Co-expression of the capsid proteins of Cowpea mosaic virus in insect cells leads to the formation of virus-like particles

Michael Shanks and George P. Lomonossoff

Department of Virus Research, John Innes Centre, Colney Lane, Norwich NR4 7UH, UK

Cowpea mosaic virus (CPMV) is the type member of the comovirus group of plant viruses, which are classified together with faba- and nepoviruses as genera of the family Comoviridae. CPMV has a genome consisting of two molecules of positive-strand RNA (RNA-1 and RNA-2) which are separately encapsidated in icosahedral particles. Both genomic RNAs are expressed through the synthesis and subsequent processing of large precursor polyproteins (for a review, see Goldbach & Wellink, 1996). RNA-1 directs the synthesis of a single polyprotein of approximately 200 kDa (the ‘200K’ protein) which is processed in cis by the 24K proteinase domain to give rise to proteins involved in RNA replication. RNA-2 directs the synthesis of two carboxyl co-terminal polyproteins (the 105K and 95K proteins) which are processed by the RNA-1-encoded 24K proteinase in trans at two sites to give the 58K/48K pair of proteins and the Large (L) and Small (S) coat proteins (Fig. 1a).

CPMV particles contain 60 copies each of the L and S proteins arranged with pseudo $T = 3$ ($P = 3$) symmetry (Lomonossoff & Johnson, 1991). These are situated around the 3- and 5-fold symmetry axes and contain two and one $\beta$-barrels, respectively. Though a high resolution structure of the mature virus particle is available (Lin et al., 1999), little is known about the mechanism of virus assembly. It has proved impossible to develop an in vitro assembly assay since the L and S proteins isolated from virions are insoluble in the absence of denaturants (Wu & Bruening, 1971). In vivo studies have been hampered by the fact that CPMV moves from cell-to-cell in the form of particles; thus mutations which perturb assembly also have dramatic effects on virus viability (Wellink & van Kammen, 1989). For many years, it was believed that a 60 kDa protein (the 60K protein of Franssen et al., 1982), consisting of the fused L and S proteins, played an obligate role in the assembly process (see Lomonossoff & Johnson, 1991). However, experiments using transgenic plants (Nida et al., 1992) or hybrid comoviruses (Clark et al., 1999) have shown that the uncleaved 60K protein cannot form virus-like particles. Further doubts on its role were cast by the observation that the individual (unfused) L and S proteins are able to form virus-like particles when expressed transiently in protoplasts (Wellink et al., 1996).

Expression of the coat proteins of isometric plant viruses in heterologous systems has proved a valuable approach to the analysis of particle formation (Bertioli et al., 1991; Singh et al., 1995; Zhao et al., 1995; Yusibov et al., 1996; Sastri et al., 1999). One common feature of these previous studies is that they all involve the assembly of particles which consist of only a single viral coat protein. In addition, in most cases the coat protein subunits isolated from virions were known to be capable of self-assembly in vitro. To extend the use of heterologous expression systems to study the assembly of a plant virus with more than one capsid protein and for which no in vitro or in planta assembly system is available, we have investigated the ability of the coat proteins of CPMV expressed in insect cells to assemble into virus-like particles.

The sequences of the individual CPMV L and S proteins were obtained from plasmids pMMVP37 and pMMVP23, respectively. These plasmids were previously used for the transient expression of the individual coat proteins in proto-
Fig. 1. Diagrammatic representation of CPMV RNA-2 and the constructs used to express the viral coat proteins. (a) Genetic organization of RNA-2, indicating the regions of the open reading frame (ORF) encoding the 58/48K proteins and the L and S coat proteins. The amino acid pairs indicate the sites at which cleavage to release the L and S proteins occurs. The numbers below the genome indicate the positions of the two in-frame initiation sites for 105K and 95K precursor polyproteins and the stop codon (TAA) at the end of the ORF. VPg is the small protein attached to the 5′ end of the RNA and the poly(A) tail at the 3′ end is indicated by (A)n. The BamHI site (position 1504) which was used in the construction of the various plasmids (Wellink et al., 1996) is also shown. (b) Structures of the donor plasmids pFB-L, pFB-S and pFB-LS used to express the CPMV coat proteins either singly or together. The restriction sites used to insert DNA fragments from pMMVP37 (for the L protein) and pMMVP23 (for the S protein) are indicated. Solid arrows indicate relative position of the promoters (Polh is the polyhedrin promoter) which drive the expression of the proteins. Numbers and thin arrows indicate position and direction of transcription with respect to the parental donor vector. * Indicates the position of a stop codon.

In the case of pMMVP37, a TGA stop codon had been introduced into the RNA-2 sequence immediately after the region encoding the L protein, initiation occurring at the natural N-terminal methionine. For pMMVP23 a single methionine had been added upstream of the natural N-terminal glycine of the S protein to allow initiation, termination occurring at the TAA found at the C terminus of the RNA-2-encoded polyproteins. To obtain pFB-L, a baculovirus donor plasmid containing the sequence of the L protein under control of the polyhedrin promoter (Polh), the BglII–EcoRI fragment from pMMVP37 was ligated into BamHI/EcoRI-digested pFastBacDual (Life Technologies). To obtain pFB-S, a donor plasmid containing the sequence encoding the S protein downstream of the p10 promoter, the BglII–KpnI fragment from pMMVP23 was ligated into BbsI/KpnI-digested pFastBacDual. To obtain pFB-LS, a plasmid that contained the sequences of both the L and S proteins under the control of different promoters, the BglII–KpnI fragment obtained from pMMVP23 was ligated into BbsI/KpnI-digested pFB-L. All constructs were propagated in E. coli strain DH5α. The structures of the plasmids were verified by restriction enzyme digestion and DNA sequence analysis and are shown in Fig. 1.

Site-specific transposition of the various plasmids into competent DH10Bac E. coli cells, isolation of recombinant bacmid genomic DNA and subsequent transfection of Spodoptera frugiperda (sf21) insect cells was carried out as recommended by the manufacturers of the Bac-to-Bac Baculovirus Expression Systems (Life technologies Ltd). The recombinant baculoviruses, termed bv-L, bv-S and bv-LS, were harvested from the sf21 cells 3 days post-transfection.
The ability of the recombinant baculoviruses to direct the expression of the CPMV coat proteins was examined by infecting monolayers of sf21 cells with either bv-L, bv-S or bv-LS as they approached confluent growth. After 3 days incubation at 28 °C, the cells were resuspended in the culture medium and harvested by low speed centrifugation. The pellets were resuspended in 62.5 mM Tris–HCl, pH 6.8, 2% (w/v) SDS and analysed by electrophoresis in 15% (w/v) SDS–polyacrylamide gels (Laemmli, 1970). Coomassie blue staining of the gel (Fig. 2a) showed that cells infected with either bv-S or bv-LS contained an additional band of the size expected of the S protein (lanes S and LS) that was not present in cells infected with bv-L (lane L). The L protein was less readily detected in the Coomassie blue-stained gel as it was partially masked by other proteins of a similar size in the cell extracts. However, a band running just behind the 36.4 kDa marker, the expected size of the L protein, can be seen in lanes L and LS. Western blot analysis using an antiserum raised against CPMV virions (Fig. 2b) clearly identified the polypeptide seen in lanes S and LS as the CPMV S protein and confirmed the presence of the L protein in cells infected with either bv-L or bv-LS. The baculovirus-expressed S protein had the size (approximately 24 kDa) expected of the C-terminal non-processed form of the S protein (Ss; Lomonossoff & Johnson, 1991). Comparison with standards containing known amounts of proteins isolated from virus particles indicated that
the level of expression obtained was approximately 1 ng of each of the L and S proteins per 10^6 sf21 cells.

To determine whether the proteins expressed in sf21 cells were capable of assembling into virus-like particles, monolayers of cells infected with the various recombinant baculoviruses were washed with serum-free medium and lysed by addition of NP40 to 0.5% (v/v). The mixture was left at 4 °C for 15 min and cell debris was removed by centrifugation at 10,000 r.p.m. for 15 min. Portions of the lysates were examined by immunosorbent electron microscopy using grids coated with rabbit polyclonal antiserum raised against CPMV particles diluted 1:1000 in 0.06 M sodium phosphate pH 6.5. After incubation for 1 h at 4 °C, the grids were washed with buffer and negatively stained with 2% (w/v) uranyl acetate. As a control, a sample of 50 ng/ml of purified CPMV virions in 0.5% (v/v) NP40 was examined in the same way. In the sample from cells infected with construct bv-LS many virus-like particles, similar in appearance to wild-type CPMV particles obtained from plants, could be seen (Fig. 3). No such particles were observed in extracts from cells infected singly with either bv-S or bv-L or co-infected with these two recombinant baculoviruses. These observations show that the CPMV particles can be assembled from the individually synthesized L and S proteins when both are expressed within the same cell from bv-LS; the lack of observable particles found in extracts of cells doubly infected with bv-S and bv-L probably reflects the fact that many of the cells were not infected with both constructs.

Electron micrographs of virus-like particles produced in insect cells infected with bv-LS showed the presence of both ‘full’ (stain excluded) and ‘empty’ (stain permeable) particles, a situation similar to that found with natural CPMV preparations (Fig. 3). Stain penetration only occurs when particles are devoid of RNA (Top component) though not all protein-only shells are permeable. The degree of permeability is governed, at least in part, by the presence or absence of the C-terminal 24 amino acids of the S protein (J. E. Johnson, personal communication). Thus the appearance of apparently ‘full’ particles in the micrographs does not necessarily imply that these particles contain RNA, especially since S protein produced in the insect cells is unprocessed. To resolve this issue, virus-like particles present in extracts of cells lysed with NP40 were concentrated by centrifugation at 27,000 r.p.m. in a Beckman type 30 rotor for 4 h at 4 °C. The resulting pellets were resuspended in 400 μl 100 mM NaCl, 50 mM Tris–HCl pH 7.6, 0.5% (v/v) NP40 and layered onto 5 ml 15–30% (w/v) sucrose gradients buffered with 100 mM NaCl, 50 mM Tris–HCl pH 7.6. The gradients were centrifuged for 2 h at 45,000 r.p.m. in a Beckman SW 50.1 rotor at 4 °C after which approximately 0.5 ml fractions were collected. The samples were diluted with 1 ml dH₂O and proteins in each fraction precipitated by the addition of 20 μg BSA, 100 μl 10% (w/v) SDS and 5 ml acetone. After standing for 2 min at room temperature the mixture was centrifuged for 5 min at 12,000 r.p.m. The pellets were analysed by Western blotting using anti-CPMV antibodies as described above. The analysis of fractions from the gradient showed that the L and S proteins exactly co-sedimented on the gradient and formed a relatively sharp peak. This peak corresponded with the position of sedimentation of purified CPMV Top component (empty capsids) centrifuged in a parallel gradient (Fig. 2c). The co-sedimentation of the L and S proteins is consistent with the formation of authentic virus-like particles. Their position in the gradient suggests that the virus-like particles are substantially free of nucleic acid since the nucleoprotein components sediment substantially more quickly in the sucrose gradients.
used for the analysis. However, the incorporation of small amounts of nucleic acid cannot be ruled out. When extracts from cells expressing the L and S proteins individually were analysed in the same way, but omitting the initial high speed centrifugation step designed to concentrate virus-like particles, the immuno-reactive material was evenly distributed across the gradients, with no clear peak of sedimentation visible (data not shown). This material probably represents aggregates of the individual L and S proteins of undefined size which may or may not be relevant to virus assembly.

We have demonstrated that CPMV virus-like particles can be obtained by the co-expression of the L and S proteins in insect cells. The results presented here are consistent with those obtained with transient expression of the CPMV coat proteins in protoplasts (Wellink et al., 1996) and effectively rule out the obligate involvement of 60 kDa L–S fusion protein in particle assembly. However, it is still perfectly possible that association between the L and S proteins occurs most rapidly when both are processed from the same polyprotein. The results presented here also make it less likely that host components might be involved in the assembly process (Wellink et al., 1996) since such host components would have to be common to both insect and plant cells.

The development of the heterologous system for CPMV morphogenesis reported here will be very useful for studies into the mechanism of virus assembly. Unlike the transient expression studies, the expression in insect cells can readily be scaled up, potentially permitting structural studies of any assembly intermediates. In this regard, it is important to note that, in contrast to the situation in cowpea protoplasts (Wellink et al., 1996), the individually expressed L and S proteins are stable in insect cells. Thus the baculovirus system reported here can be used to examine the structures of any intermediates formed by the individual coat proteins.

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References


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