Structural and Functional Characterization of Sesbania Mosaic Virus

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Abstract

The genus *Sobemovirus* includes isometric, single-stranded positive sense RNA plant viruses that infect monocots and dicots. Sesbania mosaic virus (SeMV) is the only sobemovirus, which has been studied extensively in India. SeMV, which was identified in southern India, is highly stable and multiplies to high concentration causing mosaic symptoms on the leaves of *Sesbania grandiflora*. SeMV has served as a good model for understanding the molecular mechanism of assembly, replication and movement of sobemoviruses. The structure of the virion and other chimeric VLPs, along with biochemical investigations has resulted in the elucidation of the mechanism of assembly of SeMV. The complete genome sequence, structure and function of non-structural proteins have also been determined. The review presents a summary of all these findings. SeMV being non-toxic and biodegradable and of low immune response, has been shown as a good candidate for biomedical applications.

Keywords

Sesbania mosaic virus • Structure of SeMV • Virus-like particle • Infectious clone

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18.1 Introduction

Sobemoviruses (genus *Sobemovirus*, family unassigned) constitute a group of isometric plant viruses named after the type species *Southern bean mosaic virus* (SBMV). In 1969, Walters proposed classification of all single stranded RNA viruses that are transmitted by beetles as a single group (Walters 1969). Hull (1977) recommended the establishment of sobemovirus group as a separate genus based on the sedimentation coefficient, stability of capsids, distribution of particle morphology and the properties of the capsid protein. International Committee for the Taxonomy of Viruses identified sobemovirus group as an unassigned genus in 1995 and the same taxonomic status has been retained thus far (King et al. 2012). Sobemoviruses have a very narrow host range infecting 15–20 plant families that include both monocots and dicots. Their geographic distribution is wide spread and they are of considerable economic importance, as they cause disease in several commercial crops such as legumes and rice in most parts of the world. Sobemoviruses under natural conditions are transmitted by insects or seeds. Different types of insects, chrysomilidae beetles, aphids, myrids, leafhoppers and leaf miners, have been reported to transmit sobemoviruses, however, the transmission may be due to contamination of insect mouth parts. Under the greenhouse conditions, sobemoviruses can readily be transmitted by mechanical sap inoculation. The infection of sobemoviruses leads to accumulation of large amount of virus in mesophylls and vascular tissues (Tamm and Truve 2000a). The genus *Sobemovirus* contains as many as 19 members of which only sesbania mosaic virus (SeMV) is known to occur in India. SeMV is not a significant viral pathogen in India, although, a considerable body of literature has been generated on virion structure, assembly and gene function.

18.2 Biological Properties

SeMV was identified in *Sesbania grandiflora* showing mosaic disease in the farmer's field around Tirupati, Andhra Pradesh, India (Solunke et al. 1983). SeMV infected sesbania plants show systemic yellowish green irregular patches within 10 days post sap inoculation (Fig. 18.1). At the later stage of infection, the lower leaves appear to be asymptomatic although the younger leaves continue to show symptoms. However, on *Cyamopsis tetragonoloba* plants, chlorotic lesions appear on the primary inoculated leaves but the infection is not systemic in nature. The dilution end point of this virus ranges from 1:300,000 to 1:500,000 and the thermal inactivation temperature is between 80 and 90 °C. The longevity in vitro is up to 21–28 days at 33 °C and 87 days at 8 °C indicating that the virus particles are very stable (Gopinath et al. 1994).

SeMV was purified from the infected sesbania leaves (100 g) harvested 20–30 days post inoculation by sucrose gradient centrifugation. The peak fractions containing the CP of size 29 kDa were pooled, re-centrifuged at 40,000 g for 3 h, resuspended and stored at 4 °C. The virus yield was 70–80 mg/100 g leaf material. Electron microscopy of the purified virus revealed spherical particles of 30 nm diameter (Gopinath et al. 1994).

Fig. 18.1 Symptoms of *Sesbania mosaic virus* on *Sesbania grandiflora*

18.3 Genome Sequence Properties

The genomic RNA isolated from the purified virus is of size ~4 Kb. As the genome lacked a poly(A) tail, cDNA synthesis was carried out using an oligo(dT) primer on polyadenylated SeMV genomic RNA as the template. cDNA synthesis was also carried out using various sequence specific primers. The cDNA clones were sequenced either manually or by automated sequencing and the sequences were compiled using the FRAGMENT ASSEMBLY program of Wisconsin GCG package. Figure 18.2 shows the cladogram obtained from the complete genomic sequence of all the 19 species of sobemoviruses annotated thus far. The cladograms obtained using different ORFs are nearly identical suggesting that recombination events are probably not frequent or significant in the sobemovirus group. Also, within the sobemovirus group, viruses that infect dicotyledons cluster as a closely related group (I), while those that infect monocotyledons form another distinct group (II). The analysis clearly indicated that SeMV is a new member of the genus sobemovirus and is not a strain of SBMV (Lokesh et al. 2001).

The SeMV genome is 4148 nts long with a viral protein genome linked (VPg) at the 5′ end and it lacks a poly(A) tail or a tRNA-like structure at the 3′ end (Fig. 18.3). The genome is very compact and encodes for three overlapping open reading frames (ORFs). The 5′ proximal ORF (ORF-1) codes for the movement protein (MP), that helps in the cell to cell movement of the virus. The coat protein (CP), encoded by the 3′ proximal ORF (ORF-3) is involved in the encapsidation of the viral genome. The central ORF (ORF-2) codes for two polyproteins, 2a and 2ab. The ORF-2a codes for polyprotein 2a with the domain arrangement: membrane anchor (TM) – protease – VPg-P10-P8 domain. The ORF-2b codes for RNA-dependent RNA polymerase (RdRp) which is expressed as polyprotein 2ab with the domain arrangement: TM – protease-VPg-RdRp.

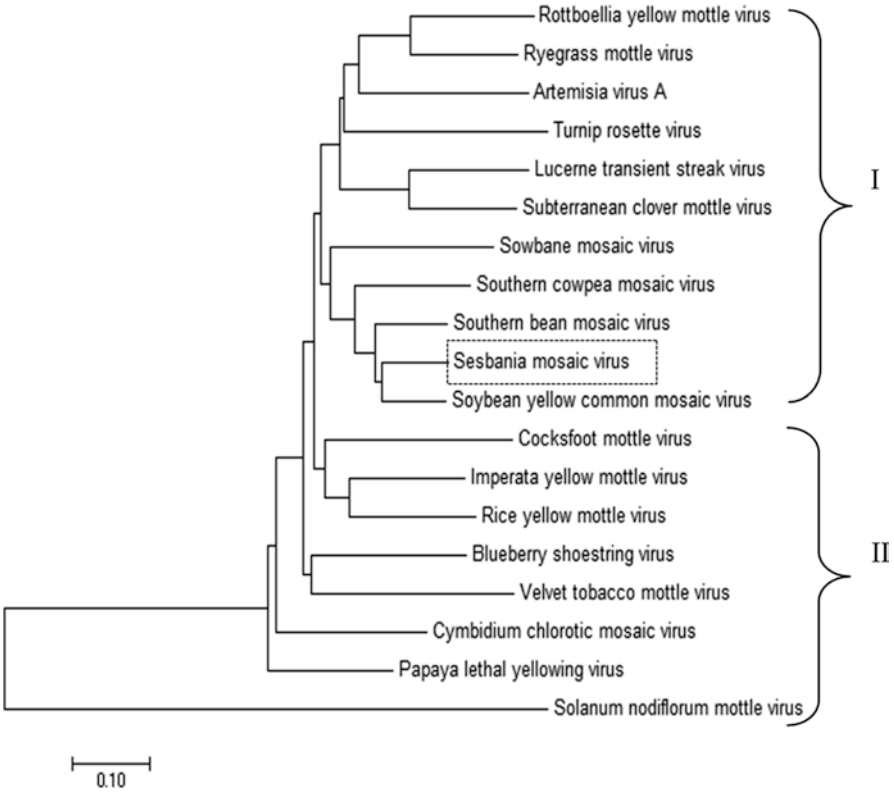

Fig. 18.2 Phylogenetic tree of sobemoviruses based on the complete nucleotide sequence of their genomes obtained using neighbour-joining method. The tree illustrates the relationship between the nineteen species of sobemoviruses

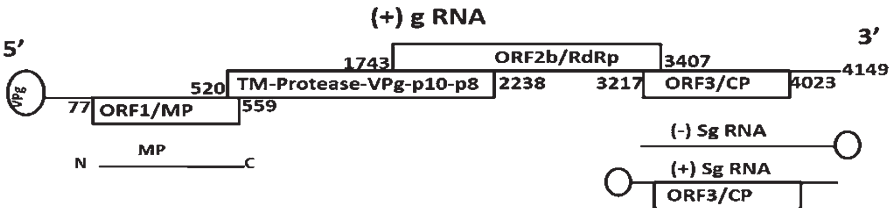

Fig. 18.3 Genome organization of Sesbania mosaic virus

18.4 Translation Mechanism

It has been proposed that only the 5′proximal ORF is translated in a eukaryotic mRNA (Kozak 1991). A comparison of the nucleotide sequence surrounding the ORF1 with the Kozak consensus sequence (A at −3 position and G at +4 position),

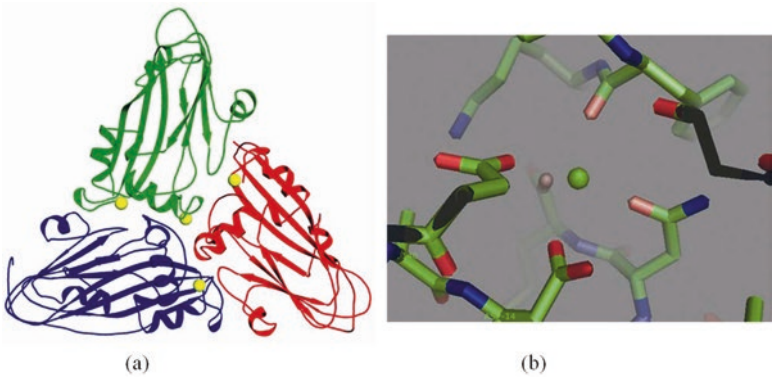

Fig. 18.4 (**a**) Organization of the three subunits A, B and C in the icosahedral asymmetric unit of SeMV capsid. The polypeptide folds are shown as ribbon diagrams. The amino terminal segment ordered only in C subunits is at the left lower edge of the Figure. *Spheres* correspond to ions *bound* to the capsids. The ions bound at the inter-subunit interfaces are calcium ions. (**b**) The geometry of calcium binding. This geometry is common to both T = 3 and T = 1 capsids of SeMV (Savithri and Murthy 2010)

that is suggested to yield a strong initiation site, revealed that the start codon of ORF1 is in a poor context for translation when compared to that of ORF2. ORF1 has a pyrimidine at the −3 position and does not have a G at the +4 position (Lutcke et al. 1987; Cavener and Ray 1991; Lokesh et al. 2001). In contrast, ORF 2 has a purine at the −3 position (Demler and de Zoeten 1991; Miller et al. 1995; Lokesh et al. 2001). Therefore, ribosomes which skip the first AUG codon, scan through the genomic RNA for the next potential start codon for translation initiation (leaky scanning mechanism) (Sivakumaran and Hacker 1998; Tamm and Truve 2000b).

The RdRp (coded by ORF 2b) is translated by a −1 ribosomal frame shift mechanism wherein the tRNAs in the ribosomal A and P sites simultaneously slip back one base on the mRNA before peptide bond formation, and the translation is resumed in the new (−1) reading frame (Fig. 18.4a, b). Pausing of ribosomes required during −1 ribosomal frame shifting is induced by the adjacent pseudoknot or stem-loop structure of genomic RNA. The slippery sequence, UUUAAAC and a stem-loop structure were found to be conserved at the −1 ribosomal frame shift site across all the members of sobemoviruses indicating that this mechanism of frame shifting is common to all sobemoviruses. However, the frequency of the frame shift appears to be very low and it is probably a regulatory mechanism for low expression of the viral encoded RdRp (Makinen et al. 1995; Tamm et al. 2009).

The CP is translated from the 3′ terminal ORF3. The start codon of this ORF is far from the 5′ terminus of the genomic RNA. The initiation of translation is efficient only when the initiation codon is proximal to the 5′ end. Therefore, many viruses follow a specialized mechanism involving synthesis of subgenomic RNAs (sgRNA) for efficient translation of the 3′ proximal ORFs. Sobemoviruses express sgRNA for the translation of CP, which is required in high amounts for encapsidation of the virus genome. Translation of CP via the sg RNA also leads to temporal

regulation of the CP during the viral life cycle. Although presence of sgRNA has been reported for sobemoviruses, the mechanism of its expression has not been reported so far. Another strategy used by SeMV and other sobemoviruses is polyprotein processing in which the polyprotein 2a and 2ab are cleaved into functional products by the protease domain autocatalytically. The mechanism by which this processing is regulated is discussed later.

18.5 Structure and Assembly of SeMV

18.5.1 Three-Dimensional Structure of Native Virus

Structural studies on SeMV carried out at IISc, Bangalore using single crystal X-ray diffraction has provided essential details on the size of the virus particles, radial distribution of protein, three dimensional structure of the coat protein, capsid architecture, protein-protein, RNA-protein and metal ion mediated interactions, that account for the stability of the capsids. The three-dimensional structure of SeMV is the first viral structure reported from Asia. SeMV viral capsid comprises of 180 chemically identical subunits arranged in three different conformations/molecular environments termed as A, B and C. The A type subunits form 12 pentamers at the vertices of the icosahedral particle, whereas the B and C subunits form 20 hexamers resulting in icosahedral T = 3 symmetry (Caspar and Klug 1962).

There are two types of dimers, the C/C dimers related by the exact icosahedral twofold symmetry and the A/B dimers related by the quasi- two- fold symmetry. The coat protein consists of two domains viz. the N terminal random (R) domain which is followed by a well ordered shell domain (S domain) consisting of eight antiparallel β-strands connected by loops and a few helices. The amino terminal 71 residues are disordered in the A and B subunits (shown in green and red, respectively), whereas the amino terminal arms of the C subunit shown in blue are ordered from residue 44 (Fig. 18.4a). These additional ordered residues result in the formation of a β-annulus like structure at the quasi -six fold axes. The combination of β-annulus and the dimeric interactions of two- fold related protein subunits result in a scaffold that connects all the C subunits of the capsid. Therefore, it was proposed that the formation of β-annulus might initiate the assembly of T = 3 particles. The asymmetric unit of these icosahedral particles consists of the three CP subunits, A, B and C along with three interfacial calcium ions, resulting in a total of 180 calcium ions per capsid. These calcium ions of the icosahedral asymmetric unit located at A-B, B-C, C-A interfaces are related by the quasi- three- fold symmetry of the T = 3 particles. The calcium ions are coordinated by six ligands (D146 and D149 from one subunit and Y203, N267 and N268 from a neighbouring subunit and a water molecule hydrogen bonded to S116; Fig. 18.4b). The calcium binding motif was found to be conserved across various other sobemoviruses indicating its crucial role in the overall integrity and assembly of the capsids (Subramanya et al. 1993; Bhuvaneshwari et al. 1995).

18.5.2 Biophysical and Structural Studies on the Recombinant Capsids

Over expression of the SeMV CP in *E. coli* resulted in the formation of T = 3 virus-like particles (VLPs) that resembled the native virus particles and were also found to encapsidate CP mRNA as well as *E. coli* 23S rRNA. The thermal stability of the VLPs (Tm ~87 °C) was comparable to that of the wild type particles (Tm ~91 °C) (Lokesh et al. 2002). The three-dimensional structure of these VLPs was found to be very similar to that of the native virus (Sangita et al. 2005). In order to dissect the role of the N-terminal domain of the CP in viral assembly, deletion mutants of CP, CP-NΔ36 and CP-NΔ65 were over expressed *in E. coli* and purified. Electron microscopy of CP-NΔ36 capsids revealed the presence of predominantly T = 1 capsids (with 60 subunits) and a few pseudo T = 2 particles (with 120 subunits). However, deletion of 65 residues from the N terminus of CP (CP-NΔ65) resulted in exclusive formation of T = 1 particles which were less stable compared to the native virus with a Tm of 83 °C (Lokesh et al. 2002). The structure of these T = 1 particles was determined (Sangita et al. 2004) and the recombinant T = 1 particles were found to bind calcium ions in a manner identical to those of native capsids. The organization of the icosahedral dimeric unit in the T = 1 structures is similar to that of the quasi-dimer (AB dimer) of the T = 3 structure. Further, the structure of CP-NΔ31and CP-NΔ36T = 1 particles, were very similar to those of CP-NΔ65 particles (Sangita et al. 2004, 2005). These observations indicated the possible role of the N-terminal disordered domain of CP in the interaction with the RNA, which might be critical for the assembly of T = 3 particles.

There is an arginine rich motif (RRNRRRQR, residues 28–36; N-ARM) within the N-terminal R domain of SeMV CP, which is conserved across sobemoviruses. In order to investigate the role of the N-ARM in viral assembly, substitution mutations were carried out where some (CP-R32-36E) or all of the arginines (CP-R28-36E) were mutated to glutamate residues. These mutant CPs assembled in to empty T = 3 particles and were less stable. These results confirmed the role of the N-ARM in the encapsidation of the RNA (Satheshkumar et al. 2005a). Further, the N-terminal 65 residues of SeMV CP were replaced with polypeptides of similar length, such as the SpA-B-domain (SeMV-B; R-domain replaced by *Staphylococcus aureus* B-domain of Protein A), SeMV-P8 and SeMV-P10, but possessing significantly different structural features. Interestingly, all the chimeric CPs assembled into VLPs, but the particles were heterogeneous. However, TEM analysis of NΔ65-P8 revealed more uniform T = 3 particles. There is no sequence similarity between P8 and the R-domain, however, both these domains are intrinsically disordered and have similar pI and are rich in basic residues. These results suggest that the presence of a positively charged disordered domain promotes the least error-prone particle assembly. The crystal structure of NΔ65-BCP showed that substitution of the R-domain of SeMV CP with the B-domain lead to assembly of T = 1 particles (and not T = 3 particles) and that the B-domain was also disordered. Thus, particle assembly can also induce disorder in the otherwise ordered B-domain (Gulati et al. 2016).

In SeMV, the hydrogen bonding interactions between residues 48–52 from one C-subunit and residues 55–59 of the neighbouring three fold related C-subunit leads to the formation of β-annulus. To examine the importance of the β –annulus in viral assembly, the residues involved were (CP-Δ48–59) deleted. Interestingly, this mutant could assemble into T = 3 particles and the capsid stability was also unaffected. The structure of the CPΔ48–59 capsids showed the absence of the β-annulus in the structure. These results indicated that β-annulus is not essential for capsid assembly. It is plausible that the formation of β-annulus is a consequence of the viral assembly (Pappachan et al. 2008). Further, mutations targeting the interfacial residue (W170) occurring near the five fold axes resulted in complete disruption of T = 3 particle assembly and formation of dimers. Crystal structure of these dimers showed that they are of type A/B related by quasi two fold axes and not of type C/C related by icosahedral two fold symmetry in the T = 3 particles (Pappachan et al. 2009).

The three-dimensional structure of the native virus indicated that D146 and D147 are crucial for calcium binding. Interestingly, CP-NΔ65-D146N-D149N capsids were similar to those of CP-NΔ65 T = 1 capsids. However, similar mutations in the full length CP resulted in heterogeneous particles suggesting that metal ion mediated interactions are more crucial for the stability of the T = 3 particles than for the T = 1 particles (Satheshkumar et al. 2004a).

18.6 Model for SeMV Assembly

Based on extensive mutational analysis of SeMV rCP, a possible mechanism for capsid assembly was proposed. Initially a pentamer of A/B type dimers in which β-annulus is disordered could be formed at one of the icosahedral five-fold axes. Further, assembly could proceed by interaction of ARM with the RNA. This could result in ordering of the amino terminal segments leading to CC dimers added to the 10-mer complex and the formation of β-annulus. Further addition of CP dimers could lead to the formation of swollen T = 3 particles. Calcium binding to the assembling or the assembled particles results in compact T = 3 particles (Savithri and Murthy 2010).

18.7 Polyprotein Processing in SeMV

The ORF 2 of SeMV contains a protease domain which is involved in processing of the polyproteins 2a and 2ab. In order to identify the cleavage sites and also to characterize the products of processing, the gene sequences corresponding to polyprotein 2a and its deletion mutant were cloned from the SeMV full-length cDNA and over expressed in *E. coli.* The cleavage products were identified by western blot analysis using antibodies to P8 and protease. It was concluded that polyprotein 2a undergoes cleavage at four sites E132-S133, E325-T326, E402-T403 and E498-S499 (Satheshkumar et al. 2004b).

Based on the sequences of all four identified cleavage sites, a consensus sequence was derived for the SeMV serine protease cleavage site, i.e., N/Q-E-T/S-X (where X is an aliphatic residue). Interestingly, the mutation of cleavage sites at positions other than E132-S133 did not affect the protease function, whereas the mutant E132A was found to be defective in processing suggesting that the removal of N-terminal membrane anchoring domain from the protease/polyprotein might be essential for the subsequent processing of the polyprotein 2a at the other sites (Nair and Savithri 2010a).

ORF2b is expressed using the −1 reading frame and therefore to translate ORF2a and ORF2b as a single polyprotein 2ab in *E. coli*, an additional nucleotide T was introduced at position 1743 (region of ribosomal frame shifting) by site directed mutagenesis (Fig. 18.4b). Even though the cleavage sites E132-S133, E325-T326 and E402-T403 were retained in the polyprotein 2ab, interestingly, no cleavage was observed between VPg and RdRp (E402-T403). Therefore, the conformation of the domains that determine the context of the cleavage site and not just the sequence could be the determinant of polyprotein processing. The unprocessed VPg-RdRp could be of functional significance in negative (−) strand RNA synthesis. A subsequent cleavage between VPg-RdRp would release RdRp that might perform the positive (+) strand synthesis (Nair and Savithri 2010a).

18.8 Structure and Function of SeMV Protease

As the protease domain of the ORF-2 of SeMV is essential for polyprotein processing, structural studies on the SeMV protease were initiated. Though SeMV protease was identified to be a serine protease of trypsin fold, it showed negligible sequence identity with any other proteases including viral proteases. The crystal structure of SeMV protease was determined at a resolution of 2.4 Å by multiple isomorphous replacement combined with anomalous scattering (MIRAS). The polypeptide fold revealed that it is indeed a serine protease of trypsin fold with a catalytic triad formed by the residues His181, Asp 216 and Ser 284 (Gayathri et al. 2006). Mutation of any of the active site catalytic triad residues completely abolished the polyprotein processing (Satheshkumar et al. 2004b). As depicted in the Fig. 18.5a, the protease structure consists of two β-barrels (domains I and II) that are connected by a long inter-domain loop. The active site and the substrate binding pocket occur in between the two domains and are solvent exposed. Interestingly, four aromatic residues are also solvent exposed. The functional significance of these residues will be discussed later. The crystal structure of SeMV protease is the first report of the structure of a non-structural protein from a plant virus (Gayathri et al. 2006).

As mentioned earlier, the serine protease domain cleaves the polyprotein at four specific E-T/S sites and not only the P1-P1' (E-T/S) residues at these sites, but also the flanking P2 (N/Q) and P2' (A/V/L) residues are conserved or conservatively substituted. In order to identify the structural basis for such stringency in substrate recognition, structure based alignment of SeMV protease was carried out with other Glu/Gln specific proteases. Based on such a comparison, residues T279, A280,

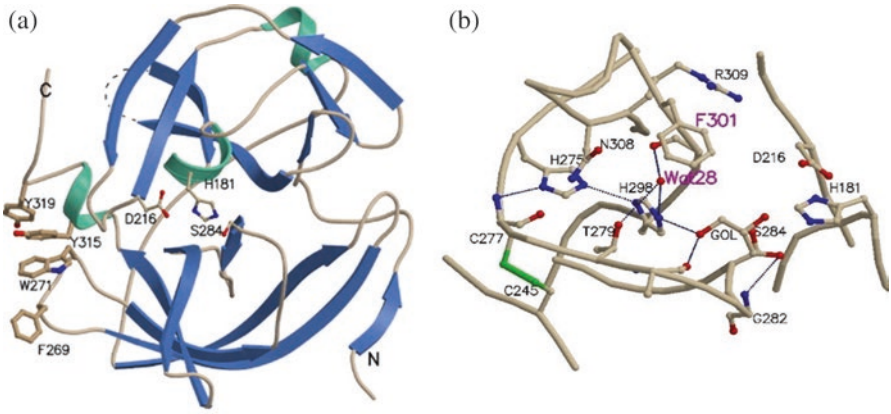

Fig. 18.5 Polypeptide fold of SeMV protease. (**a**) Ribbon drawing of the SeMV serine protease. The active site residues H181, D216 and S284 and the surface exposed aromatic residues are shown in ball-and-stick representation. The stretch of residues 171–173, that could not be traced, is shown as a *broken line*. (**b**) Stick diagram of SeMV serine S1-binding pocket. Residues H298, T279, H275 and N308 form the S1-binding pocket and residue R309 forms the S2-binding pocket (Reproduced with permission)

H298, F301 and N308 were proposed to form the S1-binding pocket that could accommodate the Glu of P1 position in the substrate (Fig. 18.5b). It was also found that these residues were highly conserved across known sobemoviral proteases. The S2-binding pocket of SeMV protease is formed by residue R309 that can recognize Asn/Gln at the P2 position (Gayathri et al. 2006)

It was observed that the protease was inactive, although the catalytic triad residues could be overlaid very precisely with those of trypsin. Interestingly, the protease-VPg fusion protein was found to be active. Therefore, mutational studies were carried out using the protease-VPg cleavage site mutant. These studies clearly indicated that H298, T279 and N308 of the S1 binding pocket were crucial for the protease activity. Also, it was found that the P2 (Asn/Gln) residue recognized by R309 plays an important role in determining the substrate specificity. Further, the crystal structure of the SeMV protease shows the presence of a disulphide bond between C248 and C277, of which C277 lies on the strand forming the S1 specificity site. Disulphides are generally implicated in the stability of the serine proteases. However, the mutation of C277 did not affect the protease activity indicating that the disulphide bond is not important for the protease function or structural stability unlike in the case of trypsin and chymotrypsin. These studies clearly indicate the differences in the structure of the SeMV protease that dictate the substrate specificity (Gayathri et al. 2006) (Fig. 18.5).

18.9 Viral Encoded Intrinsically Disordered Domain and Their Interaction with Other Globular Domains: Functional Implications

Intrinsically disordered or natively unfolded proteins lack defined secondary or tertiary structure. They are rich in charged residues and contain relatively few hydrophobic residues. These proteins attain folded conformations upon interaction with their specific partners or ligands. Their higher structural flexibility permits interaction with a large number of targets and thereby allows such proteins to regulate a number of cellular processes. Viral encoded intrinsically disordered domains/proteins can interact with other viral proteins/host factors and modulate their function. Initially, a bioinformatics analysis of all the proteins encoded by SeMV was carried out to identify the intrinsically disordered domains.

Figure 18.6 shows the fold index analysis of the ORF-1 encoded movement protein (MP), ORF-2 encoded polyproteins 2a and 2ab, and the ORF-3 encoded CP. The N- and C-terminal segments of MP which have negative fold index are predicted to be unfolded/disordered. Similarly, VPg and P8 in polyprotein 2a and VPg and the C terminal domain of RdRp in polyprotein 2ab are predicted to be disordered (Nair et al. 2012). The N-terminal R domain of CP which is structurally disordered as described earlier is indeed predicted to be disordered by the fold index analysis. In this section, the structure, interactions and functions of two of these domains, namely VPg and P8 are discussed.

18.10 Intrinsically Disordered VPg: Implications on Protease Activity

SeMV encoded VPg is a 9 kDa protein and consists of 77 amino acids. Analysis of the sequence using the software PONDR (Predictors of Natural Disordered Regions, available at http://www.pondr.com/) showed that it also falls in the category of disordered domains. (Satheshkumar et al. 2005b). The CD spectral studies with purified recombinant VPg revealed maximum negative ellipticity at 200 nm and absence of the same at 222 nm, typical of random coil structures, consistent with the structural predictions. Furthermore, the fluorescence spectrum of VPg showed a maximum emission at 357 nm even in the native state indicating lack of significant tertiary structure in VPg. In addition, VPg eluted abnormally on size exclusion chromatography as a polypeptide of higher molecular size, confirming the disordered nature of the protein. Interestingly, the CD spectral profile of ΔN70PV-E325A (protease-VPg cleavage site mutant) was characterized by the presence of a positive peak at 230 nm that was absent in the protease as well as the VPg CD spectra suggesting a conformational change in the fusion protein. As mentioned earlier, the protease was active only when expressed as protease-VPg fusion protein. Thus, the disordered VPg, upon interaction, could activate the protease. The positive CD at 230 nm was used as a probe to decipher the nature of interaction between the two domains.

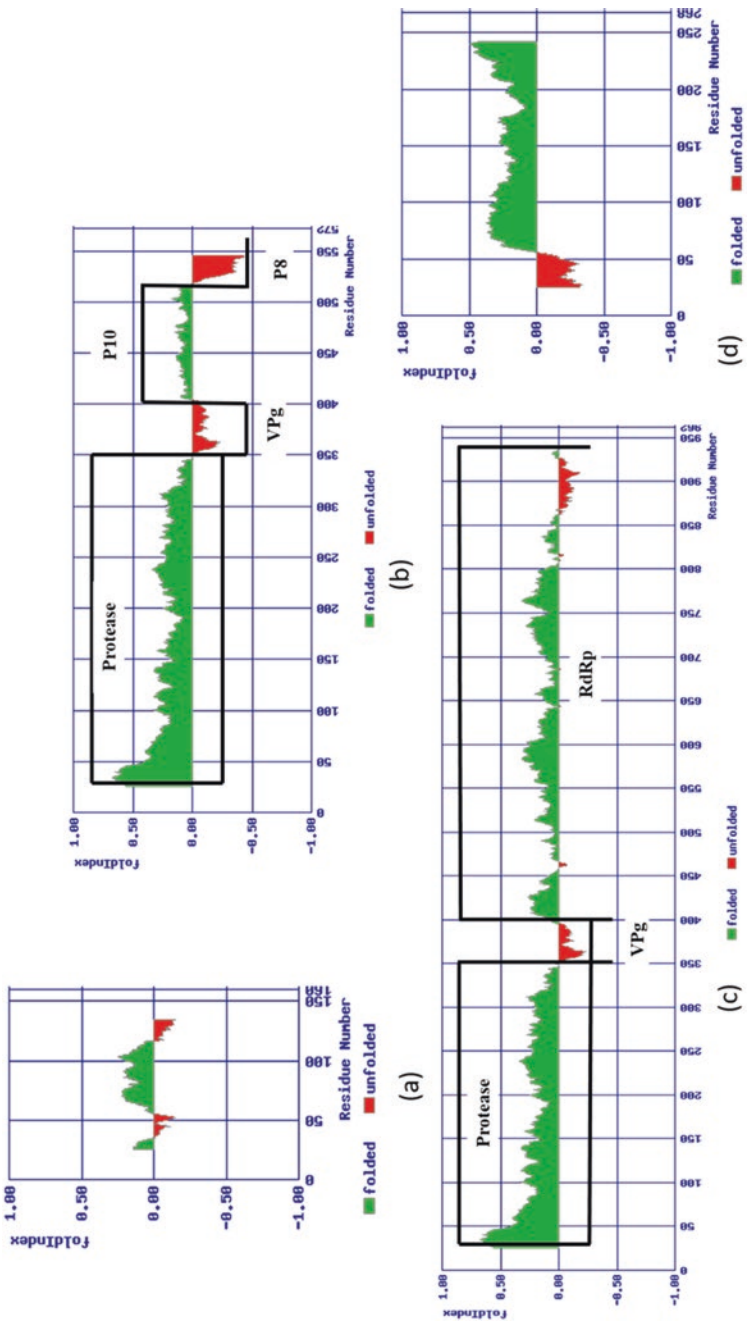

Fig. 18.6 Prediction of disordered domains in SeMV encoded proteins by fold index analysis (**a**) MP, (**b**) polyprotein 2a, (**c**) polyprotein 2ab and (**d**) CP

The presence of positive CD band at 230 nm could be either due to the presence of polyproline II (PPII) like helix (Parrot et al. 2002) or aromatic amino acids (Grishina and Woody 1994; Woody 1994). However, deletion of the C-terminal 28 amino acid residues encompassing a proline-rich sequence did not abrogate the 230 nm peak. VPg has two tyrosine and three tryptophan residues in its amino acid sequence. CD spectral studies of site-directed mutants of all these aromatic residues in the fusion protein (ΔN70PV-E325A) revealed that only the mutation of W43 to F (ΔN70PV-E325A-W43F) abolished the positive CD peak. Interestingly, the protease activity was significantly affected in this mutant (ΔN70PV-E325A-W43F) compared to that of ΔN70PV-E325A. It is possible that W43 of VPg is involved in stacking interaction with an aromatic residue from the protease domain, which leads to the positive CD band at 230 nm and modulates the structure as well as function of the fusion protein (Satheshkumar et al. 2005b). This is the first report of interaction of a natively unfolded domain with the globular protease domain leading to its activation.

As shown in Fig. 18.5a F269, W271, Y315 and Y319 are solvent exposed and form a stack near the C-terminus of the protein (Fig. 18.5a). Site-directed mutants of these residues were generated in the ΔN70PV-E325A (PVEA) mutant to identify their role in mediating the stacking interactions with the W43 of VPg and hence modulate the activity of the protease. H275, was also mutated as it lies close to the W271 in sequence and is conserved in the protease domain of all the known sobemoviruses. *Cis*- and *trans*-cleavage assays suggested that residues W271 and H275 but not Y315 or Y319 are crucial for protease activity. There was a concomitant loss of 230 nm peak in the PVEA-W271A and PV-H275A mutants. Thus, aromatic stacking interactions between W43 of VPg and W271 as well as H275 of the protease results in the positive CD peak at 230 nm in protease-VPg and activation of the protease (Nair et al. 2008).

18.11 Intrinsically Disordered P8 Domain: Activator of ATPase Activity of P10

P8 is another domain in the polyprotein 2a which is predicted to be intrinsically disordered. The far-UV spectra of hexa-histidine tagged P8 showed negative ellipticity at 200 nm, a characteristic of random coil, confirming that it is also natively unfolded. In the domain arrangement of polyprotein 2a, P10 precedes P8 and the presence of P10-P8 (P18) fusion protein was detected in the processing of this polyprotein. It was demonstrated that the ATPase activity of P18 was 2.5-fold higher than that of purified recombinant P10 protein (Nair and Savithri 2010b) further confirming the role of intrinsically disordered domains in the regulation of protein function.

18.12 SeMV Encoded RNA-Dependent RNA Polymerase (RdRp)

All positive stranded RNA viral [(+) RNA] genomes encode a RNA-dependent RNA polymerase (RdRp) that is essential for the replication of their genomes. The sequence analysis of RdRp domain revealed that all the conserved motifs of viral RNA-dependent RNA polymerases are present in SeMV RdRp.

The SeMV gRNA is covalently linked to VPg at the 5′ end (Lokesh et al. 2001). Nucleotidylylation of the VPg by RdRp is the first step of primer dependent replication in positive sense RNA viruses. Although the purified recombinant RdRp failed to nucleotidylylate VPg in vitro, it was able to carry out the polymerization reaction in the absence of the VPg primer when either genomic RNA or subgenomic RNA was used as the template. Further, the active site GDD/GAA mutant was not able to synthesize RNA suggesting that the motif is crucial for the polymerase activity and that the activity was intrinsic to the purified RdRp. The denatured product moved faster compared to the non denatured product and its position corresponded to the single-stranded template showing that the product of the reaction was double stranded. Thus, SeMV RdRp initiates de novo RNA synthesis on (+) gRNA or (+) sgRNA templates. Further, the RNA synthesis with (+) sgRNA as template was significantly reduced when limiting concentrations of ATP and GTP were used, whereas, limiting concentrations pyrimidines had no significant effect suggesting that SeMV RdRp prefers purines at the initiation step (Govind and Savithri 2010).

The secondary structure of 3′ UTR of (+) SeMV RNA was predicted using the M-fold program (Mathews et al. 1999). The UTR folds into stem-loop structures with a ΔG of −29.2 kcal/mol. The *cis* acting elements in the 3′ UTR were identified by systematic deletion analysis. Interestingly, the stem-loop structure 29–57 nt from the 3′ end with 8 bp stem and 12 nt loop seems to play an important role in determining the preferential initiation on the (+) RNA. However, under in vivo conditions, viral or host factors might modulate the polymerase conformation and thereby allow primer (VPg) dependent initiation of replication (Govind and Savithri 2010).

18.13 SeMV Infectious Clone

The in vivo functions of viral encoded genes can be deciphered using an infectious cDNA (icDNA) clone. The delivery of modified Ti-plasmid containing the icDNA via *Agrobacterium* -mediated transformation into the plant cell nucleus allows the transcription of the viral genome (Annamalai and Rao 2005; Citovsky et al. 2007; Lee and Gelvin 2008). The full-length transcripts thus generated enter the cytosol and express the viral encoded proteins leading to the replication of the viral RNA followed by encapsidation and movement (Annamalai and Rao 2005). Therefore, the full-length icDNA clone for SeMV was constructed and agroinfiltrated to *Sesbania grandiflora* plants. However, the efficiency of infection was very low. Therefore, *Cyamopsis tetragonoloba*, the local lesion host, was tested and was found to be more suitable. Time course analysis indicated CP accumulation from

6 days post infection, which increased further after 9 days. Northern analysis of the total RNA revealed complete replication of in vivo generated viral RNA. The SeMV icDNA clone had 4 additional nucleotides at the 5′end and 21 nucleotides at the 3′ end when compared to the wild type SeMV sequence. However, sequencing of the viral RNA isolated from virions obtained from SeMV icDNA infected leaves indicated lack of any extra nucleotides at both 5′ and 3′ ends suggesting that viral progeny RNA was repaired in vivo (Govind et al. 2012).

In order to verify the role of polyprotein processing in planta, mutants of all four cleavage sites (E132A, E325A, E402A and E498A) were generated using the SeMV icDNA as the template and the mutant icDNAs were tested for their ability to infect *Cyamopsis tetragonoloba* plants. There was a drastic reduction in viral infection for all the cleavage site mutants, which was evident from the absence of CP accumulation in the infiltrated cotyledons at 9 days post infection. However, co-infiltration of ORF-2 construct (pEAQ 2ab) *in trans* with all the respective cleavage site mutants could rescue the viral infection and result in CP accumulation similar to that of the wild type icDNA. This further confirmed the role of correct polyprotein processing in viral replication (Govind et al. 2012).

18.14 Viral Movement

Plant viruses have to cross the cell wall barrier while moving from one cell to another. They use resident communication networks in plants that aid in transporting macromolecules through the plasmodesmata and redirect them in such a way that the viral genome is transported and viral infection spreads. ORF-1 of SeMV that codes for the MP has only a limited sequence similarity with other well characterized MPs. One of the characteristics of viral MPs is that they bind to nucleic acids. Purified GST-MP could indeed bind to genomic RNA in a concentration dependent manner. Interestingly, no interaction was observed with other types of nucleic acids such as M13 ssDNA, dsDNA and a nonspecific viral RNA. These results are in contrast to the binding studies reported with other viral MPs, which show that the interaction is non-specific in vitro. More importantly, the interaction with the genomic RNA was lost upon treatment with pronase suggesting that SeMV MP recognizes the genomic RNA via the interaction with VPg. CP is one of the proteins shown to interact with MPs of several plant viruses. It was observed that GST MP could indeed interact strongly with the native virus and that the N-terminal 49 residues of MP were crucial for this interaction (Chowdhury and Savithri 2011a). This was further confirmed by Yeast two hybrid interaction assays as discussed in the next section.

18.15 Role of Protein-Protein Interactions in SeMV Replication and Spread

18.15.1 Interaction of MP with Viral Encoded Proteins

As mentioned earlier, SeMV MP could interact with CP in vitro, it was further validated with Y2H assay using standard Matchmaker system. Yeast cells co-transformed with pGBK T7 MP and pGAD T7 CP could grow on all nutritional selection media similar to the positive control comprising p53 and T-Ag suggesting that MP and CP also interact with each other under the ex vivo conditions of Y2H system.

Similar experiments carried out using systematic deletion constructs of MP clearly indicated that the interaction of MP and CP was significantly reduced on deletion of 49 amino acids from the N-terminus of MP corroborating the results obtained by in vitro studies. However, the C-terminal deletion of MP had no effect on the interaction.

In addition to CP, other viral encoded proteins would also be required for efficient cell-to-cell spread of SeMV genome. Therefore, the interaction of MP with other non- structural proteins/domains of SeMV were also monitored using the Y2H assay. A significant growth of AH109 cells was observed when p53-T Ag (positive control), CP-MP, P10-MP, VPg-MP were co-expressed but not when P8-MP, RdRp-MP and Pro-MP were expressed together. Thus, MP, viral RNA covalently linked to VPg, P10 and CP could all be part of the movement complex (Chowdhury and Savithri 2011b).

18.15.2 Interaction of RdRp and Other Viral Encoded Ancillary Proteins

Complete replication in +sense RNA viruses requires the assembly of the replicase complex, which consists of viral encoded proteins as well as host factors (Kushner et al. 2003; Lin et al. 2009; Vidalain and Tangy 2010). Therefore, interaction of viral encoded ancillary proteins and RdRp is an integral part in the formation of the replication complex. In many viruses it has been reported that one or more of the viral encoded proteins directly interact with RdRp domain and modulate the polymerase activity (Shirota et al. 2002; Shen et al. 2008).

Hence, all the gene segments of SeMV corresponding to individual domains of polyprotein 2a (Pro, VPg, p10, p8, p18 and p27) and RdRp were cloned into the Matchmaker system. The recombinant clones in pGBK/pGAD vectors were co-transformed in pairs into *Saccharomyces cerevisiae* strain AH109 and analysed. In addition to pGBKT7-p53 and pGADT7T antigen (positive control), a significant growth was observed only when RdRp and p10 were co-expressed. Similar analysis of C-terminal deletion mutants of RdRp showed that the C-terminal disordered domain was essential for interaction with P10. It is possible that interaction of this domain with P10 might modulate the function of RdRp. To test this possibility, RdRp and P10 were co-expressed and the RdRp-P10 complex was purified. The

complex showed eight to ten fold increase in activity compared to RdRp alone, indicating that P10 may activate the RdRp by masking the C-terminal disordered domain. This was further confirmed by the observation that RdRpCΔ43 was highly active and the activity was comparable to that of the RdRp-P10 complex and it did not increase any further when co-purified with P10 as RdRpCΔ43-P10 complex. The viral replication complex (VRC) is membrane associated and in the case of SeMV, the VRC could contain RdRp, P10 as well as the MP along with the genomic RNA and other yet to be identified host factors (Govind et al. 2014).

18.16 Biodistribution and Toxicity of SeMV in Mice

It has been demonstrated that nanoparticles (NPs) have many favourable properties such as controlled and targeted drug release, improved solubility, reduced dose-related toxicity and better absorption that make them attractive candidates for detection, imaging and treatment of diseases. In order to use these NPs in biomedicine, a detailed biodistribution and toxicity analysis needs to be carried out (Powers et al. 2006). Such studies will also allow modification and optimization of their biodistribution and clearance (Bruckman et al. 2014). Recently, virus nanoparticles (VNPs) are being investigated for various biomedical applications. Plant viruses have a distinct advantage over other NPs as they are generally non-toxic and biodegradable. With a view to use SeMV as a possible nanocarrier, the toxicity and biodistribution of SeMV was examined. Different doses of SeMV were administered intravenously or orally to mice and they were examined for some important clinical signs, body weights, and for haemagglutination. Overall, the animals appeared essentially normal (Vishnu Vardhan et al. 2016). SeMV was localized in liver and spleen up to 72 h post administration in only intravenously administered mice. In the orally administered animals, most of the virus was cleared within 16 h and it was not detected in any of the organs (Vishnu Vardhan et al. 2016). Further, no pathological or biochemical changes were observed in the control as well as treated animals demonstrating that SeMV is non-toxic and does not cause any changes in the vital organs. Further, it was observed that the administration of SeMV did not result in significant immune response indicating that the SeMV can serve as a safe bio-nanoparticle for biomedical applications (Vishnu Vardhan et al. 2016).

18.17 SeMV Virus-Like Particles (VLPs) as Nanocarriers for Antibody Delivery

Antibody based clinical therapy that can disrupt protein-protein interactions and inhibit signalling pathways are considered to be invaluable in cancer therapeutics (Chames et al. 2009; Scott et al. 2012). However, since most of the antibodies cannot cross the cell membrane barrier and internalize in cells, majority of the FDA approved antibodies target the surface exposed receptors. There have been several

attempts to internalize antibodies by the use of cell penetrating peptides, liposome conjugation or the use of synthetic gold nanoparticles. The detailed biochemical and structural knowledge available on SeMV capsids prompted the development of SeMV based nanocarriers. A close examination of the structure of SeMV CP revealed a surface exposed eight residue HI loop (238–245 aa) near the five- fold and six -fold axes that might be suitable for genetic manipulations without disturbing particle assembly. The midpoint of the HI loop (Serine 242) was selected for insertion of the antibody binding B domain of *Staphylococcus aureus* protein A. The chimeric CP with the B domain in the loop region (SLB) could assemble into VLPs, although, they were heterogeneous in nature. In addition, antibodies could bind via their Fc region to the exposed B domain of SLB.

18.18 Demonstration of Entry of VLPs into Mammalian Cells

Fluorescent labelling is one of the major techniques for imaging of cellular proteins at various time intervals. SeMV CP and SLB VLPs were fluorescently labelled with Alexafluor 488. Interestingly, CP 488 and SLB 488 were able to enter into the cytoplasm of HeLa cells and the fluorescence reached a maximum in about 4–8 h (Fig. 18.7a). After 8 h, the fluorescence of the particles decreased indicating possible degradation of VLPs. Further, the presence of BSA or sheep serum had no effect on the entry of VLPs (Fig. 18.7b). Similar studies with other mammalian cell lines

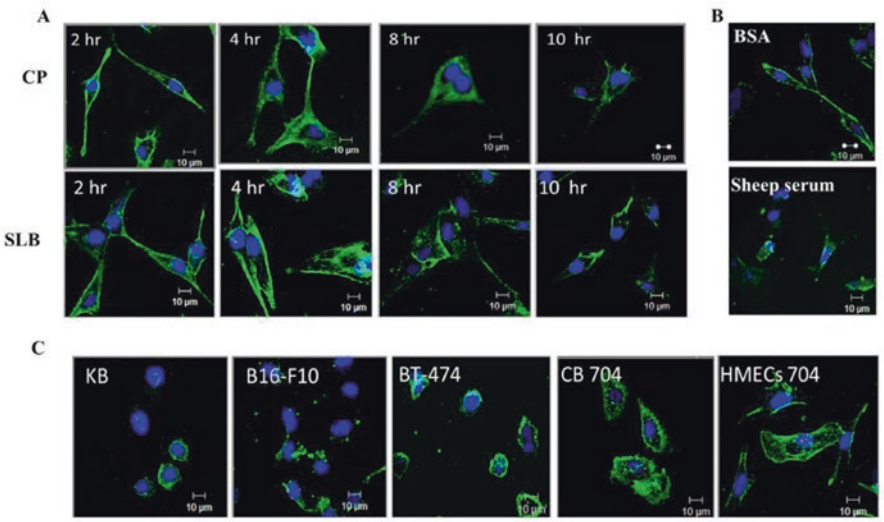

Fig. 18.7 Demonstration of virus like particle entry in to mammalian cells using confocal microscopy. (**a**) Confocal images of HeLa cells incubated with CP 488 or SLB 488 for 2, 4, 8 and 10 h at 37 °C. (**b**) Confocal images showing the entry of CP 488 in HeLa cells for 2 h in the presence of BSA/sheep serum. (**c**) Confocal images showing the entry of 1.58 nM SLB 488 in KB, B16-F10, BT-474, CB 704 and HMECs 704 cells

viz. KB, BT 474 (mammary duct cancer cells), CB 704 etc. showed that SeMV VLPs were highly versatile but non-specific in cellular entry (Fig. 18.7c).

18.19 Intracellular Delivery of Antibodies by SLB

Since SLB has the unique ability of binding the IgGs, it was interesting to check if it could deliver antibodies inside cells. D6F10 (anti-abrin), anti-α tubulin and Herclon (anti-HER2 receptor) were used as cargo for this purpose. As expected D6F10 633 by itself did not enter the cells (Fig. 18.8a). However, D6F10 was delivered inside the cells when SLB 488-D6F10 633 was incubated with HeLa cells (Fig.18.8b). In contrast, similar incubation with CP did not result in the intracellular delivery of antibodies (Fig. 18.8c) further confirming the ability of SLB, but not CP, to deliver the antibodies inside the cell. In addition, the delivered antibodies were shown to be functional as D6F10 delivered through SLB could rescue protein synthesis inhibition as well as apoptosis caused by abrin.

Tubulin is an essential cytoskeletal element. Hence disruption of tubulin network by anti-tubulin antibodies, particularly in cancer cells, could be of use in cancer therapy. It was shown that SLB could deliver anti tubulin antibodies into the cytoplasm that disrupted the tubular network. Herclon (Herceptin/Trastuzumab) is being effectively used in the treatment of HER2 positive breast cancer patients. These antibodies bind to the HER receptor and thereby inhibit downstream signalling pathways that lead to cell proliferation. Surprisingly, Herclon delivered via the SLB

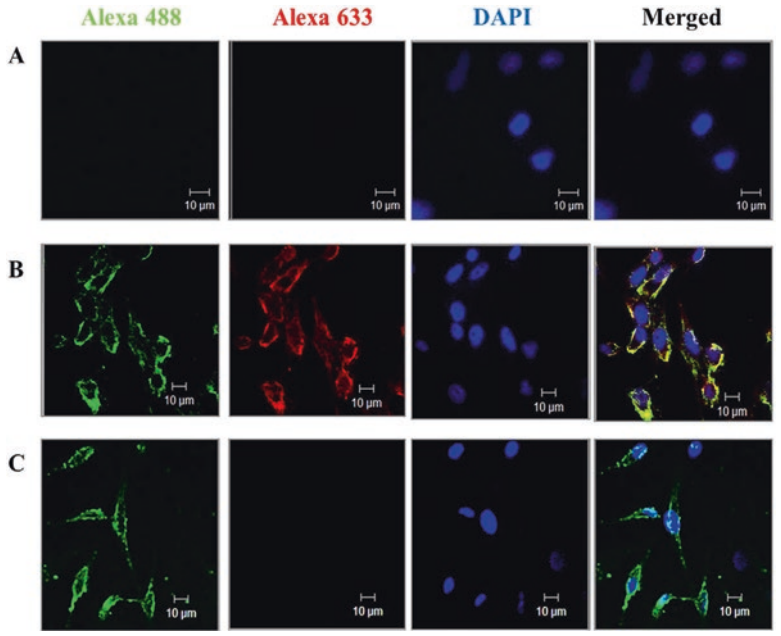

Fig. 18.8 SLB mediated D6F10 delivery in HeLa cells. Confocal images of HeLa cells treated with (**a**) D6F10 633, (**b**) SLB 488-D6F10 633 and (**c**) CP 488-D6F10 633 for 4 h

showed 83% cytotoxicity, which was to 3.33 times higher than that observed with Herclon alone. The increased levels of cytotoxicity were not contributed by CP/SLB as both had minimal effects on cell viability in vitro. These results demonstrate the potential applications of SeMV as a nanocarrier in antibody based therapeutics (Abraham et al. 2016).

18.20 Concluding Remarks

Extensive studies carried out on SeMV has resulted in a much deeper understanding of the molecular mechanism of assembly, polyprotein processing, replication, regulation of protein function by the intrinsically disordered domains encoded by the genome, cell to cell movement, and possible application of plant viruses as nanocarriers for intracellular delivery of antibodies and drugs. These studies could be further augmented by the use of SeMV icDNA which has already been used to demonstrate infectivity and importance of polyprotein processing in replication. The identification of host factors in the viral replication complexes as well as the movement complexes is another area of research that could provide deeper insights into mechanism of plant viral infection.

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