Isolation and characterization of the Mason–Pfizer monkey virus p12 protein

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Abstract

The Mason–Pfizer monkey virus (M-PMV) Gag protein, precursor to the structural proteins of the infectious virion, assembles into immature capsid-like particles when expressed at high levels in bacterial cells. Similar capsid-like particles can be obtained by in vitro assembly using a high concentration of isolated Gag. M-PMV Gag contains a p12 protein that has no corresponding analogues in most other retroviruses and has been suggested to contain an internal scaffold domain (ISD). We have expressed and purified p12 and the N- and C-terminal halves (Np12 and Cp12) that are predicted to be structurally independent domains. The behavior of these proteins was analyzed using chemical cross-linking, CD spectroscopy, and electron microscopy. The N-terminal half of p12 is largely α-helical although the C-terminal portion lacks any apparent ordered structure. Both p12 and Np12 form high-order oligomers in vitro and when expressed in E. coli produce organized structures that are visible by electron microscopy. Interestingly, Cp12, as well as the whole protein, can form dimers in the presence of SDS. The data show that both domains of p12 contribute to its ability to multimerize with much of this potential residing in its N-terminal part, most probably within the leucine zipper-like (LZL) sequence.

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The Mason–Pfizer monkey virus (M-PMV), or simian retrovirus type 3 (SRV-3), is a member of the betaretrovirus genus, which is characterized by the assembly of immature capsids from Gag polyprotein precursors within the cytoplasm of the infected cell. M-PMV Gag consists of structural domains that are proteolytically released during virus maturation to yield the matrix protein (MA; p10), phosphoprotein (pp18/24), p12 protein, capsid protein (CA; p27), nucleocapsid protein (NC; p14), and p4 protein (Bradac and Hunter, 1984). During translation of the primary viral transcript, two ribosomal frame-shift events produce additional precursors Gag–Pro and Gag–Pro–Pol. Following assembly, immature capsids are transported to the plasma membrane where they become enveloped through the combined interactions of the N-terminal myristic acid and the basic region of MA with membrane phospholipids. Through the acquisition of membrane, the particle also incorporates the viral envelope glycoproteins. Upon release, nascent particles undergo proteolytic maturation of the structural proteins and morphological rearrangement into infectious virions. As in all retroviruses, M-PMV Gag contains domains possessing characteristic motifs: the basic region in MA, the major homology region (MHR) in CA, the zinc fingers in NC, and the proline-rich motif in p4. Many retroviral Gag polyproteins contain additional sequences between the matrix and capsid domains (Yuan et al., 2000). In the case of M-PMV, these sequences consist of the pp18/24 domain, which contains a PYPY motif that is required for particle release (Yasuda and Hunter, 1998), and the p12 domain, which plays a role in efficient assembly of immature capsids and which is the subject of this report.

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Efficient assembly of immature capsids involves multiple sites of protein–protein interaction between Gag molecules. Several experimental approaches have provided support for the idea that NC plays a central role in Gag multimerization (Burniston et al., 1999; Zabransky et al., 2002) and is due to the interaction of NC with RNA (Muriaux et al., 2001). However, NC is not sufficient for efficient assembly. Interestingly, truncated forms of RSV or HIV Gag consisting of just CA-NC are capable of assembly into tubular or spherical structures in vitro (Campbell and Vogt, 1995; Gross et al., 1997). The shape of these structures depends on ionic strength and the presence of RNA. An additional critical shape determinant for assembled CA-NC for HIV and M-PMV is the N-terminal proline. Loss of this residue in M-PMV or N-terminal extension for both HIV and M-PMV CA-NC leads to assembly of spherical immature capsid-like structures (Rumlova-Klikova et al., 2000; Von Schwedler et al., 1998). The capability of CA alone to oligomerize has been confirmed for several retroviruses (Kingston et al., 2000; Lamman et al., 2002; Li et al., 2000; Mayo et al., 2003; Rumlová et al., 2001). Such CA-CA interaction is required for correct morphology of the mature virion and its infectivity (Bowzard et al., 2001; Cheslock et al., 2003; Dorfman et al., 1994; Forshey et al., 2002; Strambio-de-Castillia and Hunter, 1992). Additional Gag domains, such as M-PMV p12, may contribute to the interactions leading to the immature capsid and perhaps even to mature core formation.

The matured p12 protein consists of 83 amino acids and migrates as a 12 kDa band in SDS-PAGE, although its theoretical molecular mass is 9374 Da (Bradac and Hunter, 1984). It has been suggested that p12 contains a region, part of which must lie within residues 8–25, that is important for efficient capsid assembly in both HeLa cells (Sommefelt et al., 1992) and an in vitro translation system, and has been termed the internal scaffold domain (ISD; Sakalian and Hunter, 1999). The p12 domain can also facilitate the assembly of HIV-1 Gag in the in vitro translation system (Sakalian et al., 2002). Although several reports have described the ability of p12 to function in a scaffold-like manner, the molecular mechanism by which p12 promotes Gag–Gag interaction remains unknown. Interestingly, data from the two-hybrid system suggest that pp18/24–p12 might interact with the intracyttoplasmic chaperon TRiC (Hong et al., 2001). Molecular chaperones are important for the folding and assembly of capsid proteins in many virus systems (reviewed in Sullivan and Pipas, 2001).

In this article, we present an analysis of p12 and its N- and C-terminal halves for their propensity to self-associate into oligomers. Gel mobility, chemical cross-linking, and electron microscopy studies were used to investigate the contribution of the two p12 subdomains to oligomerization in vitro and in vivo. The predicted helical character of the N-terminal subdomain was confirmed by CD spectrometry.

Results and discussion

Sequence alignment

There are three well-characterized simian betaretroviruses: SRV-1, SRV-2, and M-PMV (SRV-3). Alignment of the M-PMV p12 sequence with the homologous sequences of SRV-1 and SRV-2 revealed that the N-terminal region, unlike the C-terminal subdomain, is highly conserved among these virus species (Fig. 1A), suggesting its importance. However, comparison with more distantly related retroviruses fails to reveal any significant sequence homology with p12. Examination of the p12 primary structure revealed a leucine zipper-like motif (LZL) that partially overlaps the region of residues 8–25 found to be necessary for ISD function (Sakalian and Hunter, 1999) (Fig. 1B). The LZL contains numerous valine, leucine, and isoleucine residues, three of which (Leu 22, Leu 29, Leu 36) are positioned in a heptad repeat. Although the residues are not precisely aligned, the similarity of this region to a leucine zipper suggests a mechanism for ISD function, as leucine zippers are an established oligomerization motif found in...

![Fig. 1. (A) Alignment of M-PMV p12 sequence with the homologous regions in SRV-1 and SRV-2 Gag (accession gene bank numbers P07567, AAC 97563 and AAA 47561, respectively). Identical amino acids are highlighted in gray fields. (B) Leucine zipper-like domain with leucine residues in a heptad repeat highlighted (bold, italics, large type). Additional adjacent hydrophobic residues are shown in bold.](image-url)
many proteins such as transcription factors (Harbury et al., 1993). Further support for the role of leucine zippers in retrovirus assembly comes from the functional replacement of HIV-1 NC by the GCN4 leucine zipper (Accola et al., 2000) and from the well-characterized functions of heptad repeats in the assembly and function of envelope glycoproteins such as gp41 of HIV, which could be further stabilized by C-terminal extension with the GCN4 sequence (Yang et al., 2000).

**Purification of recombinant proteins**

Sequences coding for p12 and both aforementioned organized domains, that is, the N-terminal conserved LZL motif comprised of amino acid residues 1–41 (Np12) and the more variable C-terminal part comprised of residues 43–83 (Cp12), were expressed and purified for investigation of their contribution to the function of p12. All p12-derived proteins were expressed in *E. coli* BL21(DE3) as fusion products including part of the phosphoprotein (p12, Np12) or CA (Cp12) extended by a hexahistidine tag (Fig. 2A). These fusions facilitated the purification of authentic p12 by use of the viral proteinase to release the desired product from the resin by cleavage. Cells were lysed and subjected to the electrophoretic analysis of supernatant and pellet from the low-speed centrifugation. It was found that the major part of all three fusion proteins was expressed as soluble proteins (not shown). Fusion proteins were adsorbed on Ni-NTA resin, washed as described in Material and methods, and cleaved by M-PMV protease to release the p12-related proteins (Fig. 2B). However, the major portion of p12 remained associated with the column probably due to hydrophobic interactions with the resin (not shown). For higher recovery, p12 was eluted with lysis buffer containing 1 M urea. The purity of isolated proteins was determined by SDS-PAGE Coomassie Blue staining and mass spectrometry and was higher than 95% for all (not shown).

**Purified p12 tends to oligomerize**

Analysis by SDS-PAGE using Tris–glycine buffer revealed that purified p12 migrates in at least three discrete bands corresponding to molecular masses of 12, 24, and 36 kDa (Fig. 3C). Western blot analysis using a monoclonal antibody against p12 confirmed that these bands correspond to proteins originating from p12 and represent SDS resistant dimers, trimers, and tetramers. Similar SDS-resistant oligomers were formed by Cp12 but the apparent efficiency was lower than for p12 (Fig. 3A). The migration of the p12 proteins is somewhat retarded in these Tris–tricine gels (Figs. 3A, B) as compared to in Tris–glycine (Fig. 3C). Surprisingly, Np12, which contains the LZL region, failed to produce any higher order complexes in the gel. Finally, formation of SDS-resistant oligomers was not affected by boiling at 100 °C (Fig. 3B).

![Fig. 2. (A) Schematic representations of the expressed fusion proteins. Black arrowheads show the cleavage sites for liberation of products from the affinity resin. (B) SDS-PAGE analysis of samples from purification steps for p12, Cp12, and Np12. Lane: M, molecular mass marker; 1, total uninduced cell lysate; 2, lysate from cells 4 h post-induction; 3, material bound to Ni-NTA after washing; 4, material released by M-PMV protease from Ni-NTA.](image)
Mass spectrometry of purified p12 was performed to rule out the presence of any higher molecular weight contaminants that could have produced the higher molecular weight bands in the gels (Fig. 4). Only one major peak corresponding to a molecular weight of 9344 Da was found, suggesting that the electrophoretic bands at 24 and 36 kDa correspond to dimers and trimers of p12. A minor difference of 3 Da was observed between the experimentally obtained (9344 Da) and the theoretical mass of p12 (9347 Da), which could be explained by an intramolecular disulfide bridge between cysteines 68 and 69. Indeed, the presence of a disulfide bond in p12 was confirmed by CD spectroscopy (not shown). The presence of this disulfide in vivo remains a matter of speculation. Curiously, the presence or absence of a reducing agent had little effect upon the oligomerization of p12 or Cp12 suggesting that the two cysteines in Cp12 do not rearrange to form an intermolecular bridge (Fig. 3A). Together, the data suggest that the SDS-resistant oligomers of isolated p12 are composed of noncovalently linked molecules.

The existence of SDS-resistant oligomers of other proteins has been described. Interestingly, similar behavior was published for the HIV-1 regulatory protein Vpr (Zhao et al., 1994), which contains a leucine- or isoleucine-rich domain. In addition, the stability of oligomers in the presence of SDS and during heating was also described for the α-toxin of Clostridium perfringens (Miyata et al., 2001), and for human epimorphin, which contains a coiled-coil region (Hirai, 1993). The formation of SDS-resistant oligomers can be dependent on protein folding as was shown with temperature-sensitive mutants of the tail spike endorhamnosidase of Salmonella phage P22 (Goldenberg et al., 1982). Nevertheless, the precise mechanism for SDS-resistant oligomerization remains unclear.

To further analyze the oligomerization properties of p12, we employed the chemical cross-linking agent DTSSP. SDS-PAGE analysis after chemical cross-linking showed the presence of a ladder of bands spanning approximately 23–60 kDa (Fig. 5A). Higher molecular weight products were visible in the gel, but were not sufficiently resolved to estimate their masses. The mobility of the lower molecular weight complexes relative to those formed by p12 in the absence of cross-linking agent suggests that these are dimers, trimers, tetramers, and so forth. Interestingly, these complexes exhibit slightly faster mobility than the corresponding SDS-resistant oligomers. This shift in mobility might be ascribed to the effect of the cross-linking agent and the position of the cross-link on the overall fold of the proteins (Westerhuis et al., 2000). Alternatively, the altered mobility is the result of amino acid modification; in particular, modification of lysines could affect binding of SDS and thus protein mobility in the gel (Aoki et al., 2003).

Fig. 3. Analysis of p12 species by SDS-PAGE. (A) Coomassie-stained Tris–tricine gel of p12, Np12, and Cp12 in the presence or absence of dithiothreitol. Arrowheads show SDS-resistant p12 and Cp12 oligomers. Molecular weight standards are indicated at left. (B) Coomassie stained Tris–tricine gel of protein samples run with and without prior boiling. (C) Silver-stained reducing Tris–glycine gel of p12 and Western blot analysis of p12 developed with an anti-p12 monoclonal antibody. The migration positions of monomer, dimer, trimer, and putative tetramer are indicated.
In contrast to the analysis above, treatment of Np12 with DTSSP resulted in a pattern of oligomer bands similar but not identical to that of p12 (Fig. 5B). This result shows that Np12 is capable of oligomerization and is consistent both with the location of the LZL region and with previous data that sequences in Np12 are essential to the scaffolding function of p12 (Sakalian and Hunter, 1999). By comparison, the cross-linking agent had no apparent affect upon the ability of Cp12 to oligomerize. The ability of Np12 to multimerize, together with the modest ability of Cp12 to form SDS-resistant oligomers, indicates that both Np12 and Cp12 likely contribute to the self-interaction of p12 and its ability to form SDS-resistant multimers.

To further confirm the role of the LZL region in oligomerization, a triple substitution mutant (LZ.A p12), where leucines 22, 29, and 36 were replaced with alanines, was created. SDS-PAGE analysis of this mutant showed a pattern of oligomerization similar to wt, but upon treatment with DTSSP, the mutant failed to form the higher ordered multimers characteristic of wt (Fig. 5A). Although some evidence of further oligomerization is apparent, the extent of these is dramatically less than for wt. How the LZL functions to facilitate large complex formation is unknown, but clearly is dependent upon the substituted leucines.

Expression of p12 in E. coli

Expression of retroviral proteins in bacteria has proven useful for the imaging and analysis of the resulting assembled structures (Gross et al., 2000; Joshi and Vogt, 2000; Klikova et al., 1995). We therefore similarly expressed p12 to investigate whether it is capable of assembling into an organized structure in the absence of the remainder of the Gag precursor. Electron micrographs of E. coli expressing p12 show accumulation of material under the cytoplasmic membrane that is clearly distinct from the inclusion bodies produced by expressed MA-p12 (Fig. 6C). Expression of Np12 resulted in the formation of structures with appearance comparable to those formed by p12. Interestingly, these Np12 structures were found inside of the cytoplasm compared to submembrane-localized p12 (Fig. 6A). Although no organized structures were observed for Cp12 (not

Fig. 4. Mass spectra of p12, Np12, and Cp12 measured by MALDI-TOF MS.

Fig. 5. Analysis of oligomerization by chemical cross-linking. (A) Coomassie stained Tris–glycine SDS-PAGE analysis of cross-linked p12 and mutant LZ.A p12. (B) Tris–tricine SDS-PAGE of cross-linked Np12 and Cp12. Concentration of DTSSP used in each reaction is given at top in millimolar. Molecular weight standards (M) are shown in kDa.
shown), its presence in full-length p12 might somehow promote its localization underneath the bacterial plasma membrane.

**Structural motifs of p12 and the implications for their interaction**

The CD spectra of p12 and Np12 (Fig. 7) show that both proteins possess α-helical characteristics. The proportion of α-helix was calculated to be approximately 70–75% and 30–35% for Np12 and p12, respectively. Parallel analysis of Cp12 suggested the absence of any periodical structural motif. Therefore, the α-helical portion of p12 is likely fully within the subdomain corresponding to Np12 in good agreement with the predicted secondary structure of p12 where the α-helix, corresponding to the LZL region, is located in the N-terminal domain and is expected to comprise 34% of the protein (Fig. 8).

The data described above suggest that p12–p12 interaction is primarily mediated through the N-terminal part of the protein. However, the C-terminal moiety that can form SDS-resistant oligomers and appears to contribute to the sub-membrane localization of p12 in bacterial cells might also contribute to this process. The presence of a heptad repeat in a region of the protein together with data demonstrating the ability of this region to oligomerize strongly supports the idea that p12 contains a leucine zipper. Leucine zippers are involved in many protein–protein interactions including transcriptional factors, regulatory and structural proteins (Burkhard et al., 2001), including those of many viruses (Pelletier et al., 1997; Perera et al., 2001). On the other hand, the presence of a leucine zipper in a retroviral Gag protein is unusual. Why does M-PMV Gag contain this unusual leucine zipper and the likely corresponding ISD? It has been suggested that the ISD acts to increase the effective concentration of Gag to facilitate intracytoplasmic assembly in infected cells, and for D-type retroviruses, substitutes for the membrane association utilized by other retrovirus Gag polyproteins during assembly (Sakalian and Hunter, 1999). This idea was supported by the observations that deletion of p12 within M-PMV Gag results in some assembly at internal cell membranes (Sommerfelt et al., 1992), and

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Fig. 7. CD spectra of purified p12-derived proteins. p12, Np12, and Cp12 spectra are shown as solid, dashed, and dotted lines, respectively, as indicated.

Fig. 8. Secondary structure prediction for p12. The amino acid sequence of p12 is presented by the single letter code. Numbers above the sequence indicate residue number and letters below the sequence indicate secondary structure. The cylinder illustrates the helical region. H, helix; E, extended helix; dashes, no structure determined.
insertion of p12 into HIV-1 Gag promotes its membrane-independent assembly in an in vitro low expression system (Sakalian et al., 2002). The placement of the LZL in the same region of M-PMV Gag as the scaffold-like function of the ISD strongly suggests the identification of a structure–function relationship. Direct evidence for the heptad repeat in ISD function will await perturbation of the structure by site-directed mutagenesis and analysis of the resulting assembly phenotypes.

Material and methods

Constructs

pET15bHis6ΔPP-p12 was prepared as follows: the DNA sequence encoding ΔPP-p12 was amplified by PCR using pG10GAG (Klikova et al., 1995), containing M-PMV gag, as the template and primers 5′-CCACCTCCTGAGAA-TTAAGCTACTCTTCCCCGAGGG-3′ and 5′-CGTGCTCGAGCTATTAGAAAATATCTTTGGG-3′ containing an XhoI cleavage site. The PCR product was cleaved with XhoI and inserted into pET-15b (Novagen). pET15b-His6ΔPPNp12 was constructed by creation of a stop codon (42Val→stop) in pET15bHis6ΔPP-p12 by site-directed mutagenesis using primers 5′-AAACAGGGGATTGAAACTTTAACCTCACCAGACAGC-3′ and 5′-GCTGTGTCTGGGTGAGTGAGCCATTTATATATATATTTATTTGGG-3′ containing an XhoI cleavage site. The pPCR product was cleaved with XhoI and inserted into pET-15b (Novagen). pET15b-His6ΔPP-p12 was constructed by creation of a stop codon (42Val→stop) in pET15bHis6ΔPP-p12 by site-directed mutagenesis using primers 5′-AAACAGGGGATTGAAACTTTAACCTCACCAGACAGC-3′ and 5′-GCTGTGTCTGGGTGAGTGAGCCATTTATATATATTTATTTGGG-3′ containing an XhoI cleavage site. The PCR product was cleaved with XhoI and inserted into pET-15b (Novagen). pET15b-His6ΔPPNp12 was prepared by PCR from pG10GAG using primers: 5′-GGGAATGAAACATATGACTCACCCA-GACACAGCAGG-3′ and 5′-GACGGCAAACTCGAGAC-CATTATGTGTCTCCAGGC-3′ containing an NdeI and XhoI site, respectively. The PCR product was cleaved with NdeI and XhoI and inserted into pET-22b (Novagen). pET22p12 was similarly constructed using primers 5′-CGGCACACCACTATGAGCCCTTTTATATATATTTATTTGGG-3′ and 5′-CGGTCTCGAGCTATTAGAAAATATCTTTGGG-3′. The PCR product was digested with NdeI and XhoI and inserted into pET22b (Novagen). pET22Np12 was prepared from pET22p12 by site-directed mutagenesis to insert a stop codon in the same way as for pET15bHis6ΔPPNp12 (see above).

Expression vector pET15bHis6ΔPP-LZ-Ap12 was constructed for PCR mutagenesis using forward primer 5′-GAGGAAACTATGAGCCCTTTTATATATATTTATTTGGG-3′ (the complementary reverse primer sequence is not shown, mutations Leu to Ala are underlined) and pET15bHis6ΔPPNp12 as template.

Purification of recombinant proteins

The His6-tagged proteins were expressed in E. coli BL21(DE3) by induction with IPTG in LB for 4 h at 37 °C. Cells (200-ml-volume) were collected by centrifugation at 5000 × g for 30 min. The cell pellets were resuspended in 20 ml of lysis buffer (100 mM NaCl, 10 mM imidazole, 50 mM Na2HPO4, pH 8.0), 100 μg/ml lysozyme, 50 μg/ml RNase, and 50 μg/ml DNase were added, and the cell suspension was incubated for 1 h at room temperature followed by disruption by sonication. The lysates were clarified by centrifugation at 40,000 × g for 30 min at 20 °C. The resulting supernatants were incubated with 1 ml Ni-NTA resin (QIAGEN) for 2 h at 4 °C. After washing with 30 ml of buffer (150 mM NaCl, 50 mM Na2HPO4, 1.5 M urea, pH 8.0), the resin was resuspended in 1 ml of M-PMV protease buffer (900 mM NaCl, 100 mM Na2HPO4, pH 6.2). M-PMV protease (0.05 mg/ml) was used for release of p12 from His6 tag fusion proteins bound to the resin. The cleavage was performed overnight at room temperature. Released protein was concentrated to approximately 0.75 mg/ml on a Centricon plus column.

Cross-linking experiments

Purified proteins were dialysed overnight at 4 °C against 50 mM phosphate (pH 7.4) and 100 mM NaCl. Cross-linking reactions were performed at a protein concentration of 0.5 mg/ml in the presence of 3,3′-dithiobis(sulfo succinimidy) propionate (DTSSP) (final 1.2 or 0.6 mM concentration) for 1 h at 25 °C. The reaction was stopped by the addition of 100 mM glycine. Products were analyzed by Tris–glycine or Tris–tricine SDS-PAGE (Schägger and Jagow, 1987).

Mass spectrometry

Molecular weights of proteins were measured by MALDI-TOF mass spectrometry (Bruker, Biflex IV) with the machine set in a linear mode. 3,5-Dimethoxy-4-hydroxy-cinnamic acid, dissolved in a mixture of acetonitrile/0.1% trifluoracetic acid (1:2), was used as the matrix.

Electron microscopy

Bacterial cells expressing the p12-derived proteins were collected by centrifugation 4 h post-induction, washed three times with PBS (phosphate-buffered saline), fixed in 2.5% glutaraldehyde in phosphate-buffered saline, and then processed for conventional thin section electron microscopy as described previously (Klikova et al., 1995).

CD spectroscopy

CD spectra were measured on a Jobin-Yvon Mark V spectrometer at a protein concentration of 0.3 mg/ml in 50 mM phosphate buffer, pH 7.4, containing 150 mM NaCl. CD spectra were presented as dependence of mean residual ellipticity on wavelength. Spectra are processed by GRAMS/Al software.
Secondary structure prediction

The p12 sequence was analyzed by jpred software using prediction by alignment, neuronal network, PSIBLAST frequency profile, hmm profile, PSIBLAST, and pssm profile.

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