amino acid motifs such as PPPY located between the MA and CA, or those mapping C-terminally to the NC, such as P(T/S)AP or YXXL, interact with the cellular proteins of the endosomal sorting pathway that promote the release of the nascent retroviral particles from the plasma membrane (for a review see Bieniasz, 2006).

Most of our knowledge on Gag functions has been provided by site-directed mutagenesis studies performed in vivo. However, when interpreting the assembly-defective phenotype of a Gag mutant in such studies it has been imperative to distinguish whether the mutation directly affected Gag–Gag interactions or instead interfered with other processes, such as Gag transport, Gag-membrane interaction or even particle budding. In this regard, in vitro assembly systems have been developed for recombinant Gag proteins of three different retroviruses: Mason-Pfizer monkey virus (Klikova et al., 1995; Rumlova-Klikova et al., 2000), Rous sarcoma virus (RSV) (Campbell and Vogt, 1997; Yu et al., 2001) and HIV-1 (Campbell and Rein, 1999; Campbell et al., 2001; Huseby et al., 2005; Morikawa et al., 1999).

We have previously investigated in feline immunodeficiency virus (FIV) the role that the MA and NC domains of Gag play in particle assembly and virus infectivity (Manrique et al., 2001, 2004a,b). We therefore decided to develop an in vitro assembly system for FIV using purified recombinant Gag protein so as to facilitate the

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analysis under defined conditions of the structural requirements for FIV Gag multimerization into particles.

For the in vitro assembly studies, the coding region for full-length FIV Gag was PCR-amplified from the FIV-14 proviral DNA and cloned into the *EcoR*V and *Sal*I sites of the pET-30b (+) plasmid (Novagen). This system allowed the expression of the Gag protein with a six-histidine tag at its N-terminus. In this regard, it has previously been shown that the presence of a His tag either at the N- or C-terminus of recombinant HIV-1 Gag protein does not interfere with the ability of this protein to self-assemble in vitro (Huseby et al., 2005; Morikawa et al., 1999). The recombinant FIV Gag protein was produced in *Escherichia coli* BL21 strain essentially as we have described before (Manrique et al., 2004b). After treating the bacterial lysates with DNase I (20 μg/ml) and RNase A (50 μg/ml) to remove nucleic acids, the His-tagged FIV Gag protein was purified by immobilized metal ion adsorption chromatography using the His Microspin Purification Module (GE Life Sciences) (Manrique et al., 2008). Protein preparations were then desalted employing microcon centrifugal filter devices (Millipore) and stored at −80 °C until further use. Protein concentrations were estimated as we have previously described (Manrique et al., 2008). Potential contamination of purified Gag protein with nucleic acids was evaluated by spectrophotometry measuring the A_{260}/A_{280} ratio as well as by agarose gel electrophoresis after phenol extraction and ethanol precipitation of protein samples. Under the conditions described above, the His-tagged FIV Gag protein was consistently found in the soluble fraction of the bacterial lysates and could be readily purified to the concentrations of 0.5–1 μg/μl and an estimated purity of approximately 90% as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 1A). Furthermore, the identity of the purified FIV Gag protein was confirmed by Western blotting with the anti-FIV CA monoclonal antibody PAK3-2C1 (Manrique et al., 2004a) (data not shown).

To investigate the assembly properties of the purified FIV Gag protein, we first used a sedimentation assay (Huseby et al., 2005). Five μg of recombinant His-tagged Gag protein was incubated for 3 h at 37 °C in a final volume of 25 μl of buffer containing 50 mM Tris–HCl (pH 8.0); 150 mM NaCl; 5 mM dithiothreitol (DTT); 10 μM $ZnCl_2$; 500 ng of RNA (see below) and 100 units of recombinant RNasin ribonuclease inhibitor (Promega). The nucleic acid added to the assembly reactions was an in vitro transcribed RNA corresponding to the encapsidation signal of the FIV genome which comprises the R and U5 regions of the 5′ long terminal repeat together with the first 320 nucleotides (nt) of Gag (nt 216–947 of the FIV-14 proviral DNA; Kemler et al., 2002; Manrique et al., 2004b). We used this RNA, referred to as R-U5-MA (Fig. 1B), because it strongly binds to recombinant FIV NC protein (Manrique et al., 2004b) and because we can easily obtain large amounts of this RNA by in vitro transcription (RiboMax Large Scale RNA Production System, Promega). After the incubation period, the assembly reactions were centrifuged at 16,000 × *g* for 60 min at 4 °C to separate the pelletable Gag-made particles from the unassembled Gag molecules that remain in the supernatant. Both the pellet and supernatant fractions were resolved by SDS-PAGE and proteins blotted onto nitrocellulose membranes. Detection of His-tagged Gag proteins was performed by Western blotting using a horseradish peroxidase-conjugated antibody specific for penta-His (QIAGEN). Western blots were developed with an enhanced chemiluminescence and chemifluorescence assay as previously described (Manrique et al., 2008). The results showed that when the FIV Gag protein is incubated under the conditions described above the majority of the protein is found in the pellet fraction, indicating that the recombinant purified FIV Gag protein is capable of assembling into multimeric complexes (Fig. 1C). By contrast, when the R-U5-MA RNA was omitted from the reaction, the FIV Gag protein was entirely detected in the supernatant fraction (Fig. 1C), demonstrating that the FIV Gag protein cannot self-assemble in the absence of RNA.

We next examined the effect of different detergents on the in vitro multimerization ability of the recombinant FIV Gag protein. When 0.1% (w/v) SDS was included in the assembly reaction, the entire FIV Gag protein was recovered in the supernatant, indicating that, as expected, a known protein-denaturing detergent such as SDS abrogates FIV Gag multimerization (Fig. 1C). By contrast, the addition to the assembly mixture of Triton X-100 at a final concentration of 0.5% (v/v) did not affect the FIV Gag assembly efficiency. Indeed, the levels of pelletable FIV Gag from the assembly assays performed in the presence of Triton X-100 were similar to those obtained when the reactions were carried out in the absence of any detergent (Fig. 1C). Interestingly, it has previously been shown

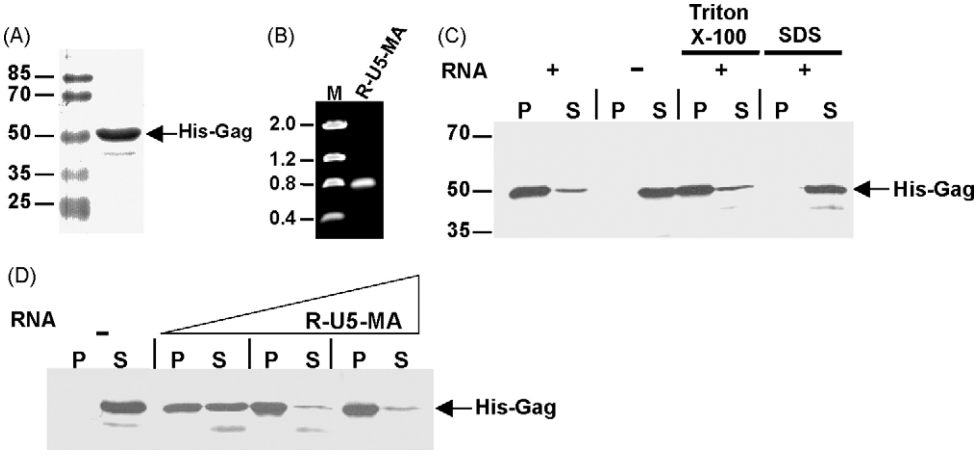

Fig. 1. In vitro assembly properties of recombinant FIV Gag protein. (A) Coomassie blue-stained SDS-polyacrylamide gel of FIV Gag protein expressed in *E. coli* with an N-terminal His tag and purified by affinity chromatography. Numbers on the left indicate the mobilities of the molecular weight standards (kDa). (B) Agarose gel electrophoresis of the in vitro transcribed R-U5-MA RNA added to the assembly reactions. This RNA corresponds to the encapsidation signal of the FIV genome (Manrique et al., 2004b). The numbers on the left correspond to the molecular weight markers (M) in kilobases. (C) Sedimentation analysis of the FIV Gag assembly reactions. FIV Gag protein was incubated in the presence (+) or absence (−) of synthetic R-U5-MA RNA. After incubation of the assembly mixtures, pellet (P) and supernatant (S) fractions were obtained by centrifugation and subjected to SDS-PAGE followed by Western blotting using a horseradish peroxidase-conjugated antibody specific for the His tag present in the Gag protein. The effect of the addition of 0.5% (v/v) Triton X-100 or 0.1% (w/v) SDS on the Gag assembly efficiency was also tested in these experiments. The arrow indicates the mobility of the FIV Gag protein, whereas the numbers on the left correspond to the positions of the molecular weight standards (kDa). (D) In vitro assembly of FIV Gag with R-U5-MA RNA at increasing RNA/protein ratios of 1, 5 and 10% (w/w). Sedimentation assays were performed as described above.

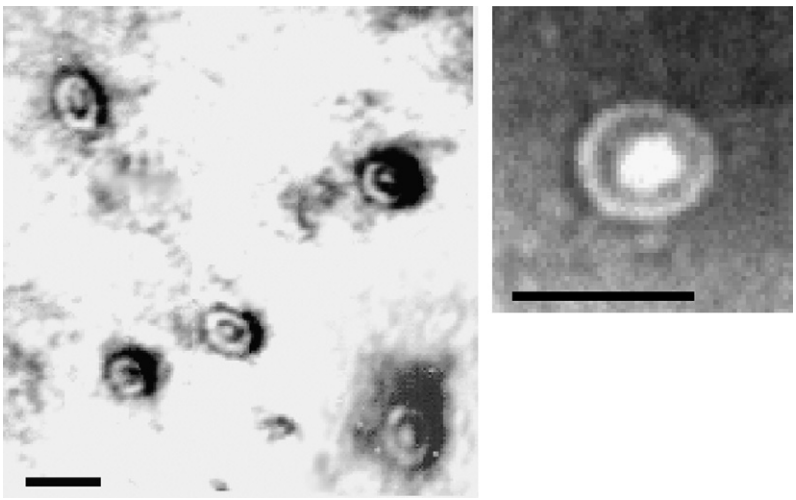

Fig. 2. Morphology of the FIV Gag particles. The assembly reactions were applied onto Formvar-coated grids (TAAB Laboratories, UK) and negatively stained with 2% uranyl acetate. Grids were visualized using a Jeol transmission electron microscope operating at 80 kV. Bars represent 50 nm.

that treatment with Triton X-100 does not disrupt the integrity of immature retroviral particles (Lee and Yu, 1998).

When we investigated how Gag assembly could be affected by varying the RNA to protein ratio in the assembly reactions, a clear correlation was observed between the amount of R-U5-MA RNA added to the mixture and the extent of FIV Gag multimerization. When the R-U5-MA RNA was present at a proportion representing 1% (w/w) of the Gag protein, Gag assembly occurred, albeit not with its highest efficiency (Fig. 1D). However, raising the RNA/Gag ratio to 5% was sufficient to recover most of the Gag protein in the pellet fraction (Fig. 1D). Of note, the RNA to protein ratio of 5% is consistent with the estimated stoichiometry of viral RNA to Gag molecules in immature retrovirus particles (Parker et al., 2001) and with in vitro measurements of the binding capacity of recombinant HIV-1 NC protein to single-stranded nucleic acids (Fisher et al., 1998).

To examine the morphology of the structures assembled in vitro by the recombinant FIV Gag protein, the assembly reactions were lifted on Formvar-coated grids, negatively stained and visualized by transmission electron microscopy. Incubation of FIV Gag under defined conditions yielded numerous particles the morphology of which resembled that of immature virions (Fig. 2, left panel). The majority of the particles were nearly spherical and had a diameter of approximately 33 nm, which is smaller than that of authentic virions (Fig. 2, left panel). Of note, if the RNA was omitted from the assembly reactions, no particles were observed. Examination of the particles under higher magnification revealed the presence of three concentric layers differing in their electron density, with the outer layer showing a series of faint striations at its periphery (Fig. 2, right panel). Our results therefore demonstrate that recombinant FIV Gag assembles into spherical particles when incubated with RNA.

We have previously shown that substitution at the FIV NC of the first cysteine residue in the amino terminal zinc finger by serine (mutation C377S) is sufficient to abrogate recombinant NC/genomic RNA interaction in vitro and to severely impair virion production in mammalian cells as well (Manrique et al., 2004b). Based on these results, we therefore investigated whether an FIV Gag protein carrying this single-amino acid substitution (C377S Gag) was capable of assembling in vitro. When the recombinant purified C377S Gag protein was incubated in the presence of the R-U5-MA RNA and then subjected to centrifugation, most of the protein remained associated to the supernatant fraction, indicating that under conditions that ensure the efficient in vitro assembly of wild-type Gag, the mutant C377S version of this protein is unable to multimerize (Fig. 3A). These results indicate that the lack of interaction between the C377S Gag protein and the R-U5-MA RNA most likely precludes Gag assembly into particles.

The experiments described here suggest that the interaction between protein and RNA is required at some point in the Gag multimerization process. It would then be expected to find the R-U5-MA RNA associated with the in vitro assembled particles. To test this assumption, we performed in vitro assembly reactions with either wild-type or C377S FIV Gag proteins in the presence of the R-U5-MA RNA. After centrifugation, the particulate fractions were disrupted by 0.1% SDS treatment, digested with proteinase

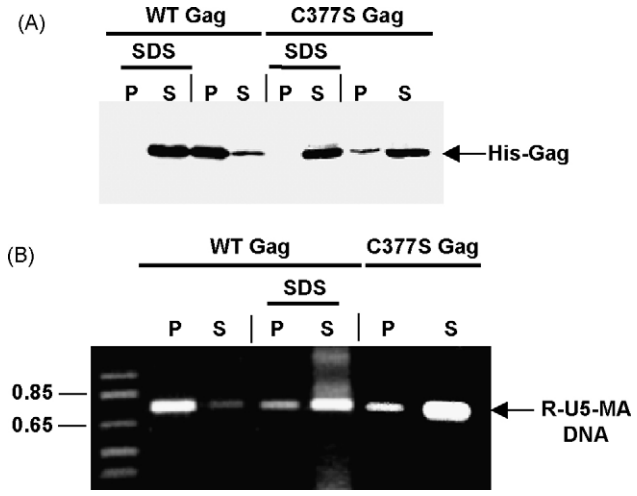

Fig. 3. In vitro assembly of mutant C377S FIV Gag protein. (A) Sedimentation assays of the assembly reactions of wild-type (WTGag) or mutant C377S (C377S) FIV Gag proteins. SDS: denotes reactions performed in the presence of 0.1% (w/v) SDS which were included as negative controls. The pellet (P) and supernatant (S) fractions were obtained and analyzed as described in Fig. 1. (B) Analysis of the presence of R-U5-MA RNA in the pellet and supernatant fractions resulting from the assembly reactions of wild-type (WTGag) or mutant C377S (C377S) FIV Gag proteins. Wild-type or C377S Gag proteins were incubated with R-U5-MA RNA as described in the text. An additional assembly reaction containing 0.1% (w/v) SDS was carried out for wild-type FIV Gag. Pellets and supernatants were digested with proteinase K and extracted with a 1:1 mixture of phenol–chloroform. RNA was concentrated by ethanol precipitation. The presence of R-U5-MA RNA in each fraction was determined by RT–sqPCR using specific primers followed by agarose gel electrophoresis and ethidium bromide staining to visualize the DNA products. The arrow indicates the mobility of the R-U5-MA DNA product. The numbers on the left correspond to the molecular weight standards in kilobase pairs.

K (50 μg/ml) and subjected to phenol–chloroform extractions. Particle-associated nucleic acids were recovered by ethanol precipitation. To analyze whether the R-U5-MA RNA was present in these samples we performed reverse transcription (RT) coupled to semiquantitative polymerase chain reaction (sqPCR; Hubert and Hentze, 2002) using specific primers. The supernatants resulting from the assembly reactions were treated similarly. As shown in Fig. 3B, the RT–sqPCR product corresponding to the R-U5-MA RNA was clearly detected in the particulate fraction from the in vitro assembly of wild-type FIV Gag. By contrast, the R-U5-MA DNA was mostly found in the supernatant when the assembly reactions of wild-type Gag were carried out in the presence of SDS (Fig. 3B). In the case of C377S Gag, the R-U5-MA product was mainly detected in the supernatant fraction, which is in agreement with the inability of this mutant Gag protein to assemble into particles (Fig. 3B). Our results therefore indicate that the R-U5-MA RNA does not simply play a catalytic role during the in vitro assembly process but that it also participates as a structural component of the Gag-made particles.

In this paper, we demonstrate that the full-length FIV Gag protein expressed in bacteria and purified by affinity chromatography is capable of assembling into spherical particles under fully defined conditions. These assemblies are morphologically similar to the virus-like particles that we have previously shown to be produced upon expression of FIV Gag in the vaccinia system (Manrique et al., 2001). However, while the virus-like particles obtained in mammalian cells have a diameter of 130–150 nm (Manrique et al., 2001), the FIV Gag particles assembled in vitro are smaller (33 nm) than those produced in vivo. In this regard, HIV-1 Gag protein lacking the p6 domain also assembles in vitro into small particles of about 25–30 nm (Campbell and Rein, 1999). Interestingly, addition to the assembly reactions of mammalian cell extracts as well as of different phosphatidylinositol phosphates, some of which are membrane components, allowed the formation of particles with a similar size to that of authentic HIV-1 virions (Campbell et al., 2001). Therefore, interaction of lentiviral Gag proteins with the plasma membrane appears to be necessary for the assembly of normal-sized particles.

We also show here that the in vitro assembly of FIV Gag into particles requires RNA. This is in agreement with the results obtained with other recombinant retroviral Gag proteins the multimerization of which has been shown to be promoted by a wide variety of RNA species both of viral and non-viral origin (Campbell and Rein, 1999; Campbell and Vogt, 1997). Importantly, our studies demonstrate that a mutation in Gag that impairs virion production in mammalian cells by interfering with the ability of the NC to interact with RNA (Manrique et al., 2004b) also abrogates particle assembly in vitro, which points to a good correlation between our studies performed with proviral DNA in mammalian cells (Manrique et al., 2004b) and those obtained here in vitro with recombinant FIV Gag protein.

In summary, we developed an in vitro assembly system for FIV Gag, which will be suitable for future studies aimed at delineating the domains in Gag whose interaction promotes FIV particle formation. In addition, this system will be useful to analyze the process of FIV RNA encapsidation into particles.

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