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In vitro analysis of the interactions of the N-terminal domain of the southern cowpea mosaic virus coat protein with RNA and membrane

Sook-Kyung Lee

To the Graduate Council:

I am submitting herewith a dissertation written by Sook-Kyung Lee entitled "In vitro analysis of the interactions of the N-terminal domain of the southern cowpea mosaic virus coat protein with RNA and membrane." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

David L. Hacker, Major Professor

We have read this dissertation and recommend its acceptance:

Jeffrey Becker, David Brian, Barry Bruce, Brad Reddick

Accepted for the Council:

Carolyn R. Hodges

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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In vitro analysis of the interactions of the N-terminal domain of the *southern cowpea mosaic virus* coat protein with RNA and membrane

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Sook-Kyung Lee
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DEDICATION

This thesis is dedicated to my parents

Mr. Jung-O Lee (이 정 오)

and

Mrs. Young-Joo Kim (김 영 주)

who have given me invaluable educational opportunities.

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ABSTRACT

Southern cowpea mosaic virus (SCPMV) is a spherical RNA virus with T=3 icosahedral symmetry. The particle is composed of 180 subunits of the CP and one copy of the viral RNA. The CP has two domains, the random (R) domain formed by the N-terminal 64 amino acids (aa) of the CP and the shell (S) domain (aa 65-260). The R domain is highly basic in charge and is localized to the interior of the particle where it is proposed to interact with the viral RNA. The R domain may also interact with membranes since it has been previously shown that the SCPMV CP binds liposomes in the presence but not in the absence of the R domain.

The RNA binding site of the R domain was identified by northwestern blot and by electrophoretic mobility shift assay (EMSA) using recombinant wild-type R domain protein (rWTR) that included aa 1-57 of the SCPMV CP and a C-terminal non-viral extension that contained two histidine tags and an S peptide tag (His-S tag domain). Deletions within the R domain revealed that the N-terminal 30 aa function in RNA binding. The R domain-RNA interaction was nonspecific with regard to RNA sequence and was sensitive to high salt concentrations suggesting that the interaction is predominantly electrostatic. The RNA binding site includes eleven basic residues, eight of which are located in the arginine-rich region between aa 22-30. It was demonstrated using alanine substitution mutants that the basic residues of the arginine-rich region but not those present at positions 3, 4, and 7 are necessary for RNA binding. None of the basic residues within the arginine-rich region are specifically required for RNA binding, but the overall charge of the N-terminal 30 aa is

important. Proline substitution mutations within the N-terminal 30 aa, and alanine substitutions for prolines at positions 18, 20, and 21 did not affect the RNA binding activity of the R domain. However, it was demonstrated by circular dichroism (CD) that the conformation of the N-terminal 30 aa of the R domain changes from a random coil to an α-helix in the presence of 50% trifluoroethanol (TFE).

The ability of rWTR to interact with artificial membrane was also analyzed *in vitro* using a dye release assay. The results demonstrated that the R domain interacts with negatively charged but not neutral liposomes. Deletion of the N-terminal 16 aa of the R domain nearly abolished the membrane interaction activity. In addition, aa 17-30 were also shown to be important for the activity. The interaction was dependent upon the presence of phosphatidic acid (PA) or phosphatidylethanol-amine (PE). The interaction was sensitive to high salt and acidic pH indicating that electrostatic interactions are involved in the liposome-R domain interaction. CD analysis and dye release assays of mutants with proline to alanine or alanine to proline substitutions within the N-terminal 30 residues of the R domain demonstrated a positive correlation between the extent of α-helical content in this region and the ability to lyse liposomes. A synthetic peptide (CP peptide$_{1-30}$) corresponding to the N-terminal 30 aa of the SCPMV CP was also able to induce lysis of liposomes. The peptide underwent a conformational change from a random coil to an α-helix in the presence of negatively charged liposomes suggesting a possible role of protein secondary structure in the membrane interaction. These experiments demonstrated that the R domain of the SCPMV CP can bind RNA and interact with membranes. The roles of these activities in the SCPMV life cycle are discussed.

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CHAPTER I

Literature Review

HOST RANGE AND CLASSIFICATION

Southern cowpea mosaic virus (SCPMV) is a member of the *Sobemovirus* genus of positive-sense RNA viruses (Fauquet and Mayo, 1999). Until recently, it was named the cowpea strain of *Southern bean mosaic virus* (SBMV-C) (Hull *et al.*, 1999). SCPMV is a 28 nm spherical virus that is composed of 180 subunits of the viral coat protein (CP) and one copy of the 4,194 nucleotide (nt) RNA genome (Hull, 1988).

SCPMV systemically infects most cultivars of *Vigna unguiculata* (cowpea) but does not infect other legumes (Shepherd and Fulton, 1962). The characteristic symptoms of infection in trifoliate leaves of *V. unguiculata* cultivar California blackeye include an initial vein clearing followed by the appearance of a mosaic pattern, mottling, rugosity, and downward cupping of the leaf margins (Shepherd and Fulton, 1962). In contrast, infected primary leaves may not show any symptoms. The virus is transmitted by leaf beetles of the family *Chrysomelidae*, through seed stock, and mechanically (breach of the epidermal layer of leaves by injury) (Tremaine and Hamilton, 1983).

Other members of Sobemovirus group include the type member Southern bean mosaic virus (SBMV), Blueberry shoestring virus (BSSV), Cocksfoot mottle virus (CfMV), Lucerne transient streak virus (LTSV), Rice yellow mottle virus

(RYMV), Subterranean clover mottle virus (SCMoV), Solanum nodiflorim mottle virus (SNMoV), Sowbane mosaic virus (SoMV), Turnip rosette virus (TRoV), and Velvet tobacco mottle virus (VTMoV) (Hull, 1988; Fauquet and Mayo, 1999).

SCPMV GENOME ORGANIZATION AND PROTEIN FUNCTION

The 5' end of the SCPMV genome is not capped but is covalently linked to a viral protein (VPg) that has an estimated molecular weight of 10 kilodaltons (kDa) (Ghosh *et al.*, 1979; Mang *et al.*, 1982). The 3' end of the genome does not have a poly (A) tail or a tRNA-like structure as observed for other positive-sense RNA viruses of plants (Wu *et al.*, 1987). The genome encodes four open reading frames (ORF) designated ORF1 through ORF4 with coding capacities of 21 kDa (ORF1), 105 kDa (ORF2), 20 kDa (ORF3), and 28 kDa (ORF4) (Fig. 1) (Wu *et al.*, 1987). ORFs 1-3 are expressed from the genomic RNA, and ORF 4 is translated from a 953 nt subgenomic mRNA (sgRNA) that is co-terminal with the 3' end of the genomic RNA and is synthesized during infection (Rutgers *et al.*, 1980; Salerno-Rife *et al.*, 1980; Ghosh *et al.*, 1981; Weber and Sehgal, 1982; Hacker and Sivakumaran, 1997). VPg is also present at the 5' end of the sgRNA (Ghosh *et al.*, 1981). Although the viral RNAs are not capped, they are translated by 5'end-dependent ribosomal scanning rather than by internal ribosome entry (Sivakumaran and Hacker, 1998). ORF2 is translated by leaky scanning of the ORF1 AUG which is present in a sub-optimal Kozak context (Sivakumaran and Hacker, 1998). ORF3 is translated by a –1 ribosomal

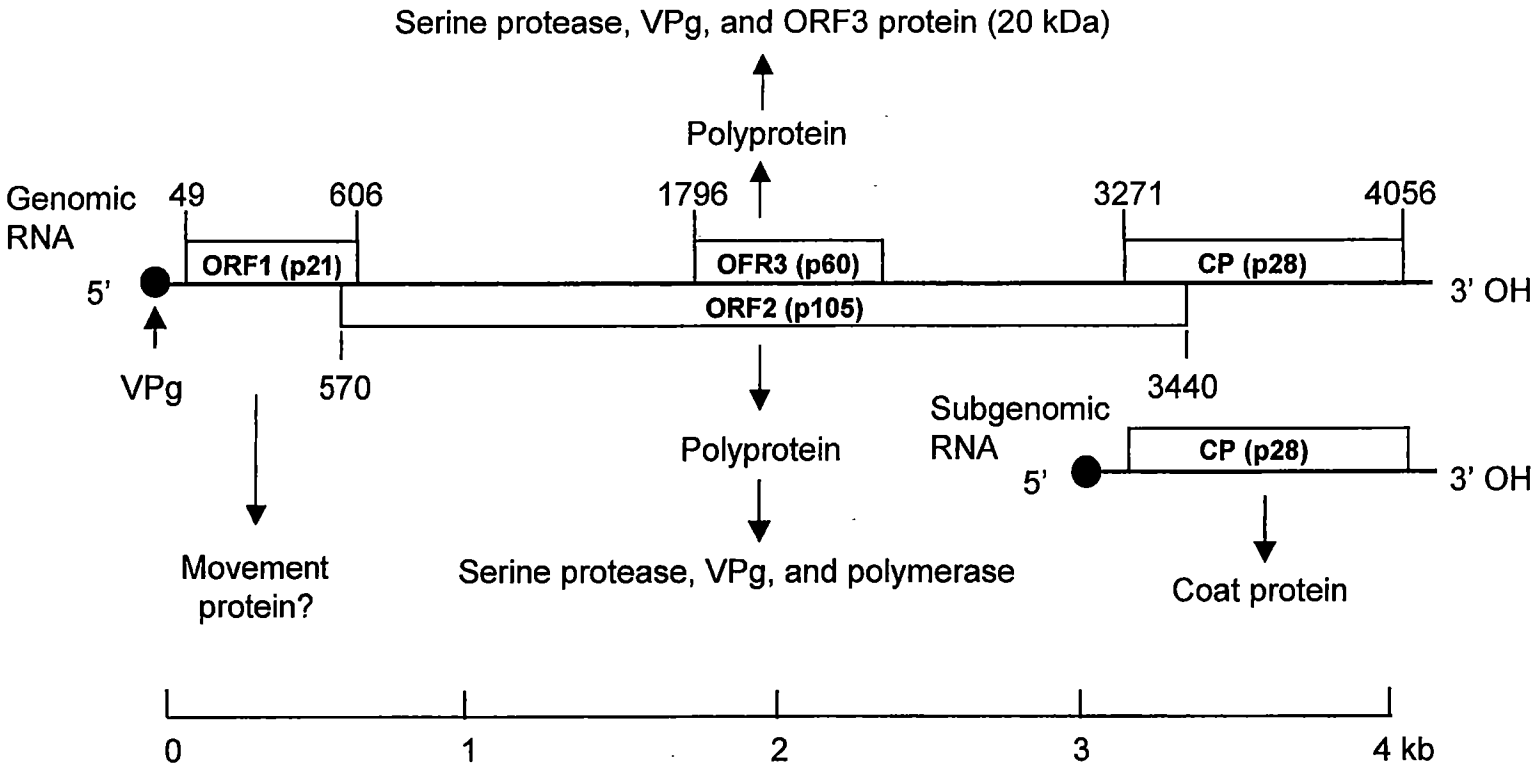


Figure 1. Schematic diagram of the genome organization of SCPMV. The ORFs are shown as boxes. The calculated molecular mass of the protein translated from each gene is indicated. The positions of initiation and termination codons are indicated (Wu et al., 1987).

frameshift within ORF2 to produce a protein with a calculated molecular weight of 60 kDa (Fig. 1) (Mäkinen *et al.*, 1995).

The 21 kDa ORF1 protein of SCPMV is necessary for cell-to-cell movement of the virus, but its role in this activity is not understood (Sivakumaran *et al.*, 1998). The ORF1 protein of RYMV has been shown to be a suppressor of post-transcriptional gene silencing in plants (Bonneau *et al.*, 1998; Voinnet *et al.*, 1999). ORF2 is translated to produce a 105 kDa polyprotein that may be processed into at least three polypeptides including a trypsin-like serine protease (20-25 kDa), VPg (10 kDa), and the RNA-dependent RNA polymerase (RdRp; 65-70 kDa) (Wu *et al.*, 1987; Gorbalenya *et al.*, 1988; Van der Wilk *et al.*, 1998). ORF3 is expected to be translated as a 60 kDa polyprotein that may be processed into a serine protease, VPg, and a 20 kDa ORF3-specific protein. The latter has been shown to be required for SCPMV cell-to-cell movement (Sivakumaran *et al.*, 1998). The SCPMV CP has also been shown to be necessary for viral cell-to-cell movement (Sivakumaran *et al.*, 1998) while that of RYMV has been shown to be required for both cell-to-cell and long-distance movement (Brugidou *et al.*, 1995).

SCPMV STRUCTURE

The structure of SCPMV has been determined to 2.8 Å resolution by X-ray crystallography (Abad-Zapatero *et al.*, 1980). The CP consists of two distinct

domains including the N-terminal random (R) domain spanning amino acids (aa) 1-64 and the shell or surface (S) domain that includes aa 65-260 (Fig. 2) (Abad-Zapatero *et al.*, 1980; Silva and Rossmann, 1987). SCPMV has T=3 icosahedral symmetry, and the capsid is constructed of 180 subunits of the CP (Abad-Zapatero *et al.*, 1980). The CP subunits can occupy one of three different packing environments termed A, B, and C that are related by quasi three-fold axes of symmetry (Abad-Zapatero *et al.*, 1980). The subunits of each different packing environment are chemically identical but structurally different (Abad-Zapatero *et al.*, 1980; Harrison *et al.*, 1978). The particle is stabilized by pH-dependent protein-protein interactions, protein-RNA interactions, and divalent metal ions (Hsu *et al.*, 1976; Hull, 1977; Abdel-Meguid *et al.*, 1981). The major Ca^{2+} binding site is located at the quasi three-fold axes between the A, B, and C subunits (Silva and Rossmann, 1987).

The R domain is localized to the interior of the particle where it may interact with the viral RNA (Abad-Zapatero *et al.*, 1980; Hermodson *et al.*, 1982; Krüse *et al.*, 1982). This domain is disordered in the crystal structure of the virus except in C subunits where residues 40-64 are ordered (Abad-Zapatero *et al.*, 1980; Silva and Rossmann, 1987). The R domain can be divided into three distinct regions. The amino-terminal 39 residues form a highly basic region that includes seven Arg and five Lys residues (Hermodson *et al.*, 1982). Residues 40-53 of three C subunits at the quasi-six-fold axes of symmetry hydrogen bond to form a structure termed the β-annulus (Fig. 3) (Silva and Rossmann, 1987).

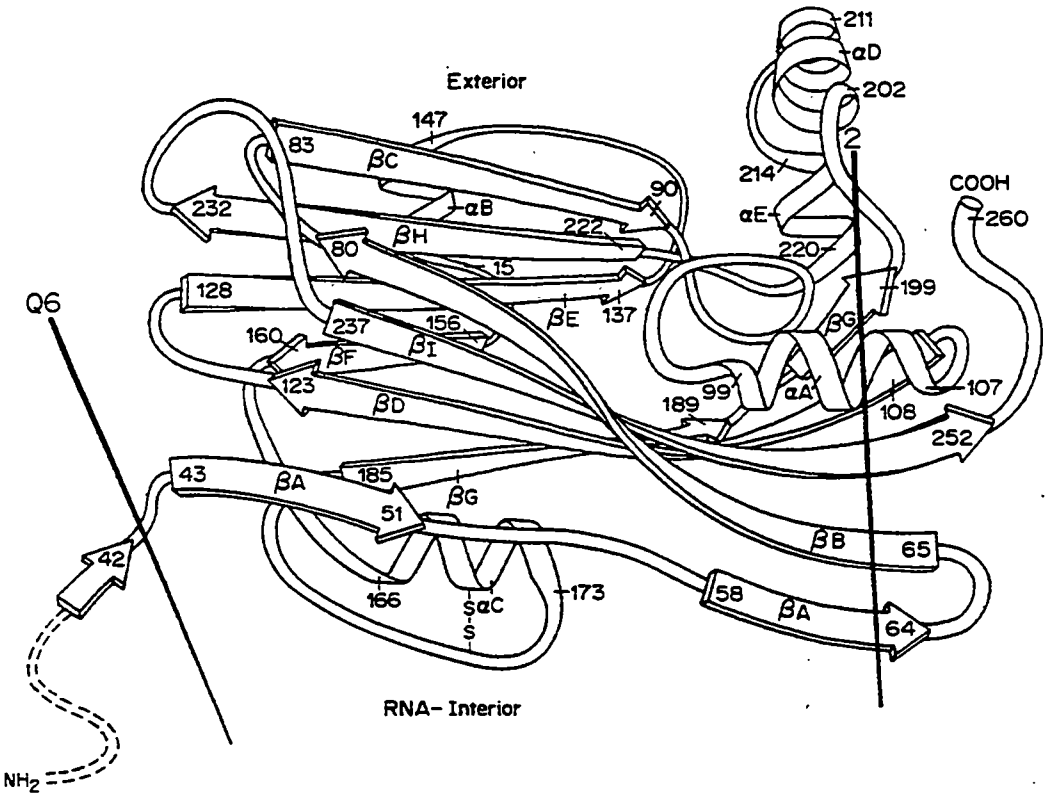


Figure 2. Diagrammatic representation of the SCPMV coat protein fold showing secondary structural elements. The α helices and the β strands are designated αA-D and βA-I, respectively. The lines designated Q6 and 2 represent the quasi six-fold and two-fold axes of symmetry, respectively. This diagram is adapted from Hermodson *et al.* (1982).

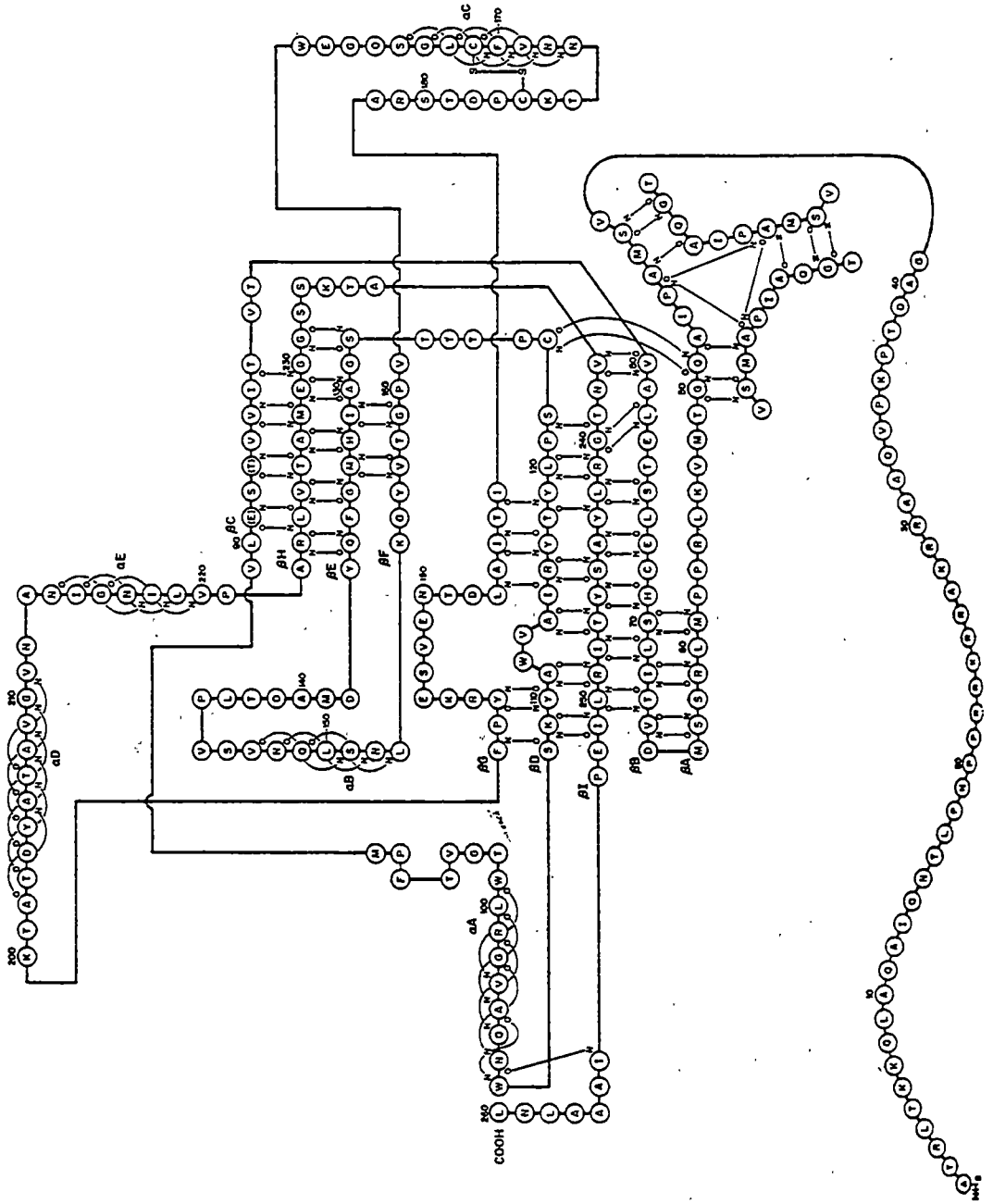


Figure 3. Complete amino acid sequence showing main-chain hydrogen bonding within the C subunit and outline of secondary structural elements. The β-annulus is shown between residues 43 and 50, which associates C subunits at icosahedral threefold axes. Adapted from Hermodson *et al.* (1982).

Finally, residues 54-64 of the C subunits form an extended arm termed βA that interacts with the S domain (Hermodson *et al.*, 1982).

The S domain folds into an eight-strand anti-parallel β-barrel to form the surface of the virus particle (Fig. 2) (Abad-Zapatero *et al.*, 1980). The S domain has a jellyroll topology that is observed in the CPs of viruses from many other families of spherical, positive-sense RNA viruses including the *Picornaviridae*, *Comoviridae*, *Tombusviridae*, and *Nodaviridae* (Rossmann and Johnson, 1989). The structural motif is also observed in the CP (hexon protein) of the *Adenoviridae*, a family of double-strand DNA viruses, and in the hemagglutinin protein of Influenza A virus, a negative-sense RNA virus (Rossmann and Johnson, 1989). Cellular proteins such as tumor necrosis factor, catabolite gene activator protein, and concanavalin A have a similar structure (Chelvanayagam *et al.*, 1992). These proteins may have diverged from a common ancestoral protein (Rossmann and Johnson, 1989; Chelvanayagam *et al.*, 1992).

SCPMV ASSEMBLY

Spherical RNA viruses must overcome two hurdles in order to produce particles. They must distinguish the viral genomic RNA from all other RNAs in the cell, and they must be able to neutralize the charge associated with a large RNA molecule. Three types of interactions are important in overcoming these barriers, specific RNA-protein interactions which may be important for the initiation and regulation of assembly, non-specific RNA-protein interactions for the stabilization of

the encapsidated RNA, and protein-protein interactions which stabilize the capsid shell (Fox *et al.*, 1994). Assembly models for T=3 icosahedral RNA viruses propose that the process is initiated by a specific interaction between one or more subunits of the CP and the viral RNA (Rossmann *et al.*, 1983; Sorger *et al.*, 1986). The nucleocapsid is then formed by the addition of CP subunits to the initiation complex to allow the condensation of the viral RNA within the growing capsid. As the subunits are added they must fold into the A, B, or C conformation depending upon their positions in the capsid.

The mechanism of conformational adaptation by the CP subunit has not been resolved, but it has been proposed for Flock house virus (FHV), an icosahedral RNA virus of insects, that the flexible N-terminus of the FHV CP and duplex regions of the viral RNA function as molecular switches to regulate the conformation of the subunits (Fisher and Johnson, 1993). The N-terminal 54 residues of the A and B conformations of the FHV CP are disordered, but residues 20-30 of C subunits are ordered and occupy a cleft on the interior surface of the capsid that is formed at the interface between C and B subunits at the two-fold axis of symmetry (Fisher and Johnson, 1993). The cleft is also occupied by about 10 base pairs of duplex RNA (Fisher and Johnson, 1993). The presence of the ordered N-terminal region and the duplex RNA in the cleft produces a flat intersubunit contact between the C and B subunits. In contrast, duplex RNA and the N-terminus of the CP are not present at the interior surface formed by the C and B subunits at the quasi two-fold axis. As a result the intersubunit contact at this site is bent (Fisher and Johnson, 1993). These observations together with the assembly studies with FHV CP mutants described

below suggest that the N-terminus of the FHV CP is important for the formation of T=3 particles. The ordered N-terminal region of the R domain of the C subunit in the SCPMV particle is also localized to a cleft formed by the interaction of the C and B subunits at the two-fold axis of symmetry (Abad-Zapatero *et al.*, 1980, Silva and Rossmann, 1987).

Besides SCPMV and FHV, the CPs of other spherical RNA viruses such as tomato bushy stunt virus (TBSV), brome mosaic virus (BMV), turnip crinkle virus (TCV), and cucumber mosaic virus (CMV) are also characterized by an internally localized, highly basic N-terminus (Harrison *et al.*, 1978; Ahlquist *et al.*, 1981; Hogle *et al.*, 1986; Fisher and Johnson, 1993; Wikoff *et al.*, 1997). For SCPMV *in vitro* reassembly experiments have demonstrated that the R domain is necessary for the assembly of T=3 particles. Trypsinization of the CP results in the elimination of the R domain by cleavage at Arg61 (Erickson and Rossmann, 1982). In the absence of RNA the resulting protein fragment of 22,000 M_r (S domain) forms RNA-free T=1 particles (18 nm particles with 60 CP subunits) (Erickson and Rossmann, 1982).

The highly basic N-termini of other viral CPs have also been shown to be required for assembly. The N-terminal 21 amino acids of the BMV CP include 7 basic residues (Ahlquist *et al.*, 1981). Deletion of the N-terminal 14 residues of the BMV CP prevents viral assembly *in vivo* (Sacher and Ahlquist, 1989; Rao and Grantham, 1996). Interestingly, single alanine substitutions for the arginine residues at positions 10, 13, or 14 prevents the encapsidation of BMV RNA 4 but not RNAs 1, 2, and 3 *in vivo* (Choi and Rao, 2000). Chemical crosslinking studies have also demonstrated that a peptide containing residues 11-19 of the BMV CP binds RNA *in*

vitro (Sgro *et al.*, 1986). Similarly, the N-terminus of the FHV CP contains 12 arginines between residues 32-49 (Fisher and Johnson, 1993). Deletion of the N-terminal 50 residues of the FHV CP prevents the assembly of T=3 particles *in vivo*, while deletion of the first 31 residues of the FHV CP results in a polymorphic population of particles that includes some T=3 particles (Dong *et al.*, 1998). These results demonstrate the essential role of the highly basic N-termini of the SCPMV, BMV and FHV CPs in virus assembly.

The R domain of the SCPMV CP includes a highly basic region which extends from residue 22 to residue 30 (RRKRRAKRR) forming an arginine-rich region. Such regions are also observed in many other RNA-binding proteins including the Tat and Rev proteins of human immunodeficiency virus 1 (HIV-1), bacteriophage antiterminator N proteins, and ribosomal proteins (Lazinski *et al.*, 1989; Calnan *et al.*, 1991; Chen and Frankel, 1994; Harada *et al.*, 1996; Battiste *et al.*, 1996). For both Tat and Rev, specific arginines within the arginine-rich region are involved in specific interactions with RNA. The Tat protein (RKKRRQRRR) binds to a bulged stem-loop TAR (trans-activation responsive element) in the viral mRNA (Calnan *et al.*, 1991; Tan and Frankel, 1995). It has been demonstrated that the second arginine of the arginine-rich region recognizes TAR by forming hydrogen bonds with two structurally distinct phosphates held in a precise orientation at the bulge in the stem-loop (Tao and Frankel, 1992). This type of interaction has been called an "arginine fork" (Tao and Frankel, 1992). The arginine-rich region of the HIV-1 Rev protein is required for binding to the Rev response element (RRE) within the viral RNA (Battiste *et*

al., 1996). The interaction between a Rev peptide containing the arginine-rich region and the RRE has been resolved by nuclear magnetic resonance spectroscopy (NMR) (Battiste *et al.*, 1996). The result revealed that three arginines and an asparagine within the arginine-rich region make base-specific contacts with the RNA (Battiste *et al.*, 1996). The arginine-rich region of the SCPMV CP is similar in sequence to those of Rev and Tat. Therefore, it is possible that this region of the CP binds specifically to the viral RNA.

A specific interaction between the SCPMV CP and RNA is thought to form a nucleation complex for virus assembly (Rossmann *et al.*, 1983). The origin of assembly on the SCPMV genomic RNA, however, has not yet been identified. Dissociation of SCPMV in the presence of EDTA and high salt at pH 8 yielded an RNA-CP complex (Hacker, 1995). Ribonuclease A (Rnase A) digestion of this complex followed by recovery of the remaining RNA-CP complex demonstated that the CP protects at nts 1410-1436 of the SCPMV genome (Hacker, 1995). The RNA was predicted to fold into a bulged stem-loop as shown in Fig. 4 (Hacker, 1995). The high affinity of CP subunits for this region of the viral genome is consistent with a role for the stem-loop in the initiation of SCPMV assembly, but this has not yet been demonstrated.

The basic N-terminus of the SCPMV CP is expected to bind to viral RNA based upon its location within the virus particle and its highly basic charge (Abad-Zapatero *et al.*, 1980). It has also been shown to be required for SCPMV assembly (Erickson and Rossmann, 1982). The objectives of the research described in Chapter II were to determine if the R domain of the SCPMV CP

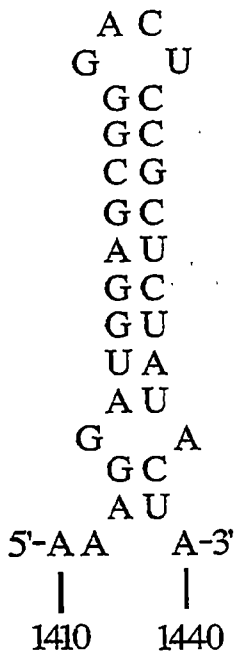


Figure 4. The predicted secondary structure of the coat protein binding site (CPBS) on the SCPMV RNA between nucleotides 1410 and 1440.

binds RNA, to determine if the RNA binding was specific or nonspecific, and to determine the regions of the R domain that were involved in RNA binding using *in vitro* assays. The results from this research are expected to contribute to the understanding of the molecular interactions involved in the assembly of SCPMV.

MEMBRANE INTERACTION OF SCPMV

SCPMV is stabilized by pH-dependent protein-protein interactions, protein-RNA interactions, and divalent metal ions (Hull, 1977). Incubation of virus particles in EDTA at neutral pH results in their expansion from 28 nm to 48 nm, and further addition of NaCl (> 0.5 M) results in dissociation of the virus (Hsu *et al.*, 1976; Tremaine *et al.*, 1982; Fitch and Garcia-Moreno, unpublished data). It has also been demonstrated by multiple angle, time resolved static light scattering that SCPMV expands when incubated at neutral pH in the presence of NaCl at a concentration of 100 mM or higher (Fitch and Garcia-Moreno, unpublished data). SCPMV expansion results in the appearance of the R domain on the exterior of the particle as demonstrated by binding of an R-specific monoclonal antibody to expanded but not native SCPMV (MacKenzie and Tremaine, 1986). Treatment of expanded SCPMV with trypsin reduces the diameter of the particles from 48 nm to 28 nm (Fitch and Garcia-Moreno, unpublished data). One explanation for these results is that trypsin digestion removes the externalized R domains from the expanded particles.

It is not known if particle expansion is necessary for the SCPMV infection cycle, but the addition of native or expanded SCPMV to wheat germ extract or rabbit reticulocyte lysate results in the translation of the viral RNA (Brisco *et al*, 1985; Brisco *et al*, 1986; Shields *et al*, 1989; Sivakumaran and Hacker, unpublished data). Based on these observations and the results from expansion studies it was concluded that SCPMV is disassembled *in vitro* by a co-translational mechanism.

The SCPMV structural studies revealed the presence of pores at the twelve five-fold axes of symmetry (Silva *et al.*, 1987). It was proposed that these pores function as ion channels (Silva *et al.*, 1987). Pores were also observed at the five-fold axes of many other sperical RNA viruses including Poliovirus (PV), Mengovirus, Human rhinovirus, TBSV, TCV, and Satellite tobacco necrosis virus (STNV; a T=1 icosahedral virus) (Kalko *et al.*, 1992). The conservation of pores in the capsids of many spherical RNA viruses of plants and animals suggests that these structures may be necessary for the infection cycle of these viruses. In support of this hypothesis it has been demonstrated that PV and SCPMV function as ion channels in planar lipid bilayers (Tosteson and Chow, 1997; Helrich and Pinto, unpublished data). For SCPMV the conductivity was observed at pH 7.5 and in 300 mM KCl, conditions which have been shown to result in SCPMV expansion (Helrich and Pinto, unpublished data). The SCPMV ion channel favors transport of cations over anions, and the ion conductivity is pH-dependent (Helrich and Pinto, unpublished data). The channel is not active at pH 5. These results point to a role of expanded particles in ion channel activity.

One possibility is that the externalized R domain is involved in the interaction between the expanded particle and the lipid bilayer. Consistent with this proposal it has been demonstrated that the purified SCPMV CP interacts with negatively charged liposomes in the presence but not in the absence of the R domain (Datema *et al.*, 1987).

The VP1 CP of PV has also been shown to interact with liposomes (Fricks and Hogle, 1990). The PV capsid is composed of four different viral proteins named VP1, VP2, VP3, and VP4 that are each present in 60 copies. VP1, VP2, and VP3 make up the capsid shell while VP4 is localized to the interior of the particle (Hogle *et al.*, 1985). Binding of PV to its cell-surface receptor leads to a conformational transition that results in altered particles which lack VP4 and sediment with a coefficient of 135S (versus 160S for the native particle) (De Sena and Mandel, 1977). In addition, the N-terminus of VP1, normally located on the interior of the capsid, becomes externalized (Fricks and Hogle, 1990; Hogle *et al.*, 1985). The N-terminus of VP1 is predicted to fold into an amphipathic α-helix and is responsible for binding of virus to liposomes (Fricks and Hogle, 1990). It has been shown that the160S and 135S PV particles have ion channel activity in a planar lipid bilayer (Tosteson and Chow, 1997). This activity is not dependent upon acid pH, however (Tosteson and Chow, 1997). Based on these results, it has been proposed that the N-termini of several VP1 proteins may form a channel in the plasma membrane or the endosomal membrane to allow passage of the viral RNA into the cytoplasm of the infected cell (Fricks and Hogle, 1990).

Similar observations have been made for FHV. The FHV CP is autoproteolyzed following assembly to release a C-terminal 44-residue peptide termed the γ peptide (Schneemann *et al.*, 1992). A bundle of five γ peptides is localized to the interior of the particle beneath the five-fold axis of symmetry (Fisher and Johnson, 1993). The γ peptide is predicted to form an amphipathic α-helix, and it has been shown to bind to membranes *in vitro* (Bong *et al.*, 1999; Janshoff *et al.*, 1999). In addition, insertion of the γ peptide into a planar lipid bilayer results in ion conductivity across the membrane (Bong *et al.*, 1999). It has been proposed that receptor binding and endocytosis of FHV causes a conformational changes in the particle resulting in the release of a bundle of five γ peptides at the five-fold axis of symmetry and these γ peptides insert into the endosomal membrane to form a channel for transport of the viral RNA into the cytoplasm (Bong *et al.*, 1999).

The observation that SCPMV forms an ion channel in a planar lipid bilayer suggests that the CP has membrane binding activity. It has also been shown that the SCPMV CP interacts with liposomes in the presence but not in the absence of the R domain (Datema *et al.*, 1987). The objectives of the research described in Chapter III were to determine if the R domain of the SCPMV CP interacts with liposomes, to determine the region of the R domain involved in the interaction, and to determine the lipid requirements for the interaction. The result will provide information to help to understand molecular mechanisms involved in virus-membrane interactions. The results from this research are expected to

contribute to the understanding of the role of the CP in the SCPMV infection cycle of the virus. The research is also expected to begin to elucidate the role of the SCPMV ion channel activity in the viral infection process.

CHAPTER II

In vitro analysis of an RNA binding site within the N-terminal 30 amino acids of the *Southern cowpea mosaic virus* coat protein

INTRODUCTION

Southern cowpea mosaic virus has been extensively studied as a model for the structure and assembly of small, spherical RNA viruses (Hull, 1988). The 28 nm spherical particle has T=3 icosahedral symmetry and is composed of 180 subunits of the 28,200 M_r CP and a single copy of the 4.2 kb genome. Three types of CP subunits termed A, B, and C, which are chemically identical but structurally non-equivalent, are related by quasi three-fold axes of symmetry and are involved in different intersubunit contacts (Abad-Zapatero *et al.*, 1980). X-ray diffraction studies of the virus structure revealed that the CP has two distinct domains, the N-terminal R domain (aa 1-64) localized to the interior of the particle and the S domain (aa 65-260) which forms the surface of the particle (Abad-Zapatero *et al.*, 1980; Silva and Rossmann, 1987). The S domain folds into an eight-strand antiparallel β-barrel, but the R domain is disordered in the crystal structure except for C subunits where residues 40-64 fold into a β-sheet (Abad-Zapatero *et al.*, 1980; Silva and Rossmann, 1987). Residues 40-53 from three C subunits hydrogen bond to form a structure termed the β-annulus at the quasi six-fold axes of symmetry (Abad-Zapatero *et al.*, 1980, Silva and Rossmann, 1987). The R domain is rich in basic amino acids and has been proposed to interact with the viral RNA on the interior of the SCPMV

particle (Abad-Zapatero *et al.*, 1980; Krüse *et al*, 1982). Of the N-terminal 30 residues of the CP 11 are basic, and 8 of these are clustered between residues 22-30 to form an arginine-rich region having the sequence RRKRRAKRR (Hermodson *et al.*, 1982; Wu *et al.*, 1987). *In vitro* reassembly experiments have demonstrated that the R domain is critical for the assembly of T=3 but not T=1 particles (Erickson and Rossmann, 1982; Savithri and Erickson, 1983).

Arginine-rich regions have been observed in a number of RNA binding proteins including the Tat and Rev proteins of HIV-1, bacteriophage antiterminator N proteins, and ribosomal proteins (Lazinski *et al.*, 1989; Calnan *et al.*, 1991; Chen and Frankel, 1994; Harada *et al.*, 1996; Battiste *et al.*, 1996). The R domain of the SCPMV CP is expected to bind to the viral RNA based on its location on the interior of the particle and its basic charge. If so, the R domain-RNA interaction is expected to be important in SCPMV assembly. The RNA binding activity of a recombinant R domain protein (rWTR) comprised of aa 1-57 of the SCPMV CP was investigated *in vitro*. It was demonstrated that the N-terminal 30 amino acids of the R domain function in RNA binding and that the arginine-rich region between amino acids 22-30 plays a critical role in this activity. The binding of the R domain to RNA was shown to depend largely on nonspecific electrostatic interactions. In addition, CD studies with a peptide corresponding to aa 1-30 of the SCPMV CP demonstrated that this region can form an α-helix in 50% trifluoroethanol (TFE). However, substitution mutations designed to alter the secondary structure in this region did not affect the RNA binding of the R domain.

MATERIALS AND METHODS

Construction of R domain expression plasmids

The region of the SCPMV genome encoding residues 1-57 of the CP was amplified by polymerase chain reaction (PCR) using oligonucleotides SBMV53 and SBMV70 (Table 1) as primers and the SCPMV cDNA clone pSBMV2 as a template (Sivakumaran and Hacker, 1998). The PCR product was digested with *Nde*I and cloned into the *Nde*I site of pET30b (Novagen) to produce pWTR. pΔ1-16 was generated by PCR amplification using oligonucleotides SBMV77 and SBMV70 (Table 1) as primers and pSBMV2 as a template. The PCR product was digested with *Nde*I and cloned into the *Nde*I site of pET30b. pΔ17-30, pΔ31-39, and pΔ40-53 were constructed by site-directed mutagenesis of pWTR using oligonucleotides SBMV78, SBMV79, and SBMV80 (Table 1), respectively, and single-strand pWTR DNA as a template (Kunkel, 1985). pA1, pA2, pA3, pA4, pA5, pA6, pP3, and pP4 were constructed by overlapping PCR mutagenesis using pWTR as a template (Horton *et al.*, 1990). For example, for pA1 two overlapping PCR products were generated using the oligonucleotide pairs SBMV104/107 and SBMV105/106 as primers (Table 1). The two PCR products were eluted from an agarose gel and combined in an overlap extension reaction in the presence of oligonucleotides SBMV104 and SBMV105. Similarly, overlapping PCR products for pA2, pA3, pA4, pA5, pA6, pP3, and pP4 were generated using the oligonucleotide pairs SBMV88/104 and SBMV89/105, SBMV94/104 and SBMV95/105, SBMV100/104 and SBMV101/105, SBMV104/114 and SBMV105/123, SBMV104/120 and SBMV105/119,

Table 1. Description of oligonucleotides.

Designation	Sequence (5'→ 3')[a]	Description
SBMV8	GTCCGCCCAGGCTTTCCC	Complementary to SCPMV nt 1683-1700
SBMV28	ACC*TAATACGACTCACTATAGG***CTCACACAGC-TCCAACCGC**	Same sense as SCPMV nt 1390-1408 (bold)
SBMV53	GATAAGAGCATTTcatATGGCTACCCG	Same sense as SCPMV nt 3255-3281
SBMV70	CGTAGCATatGAGGCCTAAGCTTC	Complementary to SCPMV nt 3432-3455
SBMV77	cGcATAtgCTTCCAAATCCGC	Same sense as SCPMV nt 3314-3334
SBMV78	**GCACCTGCGCAGC**AGTATTCTGAATAGC	Complementary to SCPMV nt 3307-3321 and SCPMV nt 3364-3376 (bold)
SBMV79	**CATGGATACCCCAGC**ACGCCGCTTCGCG	Complementary to SCPMV nt 3351-3363 and SCPMV nt 3391-3405 (bold)
SBMV80	**GAGGCCTAAGCTT**TTGGGTAGGCTTG	Complementary to SCPMV nt 3378-3390 and SCPMV nt 3433-3445 (bold)
SBMV88	GCCTgcGgcGgcGCGACGCGCGAAGCGG	Same sense as SCPMV nt 3333-3360
SBMV89	GCGTCGCgcCgcCgcAGGCGGATTTGGAAG	Complementary to SCPMV nt 3322-3351
SBMV94	CGGAAGgcAgcCGCGAAGCGGCGTGC	Same sense as SCPMV nt 3340-3365
SBMV95	CTTCGCGgcTgcCTTCCGCCGAGGCGG	Complementary to SCPMV nt 3331-3357
SBMV100	CGCGgcGgcGgcTGCTGCGCAGGTGCCC	Same sense as SCPMV nt 3351-3378
SBMV101	GCAGCAgcCgcCgcCGCGCGTCGCTTCCG	Complementary to SCPMV nt 3340-3368
SBMV104	GAGATATACATATGGCTACCCGC	Same sense as SCPMV nt 3271-3282 (bold)
SBMV105	GATGGTGCATATGAGGCCTAAGC	Complementary to SCPMV nt 3435-3445 (bold)
SBMV106	gcGgcGgcGgcAgcCGCGgcGgcGgcTGC-TGCGCAGGTGCCC	Same sense as SCPMV nt 3337-3378
SBMV107	CGCAGCAgcCgcCgcCGCGgcTgcCgcCg-cCgcAGGCGGATTTGG	Complementary to SCPMV nt 3325-3369
SBMV108	GAAGCGACGCcCGAAGCGGCGTGCTG	Same sense as SCPMV nt 3342-3367
SBMV109	CGCCGCTTCGgGCGTCGTCGCTTCCGCC	Complementary to SCPMV nt 3338-3362
SBMV114	cCgcCGCGgcTgcCTTCCGCCGAGGCGGATTTG	Complementary to SCPMV nt 3326-3358
SBMV119	gcGgcGAAGgcAgcCGCGgcGgcG	Same sense as SCPMV nt 3337-3360
SBMV120	cCGCGgcTgcCTTCgcCgcAGGCGGATTTG	Complementary to SCPMV nt 3326-3355
SBMV123	CGGCGGAAGgcAgcCGCGgcGgcGgcTGCTGCG	Same sense as SCPMV nt 3337-3369
SBMV128	TATACATATGGCTACCgcCTTGACCgcGgc-**GCAATTAGCACAAGC**	Same sense as SCPMV nt 3271-3308 (bold)
SBMV130	TATACATATGGCTACCCcCTTGACCAAGAAGC	Same sense as SCPMV nt 3271-3295 (bold)
SBMV132	TATACATATGGCTACCCGCTTGACCAAGcc-GCAATTAGCACAAGC	Same sense as SCPMV nt 3271-3308 (bold)
SBMV150	AGAATACTCTTgCAAATgCGgCTCGGCGGAAGCGACG	Same sense as SCPMV nt 3314-3350
SBMV151	CCGCCGAGcCGcATTTGcAAGAGTATTCTGAATAGC	Complementary to SCPMV nt 3307-3342

[a]Mutant bases are shown in lower case. *Nde*I restriction sites are underlined. The sequence of the bacteriophage T7 RNA polymerase is shown in italics.

SBMV104/109 and SBMV105/108, and SBMV104/151 and SBMV105/150, respectively (Table 1). For each construct, the two PCR products were combined to generate an overlapping PCR product using oligonucleotides SBMV104 and SBMV105 as primers. p3A, p3A/A5, and p3A/A6 were constructed by PCR amplification using pWTR, pA5, and pA6, respectively, as templates and the oligonucleotides SBMV128 and SBMV105 as primers (Table 1). pP1 and pP2 were generated by PCR using the oligonucleotide pairs SBMV130/105 and SBMV132/105 as primers (Table 1), respectively, and pWTR as a template. For all constructs, the PCR product was eluted from an agarose gel, digested with *Nde*I, and cloned into the *Nde*I site of pET30b. All mutations were confirmed by DNA sequencing.

Expression and purification of recombinant proteins

Expression of recombinant R domain proteins in *E. coli* was carried out by established protocols (Studier and Moffatt, 1986). Briefly, plasmid DNA was transformed into *E coli* strain BL21 (DE3), and recombinant protein expression was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. After incubation for 3 hr, the cells were collected by centrifugation, lysed in Tris-buffered saline (TBS) at pH 7.5 (25 mM Tris base, 3 mM KCl, and 140 mM NaCl), 1 mM dithiothreitol (DTT), 1% Triton X-100, and 1 mM phenylmethylsulfonylfluoride (PMSF), and sonicated (Zhao *et al.*, 1995). Following centrifugation, the recombinant protein was purified from the soluble fraction by affinity chromatography using a nickel-chelating column (Novagen). Briefly, the soluble fraction of the *E. coli* extract was loaded onto the column and washed with 1×

binding buffer (5 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9) and then with 1× wash buffer (60 mM imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). Bound protein was eluted from the column using 1× elution buffer (1 M imidazole, 0.5 M NaCl, and 20 mM Tris-HCl, pH 7.9). Recombinant proteins prepared in this way were first analyzed by SDS-PAGE and then used for northwestern binding. For analysis by electrophoretic mobility shift assay (EMSA), the purified recombinant proteins were dialyzed against 10 mM Tris-HCl (pH 8.0), 25 mM NaCl, and 0.1 mM PMSF and concentrated using an ultrafiltration membrane with a 10,000 MW cut-off size. The His-S tag domain protein derived from the vector, pET30b, was recovered by the same procedure from *E. coli* BL21 (DE3) cells transformed with pET30b. Following purification, the protein was concentrated using an ultrafiltration membrane with a 3,000 MW cut-off size. Protein concentration was determined by the Bradford assay (Bio-rad).

In vitro transcription

The region of the SCPMV genome (nts 1390-1700) that includes the coat protein binding site (CPBS) was amplified from pSBMV2 by PCR using the oligonucleotide primers SBMV8 and SBMV28 (Table 1). SBMV8 includes the sequence for the bacteriophage T7 RNA polymerase promoter at its 5' end. The PCR product was cloned into the *Sma*I site of pUC129 to produce pCPBS, and the plasmid was linearized by digestion with *Sal*I (SCPMV nt 1473). *In vitro* transcription resulted in an RNA of 83 nts. Transcript RNA corresponding to SCPMV nts 1-101 was synthesized from pSBMV2 DNA linearized with *Taq*I. Nonviral transcript RNA of

52 nts was synthesized from pBluescript II (KS+) DNA (Stratagene) linearized with *Sma*I. The transcription reactions contained 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM DTT, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 50 μCi α^{32}P-UTP (ICN; specific activity >3000 Ci/mmol), 1 μg linearized plasmid DNA, 50 units bacteriophage T7 RNA polymerase (New England Biolabs), and 20 units RNasin (Promega). The reactions were incubated at 37°C for 1 hr. Transcript RNA was denatured at 95°C for 2 min in the presence of 40% formamide, electrophoresed in a denaturing 12.5% polyacrylamide gel, and eluted from the gel by soaking overnight at 4°C in 500 μl of elution buffer (50 mM ammonium acetate, pH 5.7 and 5 mM EDTA) containing 50 μg of *E. coli* tRNA. The RNA was precipitated with ethanol, resuspended in water, and quantitated by Cherenkov counting.

Northwestern blot

Purified recombinant proteins (450 pmol) were electrophoresed in a denaturing 15% polyacrylamide gel and transferred to nitrocellulose by electroblotting. The membrane was preincubated with shaking for 30 min at room temperature in buffer containing 10 mM Tris-HCl (pH 7.0), 120 mM NaCl, 1 mM EDTA, 1× Denhardt's solution, and 50 μg of *E. coli* tRNA (Weiss *et al.*, 1989). ^{32}P-labeled transcript RNA synthesized from pCPBS (10^6 cpm) was then added to the buffer, and the incubation was continued for 1 hr at room temperature. The membrane was washed three times at room temperature in the same buffer containing 200 mM NaCl, and bound RNA was detected by autoradiography.

Electrophoretic mobility shift assay

The conditions for the EMSA were adapted from Skuzeski and Morris (1995). ^{32}P-labeled RNA was incubated at 65°C for 10 min in 0.2 M Tris-HCl (pH 8.0), 2 mM $MgCl_2$, and 160 mM KCl and then allowed to cool to room temperature for 15 min. Transcript RNA (10^4 cpm) was incubated with varying amounts of purified proteins in a 20 μl reaction mix containing 0.1 M Tris-HCl (pH 8.0), 1 mM $MgCl_2$, 80 mM KCl, 100 mM NaCl, 10 mM DTT, 10 units RNasin, and 5 μg of *E. coli* tRNA. After a 15 min incubation at room temperature 2 μl of loading dye was added, and the reactions were electrophoresed on a 4.5% nondenaturing polyacrylamide gel (40:1 acrylamide:bis-acrylamide) in 0.5× TBE buffer (45 mM Tris-borate, 1 mM EDTA) at room temperature for 2 hr at 20 mA. The gels were dried and analyzed with a phosphoimager.

Peptide synthesis

The peptide acetyl-ATRLTKKQLAQAIQNTLPNPPRRKRRAKRR-$CONH_2$ was prepared at the Keck Biotechnology Resource Laboratory, at the Yale University School of Medicine.

Circular dichroism

CD spectra were measured using an Aviv model 202 spectropolarimeter at 4°C with a 1 mm cell. Peptide (5 μM) was prepared in 20 mM potassium phosphate, pH 7.4 in the absence or in the presence of 50% TFE. CD spectra

were collected from 260 nm to 190 nm and reported in terms of ellipticity units per mol peptide residue [θ]. The spectrum of a reference sample with identical conditions but without CP peptide$_{1-30}$ was subtracted from the CD spectra of solutions containing CP peptide$_{1-30}$.

RESULTS

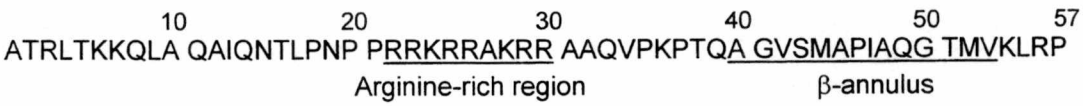
In vitro RNA-binding activity of the R domain

The *in vitro* RNA binding activity of the R domain was investigated by both northwestern blot and electrophoretic mobility shift assay (EMSA) using a riboprobe corresponding to SCPMV nucleotides 1390-1473. This region of the SCPMV genome has been identified as a high affinity CP binding site (CPBS) (Hacker, 1995). The R domain (amino acids 1-57) was overexpressed in *E. coli* with a nonviral C-terminal extension containing two histidine tags (His-S tag domain) (Fig. 5B). This recombinant protein (rWTR) with a calculated molecular weight of 14.4 kDa was purified from *E. coli* by affinity chromatography using a nickel-chelating column.

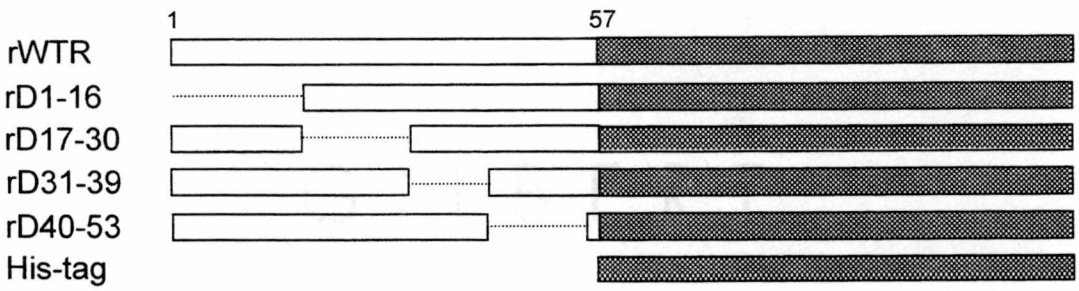
A northwestern blot was used to demonstrate that rWTR and the SCPMV CP bind the CPBS RNA, whereas the His-S tag domain does not (Fig. 6A). To confirm that equivalent amounts of protein were loaded in each lane, a separate gel was stained with coomassie blue (Fig. 6B). It was also shown by EMSA that rWTR but not the His-tag domain alone binds the CPBS RNA (Fig. 6C). To determine if the RNA binding activity of rWTR was dependent upon electrostatic interactions, the effect of salt concentration on RNA binding was analyzed by EMSA. As the

Figure 5. The schematic diagram of the R domain mutant constructs. (A) Amino acid sequence of residues 1-57 of the N-terminus of the SCPMV CP. The positions of the arginine-rich region and the β-annulus are underlined. (B) The R domain deletion mutants. The R domain and the His-tag domain are represented by white and gray boxes, respectively. The dotted lines indicate the positions of deletions. The names of the recombinant proteins are indicated at the left. (C) The R domain substitution mutants. Substituted amino acids are in bold and are underlined. Amino acids 31-57 of the R domain and the His-tag domain are represented by white and gray boxes, respectively.

A



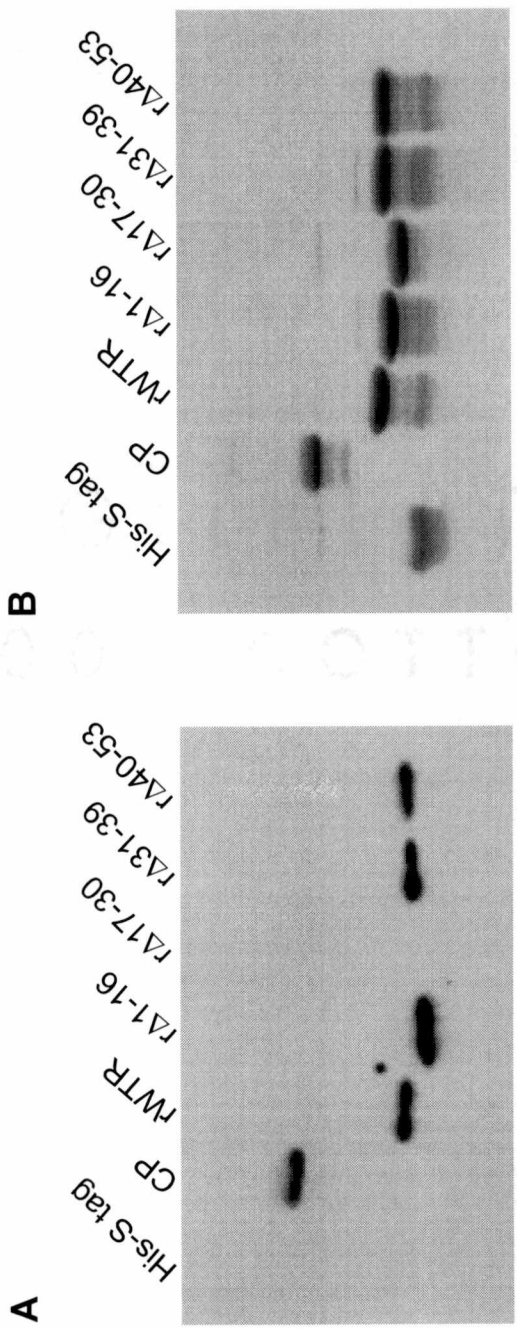
B



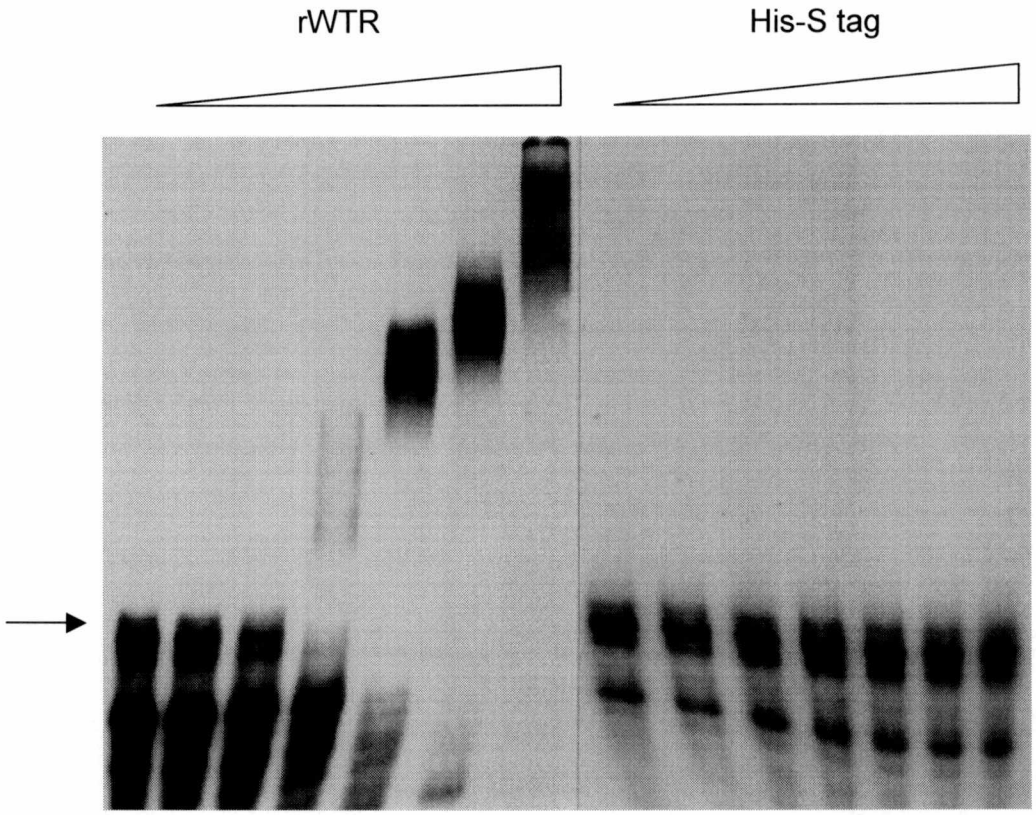
C

	1 ... 30 ... 57
rWTR	ATRLTKKQLAQAIQNTLPNPPRRKRRAKRR
rA1	ATRLTKKQLAQAIQNTLPNPP**AAAAA**A**AAA**
rA2	ATRLTKKQLAQAIQNTLPNPP**AAA**RRAKRR
rA3	ATRLTKKQLAQAIQNTLPNPPRRK**AA**AKRR
rA4	ATRLTKKQLAQAIQNTLPNPPRRKRRA**AAA**
rA5	ATRLTKKQLAQAIQNTLPNPPRRK**AA**A**AAA**
rA6	ATRLTKKQLAQAIQNTLPNPP**AA**K**AA**A**AAA**
r3A	AT**A**LT**AA**QLAQAIQNTLPNPPRRKRRAKRR
r3A/A5	AT**A**LT**AA**QLAQAIQNTLPNPPRRK**AA**A**AAA**
r3A/A6	AT**A**LT**AA**QLAQAIQNTLPNPP**AA**K**AA**A**AAA**
rP1	AT**P**LTKKQLAQAIQNTLPNPPRRKRRAKRR
rP2	ATRLTK**P**QLAQAIQNTLPNPPRRKRRAKRR
rP3	ATRLTKKQLAQAIQNTLPNPPRRKRR**P**KRR
rP4	ATRLTKKQLAQAIQNTL**A**N**AA**RRKRRAKRR

Figure 6. RNA binding by rWTR and the R domain deletion mutants. (A) Northwestern blot analysis of RNA binding by rWTR and R domain deletion mutants. Identical amounts (450 pmol) of proteins were transferred to nitrocellulose following SDS-PAGE, and the membrane was incubated with 10^6 cpm of ^{32}P-labeled CPBS RNA as described in Materials and Methods. Purified His-tag domain protein and the SCPMV CP were used as negative and positive controls, respectively. (B) Coomassie blue stained gel of the same protein samples as in (A).


A
His-S tag
CP
rWTR
rA1-16
rA17-30
rA31-39
rA40-53
B
His-S tag
CP
rWTR
rA1-16
rA17-30
rA31-39
rA40-53

C



(C) EMSA of rWTR. ^{32}P-labeled CPBS RNA (10^4 cpm) was incubated with varying concentrations (0-43 μM) of rWTR (left panel) or the His-tag domain (right panel). Following incubation the samples were electrophoresed on a nondenaturing 4.5% polyacrylamide gel. The arrow indicates the position of the unbound CPBS RNA.

Figure 6 (continued)

concentration of NaCl was increased from 0.1 M to 1.5 M, the level of RNA binding by rWTR decreased (Fig. 7). These results indicate that electrostatic interactions play a significant role in the binding of rWTR to RNA.

The specificity of the interaction between RNA and rWTR was investigated by EMSA using three different transcript RNAs including the CPBS RNA, an RNA corresponding to the 5′ end of the SCPMV genome (SCPMV nts 1-101), and a nonviral RNA of 52 nts transcribed from the pBluescript II (KS+) polycloning region. rWTR bound each RNA with about the same affinity (Fig. 8). These results suggest that the RNA binding activity of rWTR is nonspecific with regard to RNA sequence.

Deletion mapping of the RNA binding site of the R domain

To identify the RNA binding site of the R domain, the RNA binding of four deletion mutants rΔ1-16, rΔ17-30, rΔ31-39, and rΔ40-53 (Fig. 5B) was investigated by northwestern blot and EMSA using the CPBS RNA as a probe. It was demonstrated by northwestern blot that the mutants rΔ1-16, rΔ31-39, and rΔ40-53 retained RNA binding activity, but no RNA binding was observed for rΔ17-30, a mutant that lacked the arginine-rich region of the R domain (Fig. 6A). By EMSA it was demonstrated that deletion of amino acids 1-16, 17-30, or 31-39 resulted in mutant proteins with reduced affinity for RNA as compared to rWTR (Fig. 9). The deletion of amino acids 40-53, however, did not affect the RNA binding of the R domain (Fig. 9). These results demonstrated that the major RNA binding determinant of the R domain is located between amino acids 17-30. In addition, the N-terminal 16 residues and to a lesser extent residues 30-39 also contribute to the RNA binding

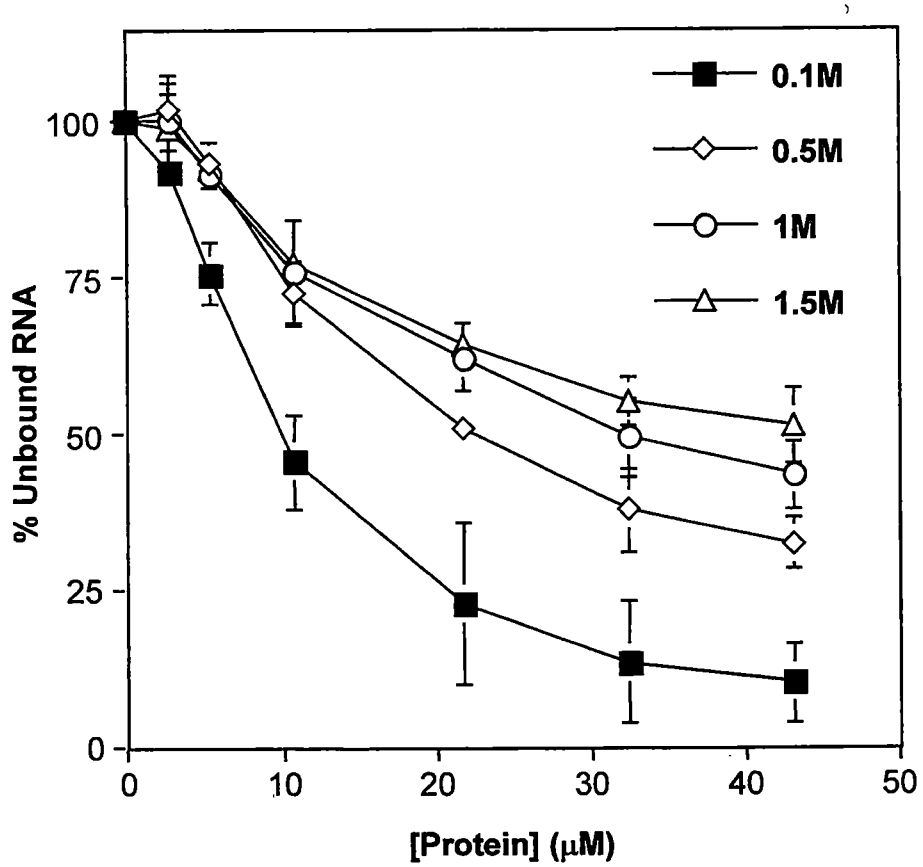


Figure 7. Effect of salt concentration on RNA binding by rWTR. Reactions containing ^{32}P-labeled CPBS RNA (10^4 cpm) and varying concentrations of rWTR (0-43 μM) and NaCl (0.1-1.5 M) were electrophoresed on a nondenaturing 4.5% polyacrylamide gel. The percentage of unbound RNA was determined using a phosphoimager. The results shown are the average of three independent experiments.

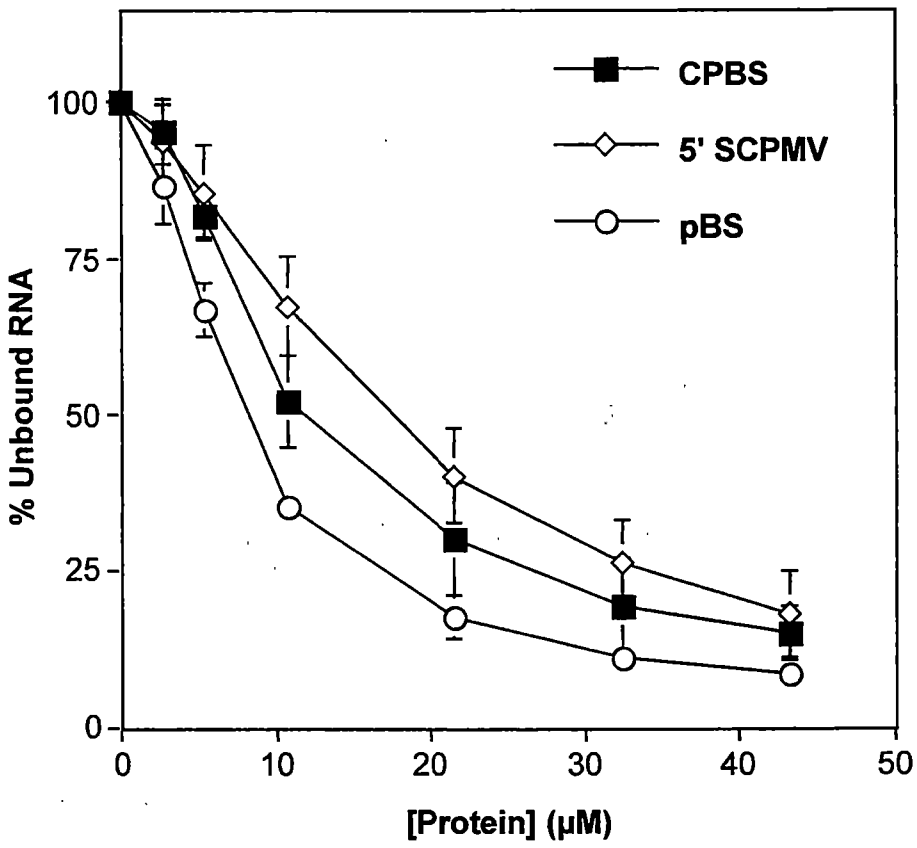


Figure 8. Specificity of RNA binding by rWTR. ^{32}P-labeled RNAs (10^4 cpm) including the CPBS RNA, the 5' end of the SCPMV RNA (5' SCPMV) and an RNA transcribed from the polycloning region of pBluescript (pBS) were incubated in the presence of varying concentrations (0-43 μM) of rWTR and analyzed as described in Fig. 7.

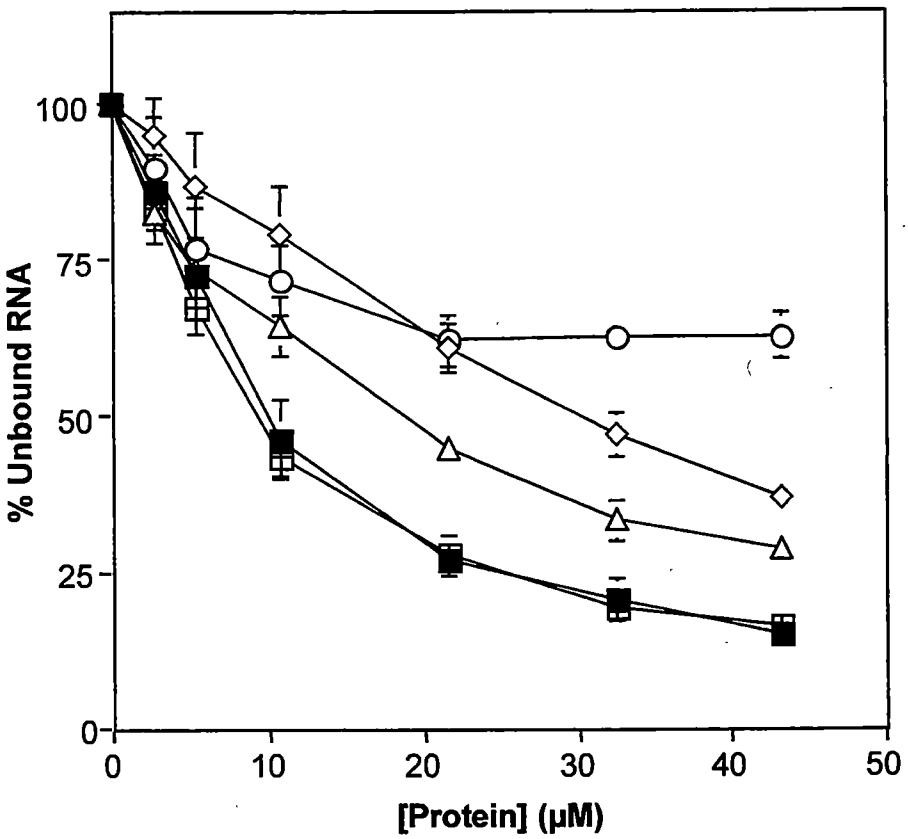


Figure 9. Deletion mapping of the RNA binding site of rWTR. rWTR (—■—), rΔ1-16 (—◇—), rΔ17-30 (—○—), rΔ31-39, (—△—)and rΔ40-53 (—⊞—) were incubated separately with ^{32}P-labeled CPBS RNA (10^4 cpm), and analyzed as in Fig. 7.

activity of the R domain.

The requirement for basic amino acids of the R domain for RNA binding

Eleven basic residues are located within the N-terminal 30 amino acids of the R domain. To determine if these charged residues are required for RNA binding, two recombinant R domain mutants (rA1 and r3A) were analyzed by EMSA. rA1 has alanine substitutions for all eight basic residues in the arginine-rich region, and r3A has alanine substitutions for the three basic residues at positions 3, 6, and 7 of the R domain (Fig. 5C). A low level of RNA binding activity was observed for rA1, but r3A bound RNA with slightly higher affinity than rWTR (Fig. 10). These results demonstrated that basic residues in the arginine-rich region but not those at residues 3, 6, and 7 are necessary for RNA binding by the R domain.

Minimal charge requirements for RNA binding by the arginine-rich region of the R domain

For other RNA binding proteins with an arginine-rich region such as the tat and rev proteins of HIV-1, RNA binding has been shown to require one or more specific arginine residues (Calnan *et al.*, 1991, Battiste *et al.*, 1996). To determine if the same is true for the R domain of the SCPMV CP, recombinant R domain proteins with alanine substitutions for two or three consecutive basic residues within the arginine-rich region were analyzed by EMSA. The mutant proteins rA2, rA3, and rA4 have alanine substitutions at residues 22-24, 25-26, and 28-30, respectively (Fig. 5C). All three proteins had approximately the same affinity for RNA as the rWTR

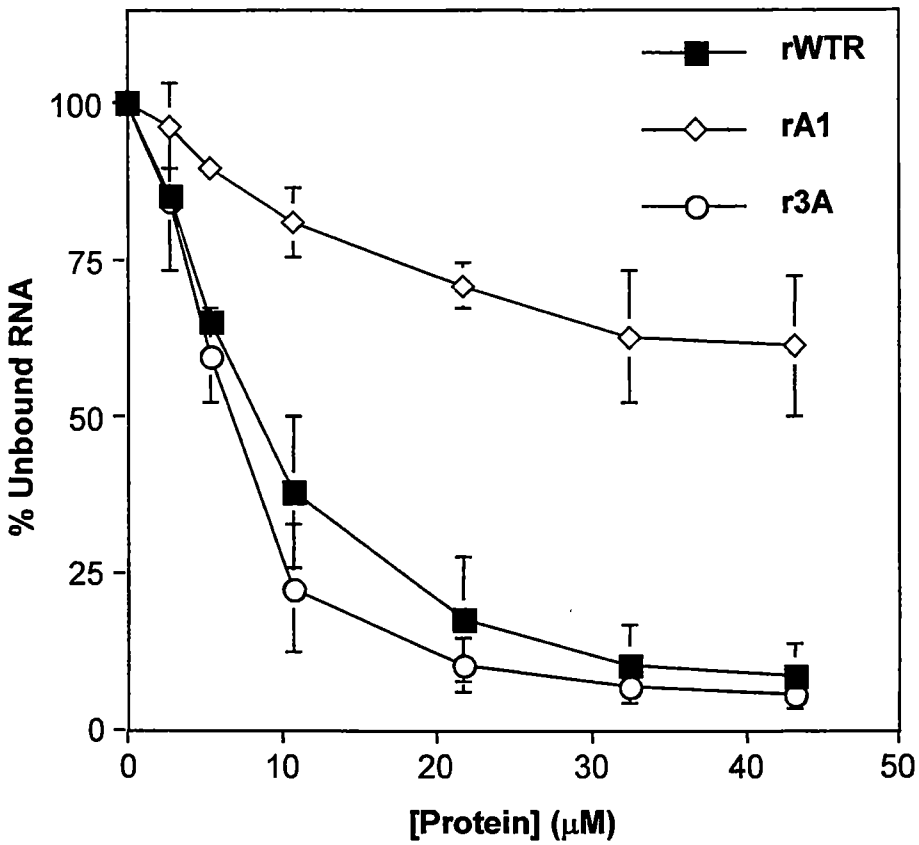


Figure 10. Requirement for basic amino acids for RNA binding by rWTR. rWTR, rA1, and r3A were incubated separately with ^{32}P-labeled CPBS RNA (10^4 cpm), and analyzed as in Fig. 7.

(Fig. 11). These results demonstrated that the basic residues within the arginine-rich region of the R domain are not specifically required for the interaction with RNA.

To determine if RNA binding by the arginine-rich region requires a minimum number of basic residues, the RNA binding activity of two additional alanine substitution mutants was analyzed. The mutant proteins rA5 and rA6 have five and seven alanine substitutions for basic residues in the arginine-rich region, respectively (Fig. 5C). It was demonstrated that rA5 had approximately the same affinity for RNA as rWTR but the RNA-binding affinity of rA6 was reduced about two-fold compared to rWTR (Fig. 12). The effects of alanine substitutions in the arginine-rich region were more pronounced, however, when combined with alanine substitutions for basic residues near the N-terminus of the R domain. The mutant proteins r3A/A5 and r3A/A6 have alanine substitutions for the three basic amino acids at residues 3, 6, and 7 in addition to the alanine substitutions in the arginine-rich region as described for rA5 and rA6 (Fig. 5C). These mutants had little affinity for RNA (Fig. 12). These results demonstrated that three basic amino acids within the arginine-rich region are sufficient for wild-type levels of RNA binding when additional basic residues are present within the N-terminus of the R domain. These results also suggest that a minimal number of basic residues is required for RNA binding by the R domain. Wild-type levels of RNA binding were observed when six but not four basic residues were present within the N-terminal 30 aa of the R domain.

The effect of prolines on RNA binding by the R domain

Residues 6-14 of the R domain were predicted by the AGADIR program to

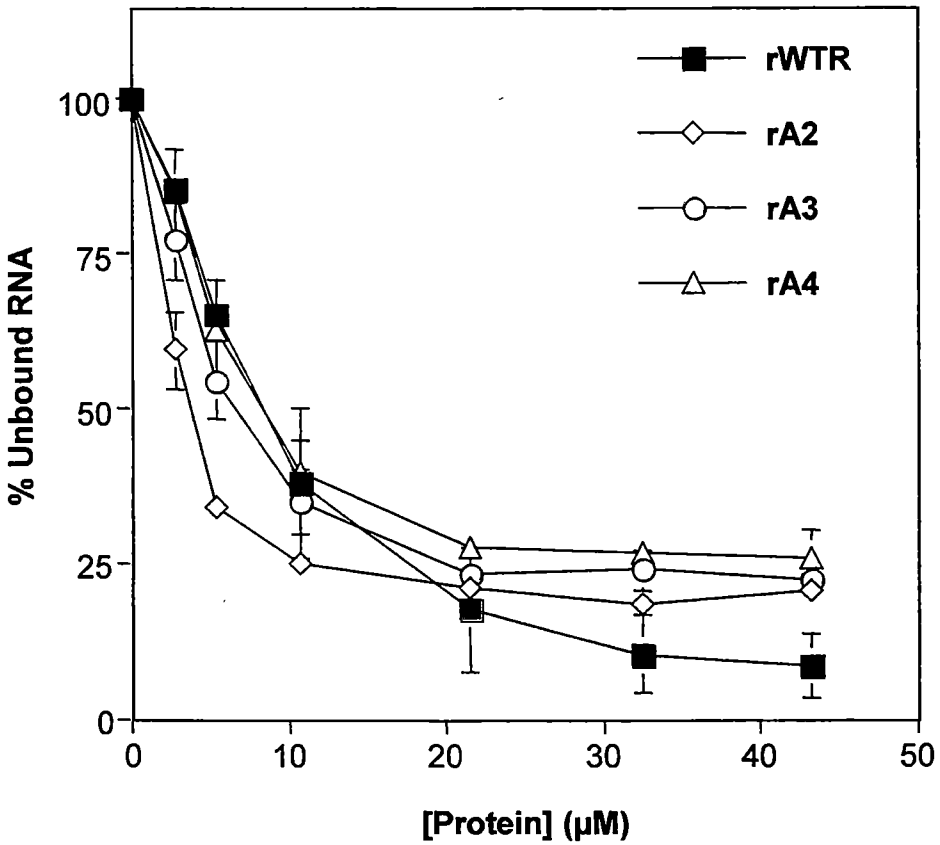


Figure 11. Specificity of basic amino acids of the arginine-rich region of rWTR for RNA binding. rWTR, rA2, rA3, and rA4 were incubated separately with ^{32}P-labeled CPBS RNA (10^4 cpm), and analyzed as in Fig. 7.

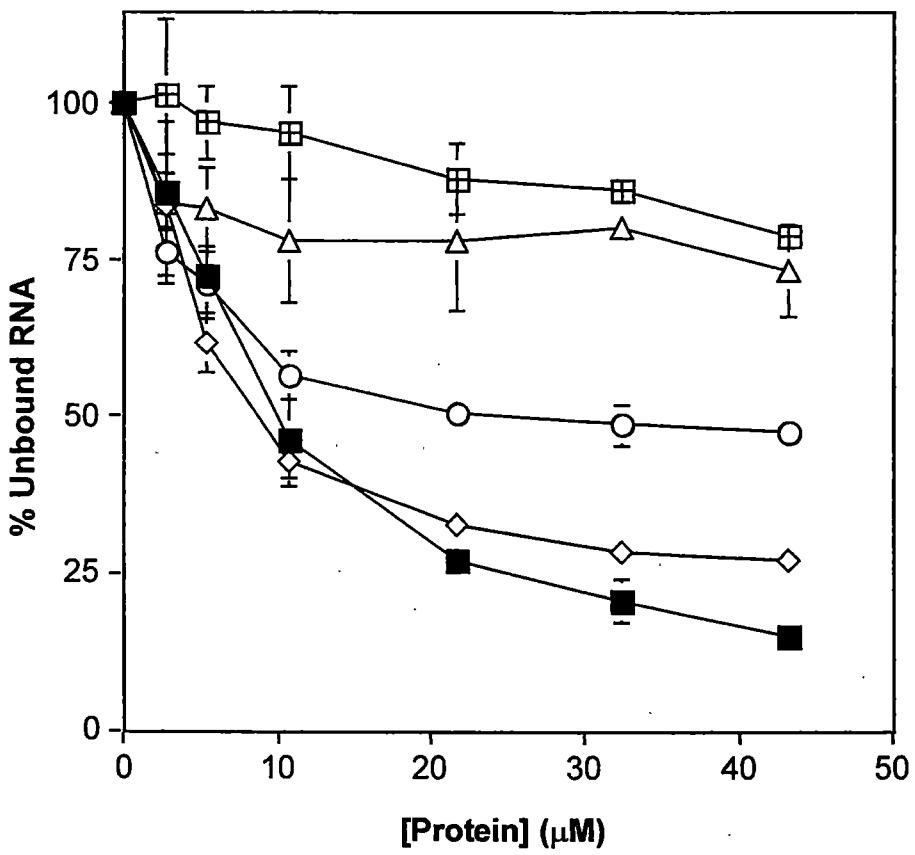


Figure 12. Minimal requirement for basic amino acids in the arginine-rich region of rWTR. rWTR (—■—), rA5 (—◇—), rA6 (—○—), r3A/A5 (—△—), and r3A/A6 (—⊞—) were incubated separately with ^{32}P-labeled CPBS RNA (10^4 cpm). Gel electrophoresis was performed as described in Fig. 7.

fold into an α-helix (Muñoz and Serrano, 1997). To determine if the N-terminus of the SCPMV CP folds into an α-helix in solution, a peptide corresponding to aa 1-30 of the CP (CP peptide$_{1-30}$) was chemically synthesized and its helicity was measured by CD in the presence or absence of 50% TFE. The peptide was found to be a random coil in the absence of TFE, but about 42% helicity was observed for the peptide in the presence of TFE (Fig. 13). This result implies that the N-terminus of the SCPMV CP might fold into an α-helix upon RNA binding.

To determine if protein secondary structure contributes to the RNA binding activity of the R domain, three R domain mutant proteins with proline substitutions for Arg3 (rP1), Lys7 (rP2), or Ala27 (rP3) (Fig. 5C) were analyzed by EMSA. In addition, the RNA binding activity of a fourth mutant (rP4) having alanines substituted for the proline residues at positions 18, 19, and 21 (Fig. 5C) was also investigated. Surprisingly, all of the mutants bound RNA with about the same affinity as rWTR (Fig. 14). The results can be interpreted in one of two ways. Either the mutations did not alter the structure of the RNA binding site or the secondary structure of the N-terminal of the CP is not important for RNA binding.

DISCUSSION

It has been proposed that the R domain of the SCPMV CP interacts with RNA on the interior of the virus particle and that this interaction is necessary for assembly of T=3 particles (Abad-Zapatero *et al.*, 1980; Erickson and Rossmann, 1982). In this report, it was demonstrated that the N-terminal 30 amino acids of the R domain

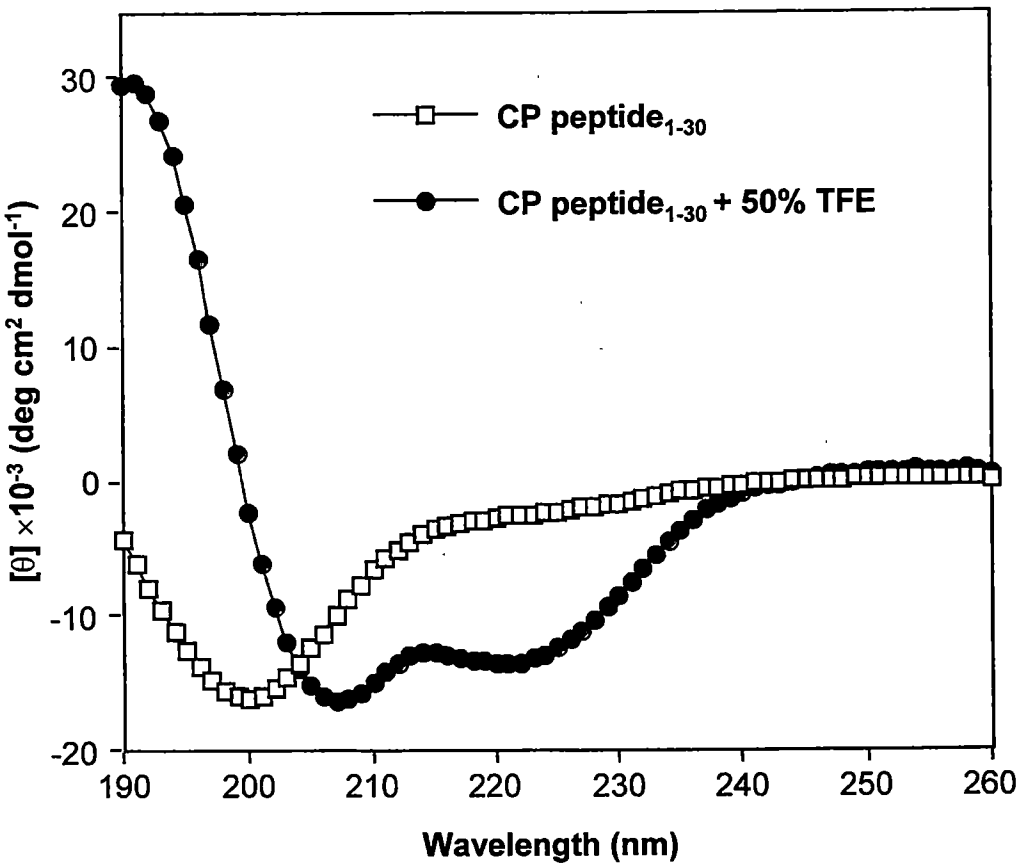


Figure 13. Circular dichroism of CP peptide$_{1-30}$. CD spectra are shown in the absence and in the presence of 50% TFE.

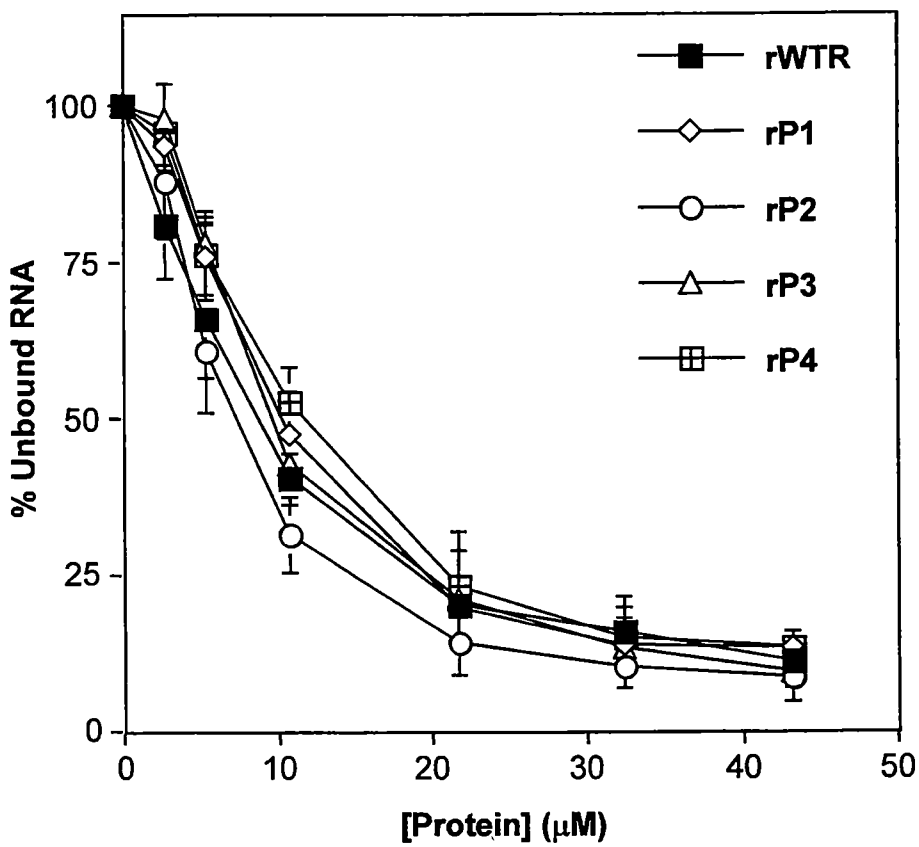


Figure 14. Effects of proline mutations on RNA binding by rWTR. rWTR, rP1, rP2, rP3, and rP4 were incubated separately with ^{32}P-labeled CPBS RNA (10^4 cpm), and analyzed as in Fig. 7.

constitute the major determinant for RNA binding *in vitro*. The most critical component of the RNA binding site is the arginine-rich region between residues 22-30. We propose that this element functions in RNA binding predominantly through electrostatic interactions because the binding was nonspecific with regard to RNA sequence, the presence of high salt interfered with RNA binding, and no basic residues within the arginine-rich region were specifically required for RNA binding. A second element of the RNA binding site is present within the 16 N-terminal amino acids of the R domain. It is not known how this element functions in RNA binding, but it is clear that the three basic amino acids at positions 3, 6, and 7 are not necessary for this activity. As discussed in more detail below, one possible function of this element is to stabilize the RNA-protein interaction by folding into an α-helix upon RNA binding. It is also proposed that the 30 N-terminal amino acids of the R domains of A, B, and C subunits participate in RNA binding in the intact particle. Although only nonspecific RNA binding by the R domain was observed in this study, it remains a possibility that the R domain is also involved in specific interactions with the viral RNA. Models for the assembly of T=3 spherical viruses propose that one or more CP subunits bind specifically to the viral RNA at the origin of assembly (OAS) (Rossmann *et al.*, 1983; Sorger *et al.*, 1986). This specific interaction, if it occurs for SCPMV, may require a multimeric form of the CP, and the OAS may only be recognized by a specific conformation of the S and R domains. Thus, the R domain alone may not be sufficient for this specific interaction with the viral RNA. The CPBS RNA used here was previously identified as a high-affinity binding site for the SCPMV CP in a CP-RNA complex recovered from dissociated virus (Hacker, 1995), but the

CPBS has not been demonstrated to be the SCPMV OAS.

Although the arginine-rich region plays an essential role in RNA binding by the R domain, none of the basic amino acids in this element are specifically required to perform this function. Arginine-rich regions are present in other RNA binding proteins including the tat and rev proteins of human immunodeficiency virus 1 (HIV-1), the N protein of bacteriophage λ, ribosomal protein L11, and the bovine immunodeficiency virus (BIV) Tat protein (Draper, 1999). Except for the tat protein of HIV-1, the structures of these proteins bound to RNA have been resolved (Battiste *et al.*, 1996; Legault *et al.*, 1998; Puglisi *et al.*, 1995; Ye *et al.*, 1995; Conn *et al.*, 1999). In all these examples, specific arginine residues contact specific bases of the RNA. The structures of the RNA binding domains of these proteins are distinct, and these proteins contact RNA in different ways. The SCPMV CP, therefore, provides another example of how an arginine-rich region can be utilized for RNA binding. In this case, the RNA binding site is located within a disordered region of the protein and functions in nonspecific RNA binding. Recently, it was shown that specific arginine residues within the arginine-rich region of the R domain of the BMV CP are necessary for the specific encapsidation of BMV RNA4 (Choi *et al.*, 2000; Choi and Rao, 2000). Thus, arginine-rich regions in disordered domains of proteins may also interact with RNA specifically.

Like the SCPMV CP, the N-terminus of the cowpea chlorotic mottle bromovirus (CCMV) CP is highly basic (NH_2-STVGTGKLTRAQRRAAARKNKRNTR-COOH) and is localized to the interior of the virus particle (Dasgupta and Kaesberg, 1982; Speir *et al.*, 1995). The N-terminal 25 residues are predicted to form a

random-coil when the lysine and arginine side-chains are charged, but when these side-chains are neutralized, residues 10-20 are predicted to form an α-helix (Vriend *et al.*, 1986). Based on these predictions, a "snatch-pull" model of RNA binding by the N-terminus of the CCMV CP was proposed (Vriend *et al.*, 1986). It was predicted that an interaction between the phosphate backbone of RNA and a basic residue of the N-terminus initiated the formation of the α-helix between residues 10-20 and that the neutralization of one basic side-chain favored additional ionic interactions between basic side-chains and the RNA so that the α-helix is extended in a C-terminal direction (Vriend *et al.*, 1986). This model was examined by studies of the conformation of a synthetic peptide corresponding to the N-terminal 25 residues of the CCMV CP using nuclear magnetic resonance (NMR) and CD. It was demonstrated that residues 10-20 form an α-helix in the presence of oligophosphates (van der Graaf and Hemminga, 1991; van der Graaf *et al.*, 1992). Interestingly, the CD results of this study showed that amino acids within the N-terminus of the SCPMV CP fold into an α-helix in the presence of 50% TFE. This finding suggests a possibility that the conformation of the N-terminal 30 aa of the SCPMV CP may change from a random coil to an α-helix upon binding to RNA. It is possible that the α-helix stabilizes the interaction between the RNA and the arginine-rich region. The low affinity of rΔ1-16 for RNA can be explained by this model. In contrast, the results of the RNA binding studies with rP1, rP2, rP3, and rP4 seemingly do not support the model. It is not known, however, if these mutations affect the secondary structure of the RNA binding site. It is possible that all or part of the predicted α-helix between aa

6-14 may form despite these mutations. To better understand RNA binding by the R domain, the conformation of its RNA binding site will need to be determined in the presence and absence of RNA using a biophysical approach.

CHAPTER III

In vitro analysis of the interaction of the N-terminus of the *Southern cowpea mosaic virus* coat protein with membranes

INTRODUCTION

The resolution of the atomic structure of SCPMV revealed the presence of a pore at the five-fold axis of symmetry (Abad-Zapatero *et al.*, 1980), and it has been proposed that this pore may function as an ion channel (Silva *et al.*, 1987). Recently, SCPMV has been shown to function as an ion channel in a planar lipid bilayer, but it is not known if this activity is due to the presence of the pore at the five-fold axis (Helrich and Pinto, unpublished data). The ion channel activity has been observed in 300 mM KCl at pH 7.4, conditions which have been shown to allow expansion of SCPMV *in vitro* (Fitch and Garcia-Moreno, unpublished data). One explanation for these results is that upon expansion, the R domain of the SCPMV CP is externalized and interacts with the lipid bilayer resulting in an ion flux across the membrane. In support of this proposal it has been shown that purified SCPMV CP binds to negatively charged liposomes in the presence but not in the absence of the R domain (Datema *et al.*, 1987).

Similarly, the N-terminus of the PV CP (VP1) has been shown to interact with liposomes (Fricks and Hogle, 1990). The N-terminus of VP1 is localized to the interior of the particle but is externalized upon expansion (Fricks and Hogle, 1990; Hogle *et al.*, 1985). The γ peptide of FHV is also able to bind and

permeabilize liposomes (Bong *et al.*, 1999; Janshoff *et al.*, 1999). The γ peptide is cleaved from the C-terminus of the FHV CP (Schneemann *et al.*, 1992). It is present within the FHV capsid, but it is externalized upon particle expansion (Bothner *et al.*, 1998). These results suggest that interactions between membranes and the SCPMV, PV, and FHV CPs may be necessary for the viral infection cycle. Since PV and FHV are animal viruses that penetrate host cells by receptor-mediated endocytosis, it is possible that the PV and FHV CPs interact with the endosomal membrane to allow transport of the viral RNA out of the endosome. This is not expected to be the case for SCPMV, however, since there is no evidence that plant viruses are endocytosed by plant cells.

In this study, the interaction between the SCPMV CP and liposomes was investigated *in vitro* using a dye-release assay. It was demonstrated that the N-terminal 30 amino acids of the CP are sufficient for this interaction. It was also demonstrated that the interaction requires a negatively charged lipid (PA) or non-bilayer lipid (PE) and is sensitive to high salt and low pH. CD studies showed that the interaction caused an increase in the α-helical content of the N-terminus of the CP. The potential role of this interaction in SCPMV transmission and replication is discussed.

MATERIALS AND METHODS

Materials

Phosphatidylcholine (PC, 99% purity), phosphatidylethanolamine (PE, 99% purity), phosphatidylserine (PS, 99% purity), 1,2-dimyristoyl-sn-glycero-3-phosphoglycerol (dimyristoyl- phosphatidylglycerol, DMPG, 99% purity), and 1,2-dilauroyl-sn-glycero-3-phosphatidic acid (dilauroylphosphatidic acid, DLPA, 99% purity) were purchased from Avanti Polar Lipids, and calcein was purchased from Sigma Chemical. A synthetic peptide corresponding to the N-terminal 30 amino acids (CP peptide$_{1-30}$) of the SCPMV CP were prepared at the Keck Biotechnology Resource Laboratory at the Yale University School of Medicine.

Plasmid construction

The construction of plamids pWTR, pΔ1-16, pΔ17-30, pΔ31-39, pΔ40-53, pA1, p3A, pP1, pP2, pP3, and pP4 was described in the Materials and Methods Section of Chapter II.

Recombinant protein purification

The recombinant proteins were purified from *E. coli* extracts by affinity chromatography using a nickel-chelating column as described in the Materials and Methods Section of Chapter II. For dye-release assay proteins were dialyzed against phosphate buffered saline (PBS) at pH 7.4 containing 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4. For CD experiments proteins were dialyzed

against 20 mM potassium phosphate buffer (pH 7.4). His-S tag protein was purified from *E. coli* BL21 (DE3) cells transformed with pET30b. Following affinity chromatography, the protein was filtered using a YM 30 membrane (Amicon) and the filtrate was concentrated using a YM 5 membrane (Amicon). Protein concentration was determined by the Bradford assay.

Coat protein preparation

SCPMV was propagated and purified as described by Johnson *et al.* (1974). The SCPMV CP was purified from virus as described by Erickson and Rossmann (1982). Briefly, the virus (10-20 mg/ml) was expanded in 0.1 M sodium phosphate buffer (pH 8.0) containing 0.01 M EDTA and 1 mM phenylmethylsulfonylfluoride (PMSF) for 2 hr at 0°C and then dissociated by adding an equal volume of 4 M LiCl and freezing overnight at –20°C. The RNA precipitate was removed by low-speed centrifugation for 20 min at 4°C. The supernatant was first dialyzed in the cold against 0.01 M phosphate buffer (pH 8.0) containing 0.01 M EDTA and 1 mM PMSF, and further dialyzed against PBS.

S domain preparation

S domain was prepared from purified SCPMV CP by mild trypsin digestion (Erickson and Rossmann, 1982). Briefly, the CP was mixed with trypsin in a ratio of 100:1 (w/w) CP to trypsin (Sigma) and incubated for 1 hr at room temperature. The protein was then dialyzed against PBS using dialysis tubing with a 6-8 kDa cut-off size to remove low molecular weight peptides.

Liposome Preparation

Liposomes were prepared as previously described with some modifications (Pinnaduwage and Bruce, 1996). The appropriate lipids (10 mM each) were mixed in chloroform. The solvent was evaporated under a stream of N_2, and the samples were vacuum desiccated overnight. The dried lipid film was hydrated for 1 hr in PBS containing 1 mM EGTA, 0.02% sodium azide, and 50 mM calcein. For CD analysis, the dried lipid film was hydrated in 20 mM potassium phosphate buffer containing 1 mM EGTA and 0.02% sodium azide without 50 mM calcein. The lipids were mixed by vortexing and then sonicated for 5 min in a bath sonicator (Laboratory Supplies Inc.). The lipids were incubated at room temperature for 4-5 hrs and then sonicated again. This step was repeated three times. Free unincorporated calcein was removed by chromatography on a Bio-Gel A column (0.5 m) equilibrated in PBS containing 1 mM EGTA and 0.02% sodium azide. Liposomes eluted in the void volume had a calcein fluorescence quenching of >70-85% (see below).

Dye-release assay

Fluorescence of liposomes was measured as described by Pinnaduwage and Huang (1992) using a Perkin-Elmer LS 50 Spectrofluorometer. Fluorescence excitation was at 490 nm. Percent fluorescence quenching was calculated from the formula

$$\% \text{ Quenching} = (1 - F_0 / F_t) \times 100$$

where F_0 and F_t are the fluorescence of the liposome samples before and after addition of 0.1% Triton X-100, respectively.

Liposomes containing entrapped calcein were mixed with various concentrations of recombinant protein in 2 ml of reaction buffer containing PBS and 1 mM EGTA. The reaction mixtures were incubated at room temperature for 2 hr. The fluorescence measurements were performed as described above. The percentage of calcein release was calculated using the formula

$$\% \text{ Release} = (F - F_0 / F_t - F_0) \times 100$$

where F_0 and F are the calcein fluorescence before and after the addition of the protein, respectively, and F_t is the total fluorescence after addition of 0.1% Triton X-100. All experiments were done in triplicate.

Circular dichroism

CD spectra were measured using an Aviv model 202 spectropolarimeter at 25°C with a 1 mm cell. Recombinant proteins and CP peptide$_{1\text{-}30}$ were prepared in 20 mM potassium phosphate (pH 7.4). CD spectra were collected at wavelengths of 260 nm to 200 nm and reported in terms of ellipticity units per mol peptide residue [θ]. The spectrum of a reference sample was subtracted from the CD spectra of solutions containing protein or peptide. The reported CD spectra are the average of at least three wavelength scans.

RESULTS

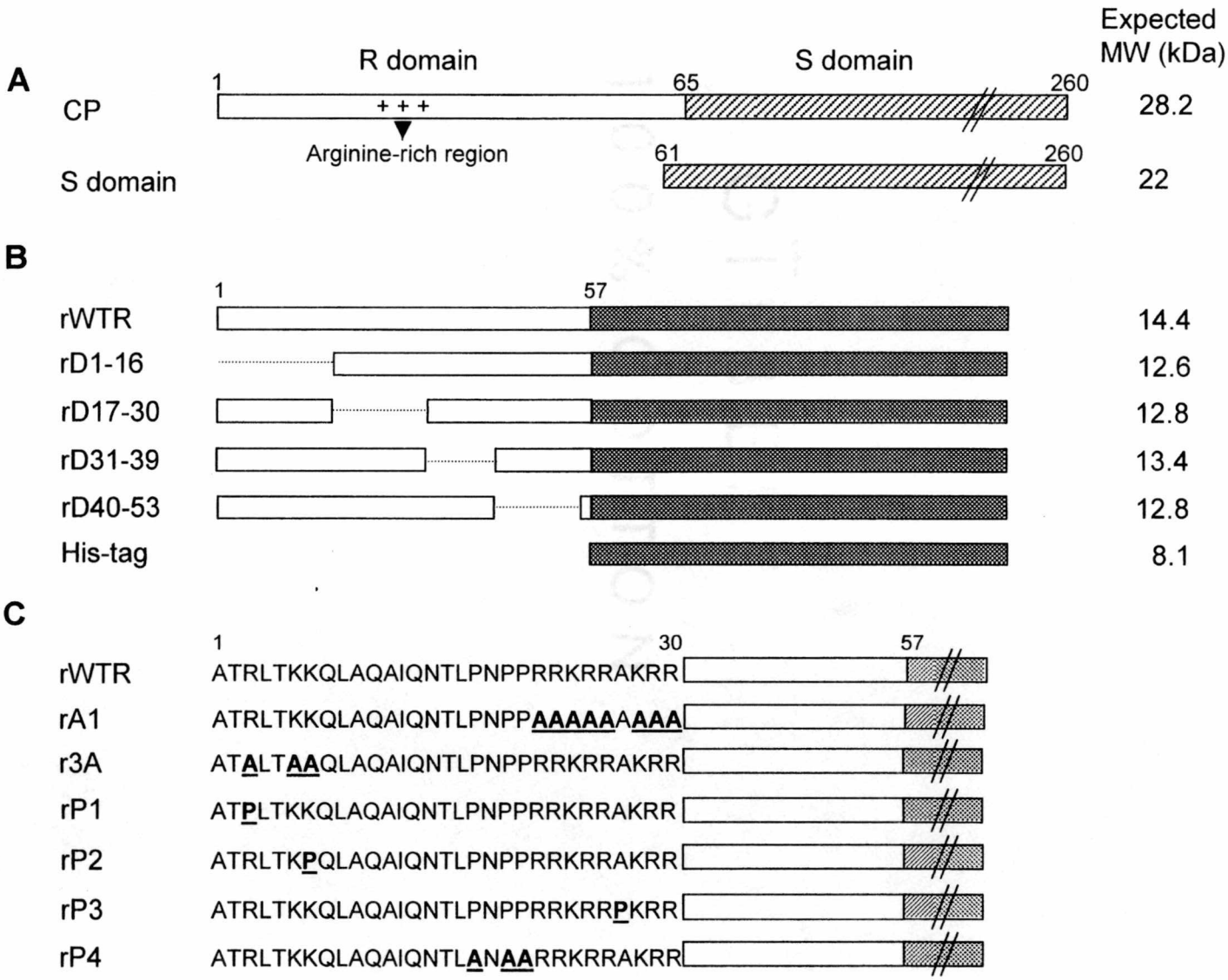
Interaction of the SCPMV CP with liposomes

The interaction between the SCPMV CP and neutral or negatively charged liposomes was investigated using a dye-release assay. When the fluorescent dye calcein is entrapped in liposomes it is self-quenched, but if it is released following liposome lysis it will fluoresce when excited at the appropriate wavelength. Using this assay the ability of the SCPMV CP to interact with membranes was investigated. A schematic diagram of the SCPMV CP is shown in Fig. 15A, and diagrams of the recombinant proteins used to analyze liposome-interaction activity are shown in Figs. 15B and 15C. The purity of the viral and recombinant proteins used in this study was determined by SDS-PAGE (Fig.16). An anomalous behavior is observed for rΔ17-30 when it migrates in the gel due to the deletion of the arginine-rich region (Fig. 16).

When the purified SCPMV CP was mixed with negatively charged PC/PA (8:2) liposomes, 100% calcein release was observed (Fig. 17A). In contrast, only ≈7.0% of the calcein was released from neutral PC liposomes in the presence of the CP (Fig. 17A). To identify the CP domain that is required for the liposome interaction, the R domain and the S domain were tested separately for the ability to induce lysis of PC/PA liposomes.

The R domain (aa 1-57) was prepared as a recombinant protein (rWTR) with a non-viral C-terminal extension of 8.1 kDa that included two histidine tags

Figure 15. Schematic representation of the SCPMV CP and the recombinant R domain proteins. (A) A diagram of the CP and the S domain. The expected molecular weights are shown on the right. The R domain and the S domain are illustrated as white and hatched boxes, respectively. The position of the arginine-rich region is indicated. The numbers refer to the amino acid residue. (B) Diagram of rWTR and the R domain deletion mutants. The His-S tag domain is shown as a gray box. The predicted molecular weight of the recombinant proteins are shown on the right. (D) Diagram of the alanine and proline substitution mutants. The sequence of the R domain residues 1-30 is shown. Residues 31-57 of the R domain are shown as a white box, and the His-S tag domain is shown as a hatched box. Substituted residues are shown in bold and are underlined.


A
R domain
S domain
Expected MW (kDa)
1
65
260
CP
+ + +
28.2
Arginine-rich region
61
260
S domain
22
B
1
57
rWTR
14.4
rD1-16
12.6
rD17-30
12.8
rD31-39
13.4
rD40-53
12.8
His-tag
8.1
C
1
30
57
rWTR ATRLTKKQLAQAIQNTLPNPPRRKRRAKRR
rA1 ATRLTKKQLAQAIQNTLPNPPAAAAAAAAAA
r3A ATALTAAQLAQAIQNTLPNPPRRKRRAKRR
rP1 ATPLTKKQLAQAIQNTLPNPPRRKRRAKRR
rP2 ATRLTKPQLAQAIQNTLPNPPRRKRRAKRR
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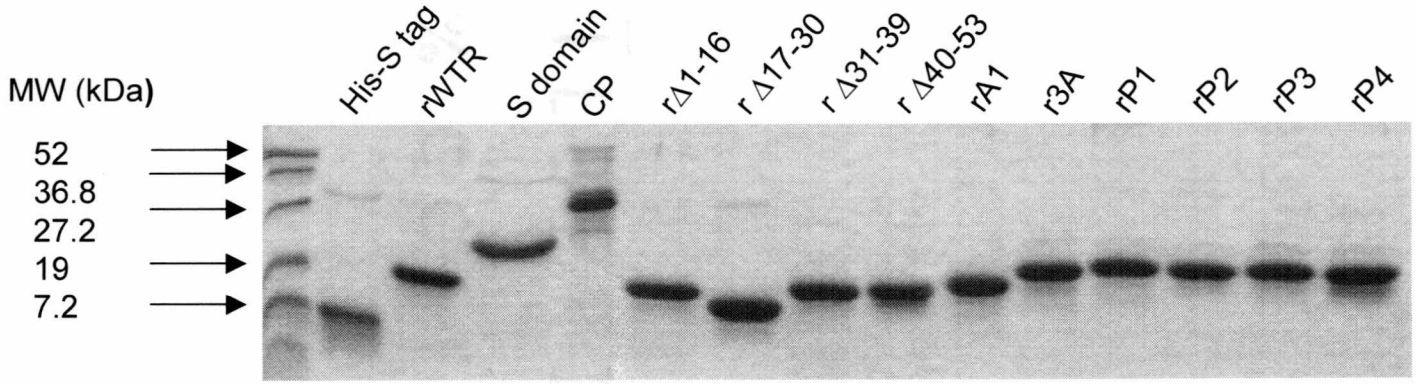
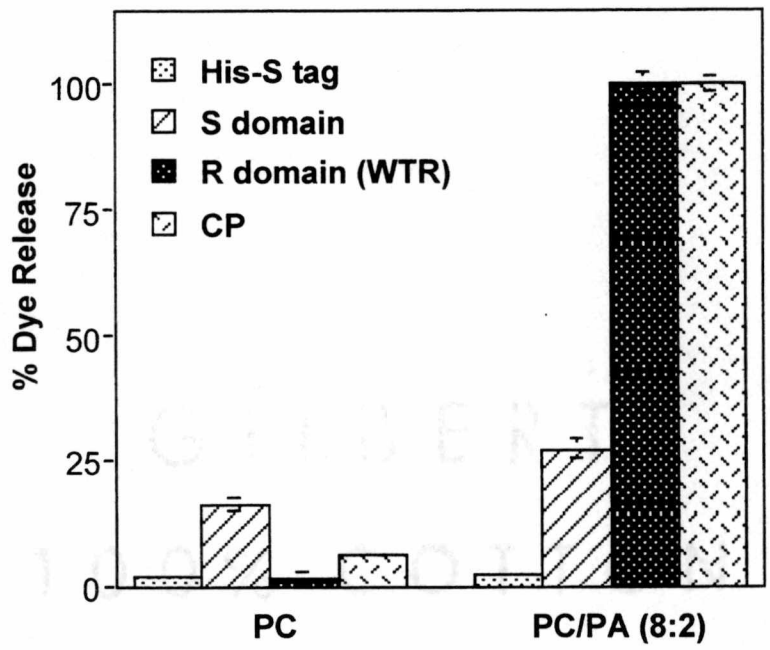


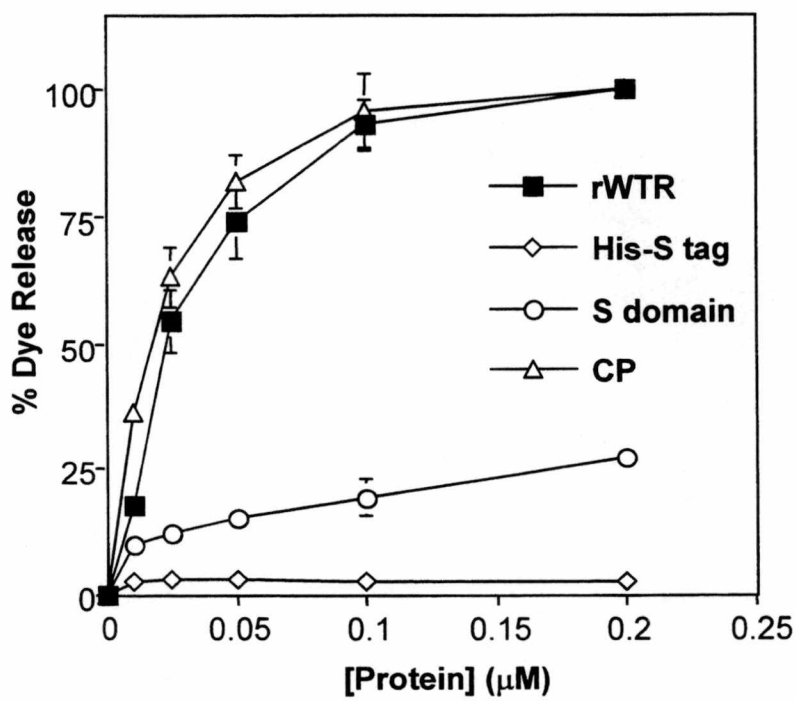
Figure 16. SDS-PAGE analysis of purified proteins. The gel was stained with Coomassie Brilliant Blue.

Figure 17. Liposome lysis by His-S tag, the S domain, rWTR, and the CP. (A) PC or PC/PA (8:2) liposomes were incubated for 2 hr at room temperature with 0.2 μM of proteins. (B) PC/PA (8:2) liposomes were incubated at room temperature for 2 hr with varying concentrations (0-0.2 μM) of proteins. Percent dye release was determined as described by Pinnaduwage and Huang (1992).

A


His-S tag
S domain
R domain (WTR)
CP
% Dye Release
100
75
50
25
0
PC
PC/PA (8:2)

B


% Dye Release
100
75
50
25
0
rWTR
His-S tag
S domain
CP
0
0.05
0.1
0.15
0.2
0.25
[Protein] (μM)

and one S tag (His-S Tag domain) (Fig. 15B). The S domain (22 kDa) was prepared from purified SCPMV CP that was treated with trypsin which cleaves the CP at Arg 61 (Fig. 15A) (Erickson and Rossmann, 1982). In the presence of rWTR, 100% of the dye was released from the negatively charged liposomes while almost no dye release was observed when neutral liposomes were incubated with rWTR (Fig. 17A). No dye release was observed when the liposomes were incubated with the His-S tag protein demonstrating that dye release in the presence of rWTR is due to residues of the R domain. About 20-25% dye release was observed from either PC or PC/PA liposomes in the presence of the S domain (Fig. 17A). Similar results were observed when dye release from PC/PA liposomes was measured as a function of protein concentration using the SCPMV CP, rWTR, the S domain, and the His-S tag protein (Fig. 17B). These results demonstrated that the SCPMV CP interacts with negatively charged liposomes but not with neutral liposomes and that the membrane interaction region of the CP is located within the R domain.

Deletion mapping of the membrane interaction region of the R domain

The region of the R domain that interacts with PC/PA liposomes was initially mapped using four deletion mutant proteins rΔ1-16, rΔ17-30, rΔ31-39, and rΔ40-53 (Fig. 15B). Dye release from PC/PA liposomes was measured as a function of protein concentration (Fig. 18). The deletion of aa 1-16 and aa 17-30 significantly reduced the interaction between the liposomes and the R domain (Fig. 18). Only about 10% dye release was observed in the presence of the

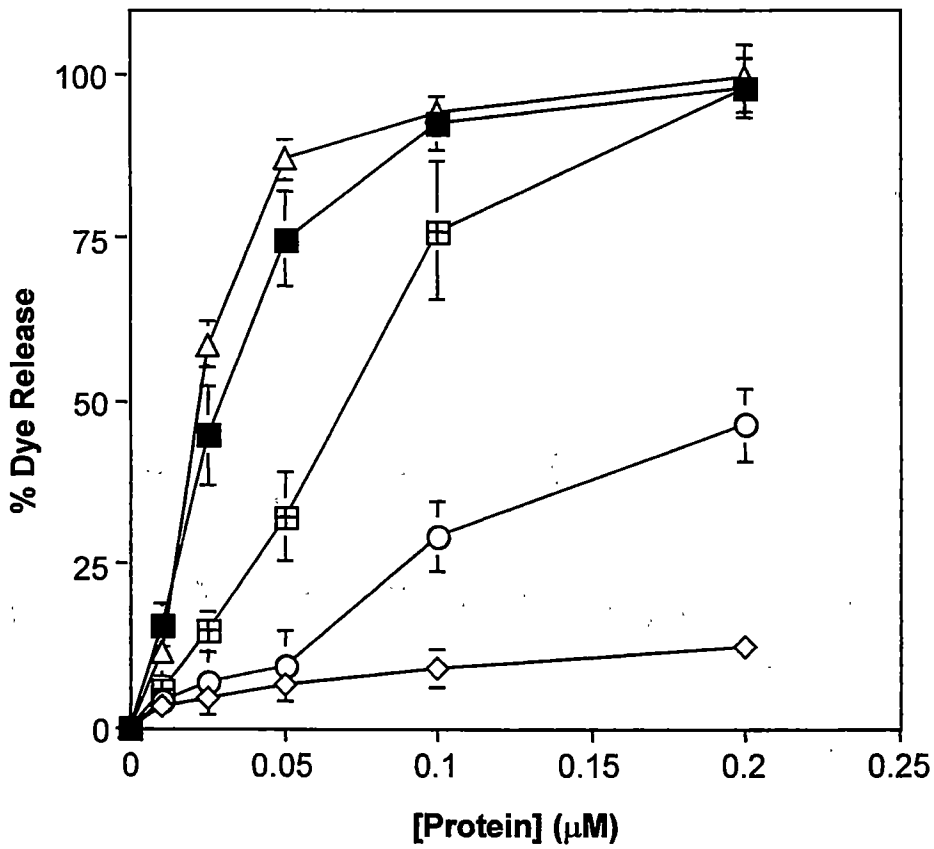


Figure 18. Deletion mapping of the membrane-binding domain of the R domain. PC/PA (8:2) liposomes were incubated at room temperature for 2 hr with varying concentrations (0-0.2 μM) of rWTR (—■—), rΔ1-16 (—◇—), rΔ17-30 (—○—), rΔ31-39 (—△—), and rΔ40-53 (—⊞—). Percent calcein release was measured as described by Pinnaduwage and Huang (1992).

highest concentration of rΔ1-16. On the other hand, the deletion of aa 31-39 did not noticeably alter the ability of the R domain to interact with PC/PA liposomes. At most concentrations of rΔ40-53 the dye release was less than that caused by the same concentration of rWTR (Fig. 18). These results demonstrated that the N-terminal 30 residues of the R domain are important for the interaction between the R domain and PC/PA liposomes and that residues 1-16 are required for this interaction.

Effect of basic residues within the R domain on the liposome interaction

The N-terminal 30 aa of the R domain includes eleven basic residues (Fig. 15C). To determine if they contribute to the interaction between the R domain and PC/PA liposomes, alanine substitution mutants (rA1 and r3A) were analyzed for their ability to induce lysis of PC/PA liposomes. rA1 has alanine substitutions for all eight basic residues in the arginine-rich region, and r3A has alanine substitutions for the basic residues at positions 3, 6, and 7 of the R domain (Fig. 15C). The percent dye release from PC/PA liposomes was measured in the presence of varying concentrations of rWTR, rA1, or r3A (Fig. 19). Both mutants caused less dye release than rWTR over all protein concentrations tested, but rA1 affected dye release more dramatically than r3A. The level of dye release resulting from incubation of liposomes with rA1 was about the same as that resulting from incubation with rΔ17-30 (cf. Fig. 18). These results demonstrated that the basic residues within the arginine-rich region are important for the interaction between the R domain and PC/PA liposomes. By comparison, the

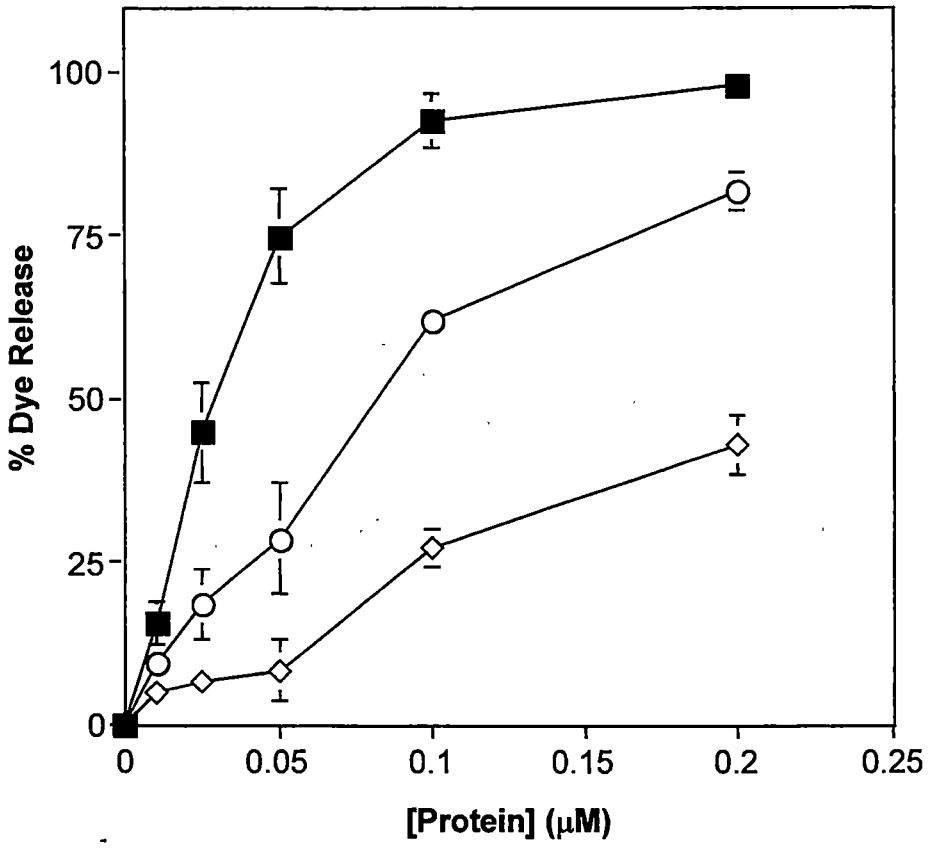


Figure 19. Effect of basic residues of the R domain on liposome lysis. PC/PA (8:2) liposomes were incubated with varying concentrations (0-0.2 μM) of rA1 (—◇—) and r3A (—○—). Liposome lysis by the rWTR (—■—) is also shown. Percent calcein release was measured as described in Materials and Methods.

basic residues within the N-terminal 16 residues of the R domain are less critical for this interaction.

Effects of salt concentration and pH on the interaction between the R domain and liposomes

Since charge interactions are involved in the R domain-liposome interaction, the effect of NaCl concentration on this interaction was analyzed. rWTR was pre-equilibrated in PBS containing supplementary NaCl (50-150 mM) and incubated with PC/PA liposomes. As concentration of NaCl increased the percentage of dye release in the presence of rWTR decreased (Fig. 20A). At 150 mM of added NaCl, dye release was almost completely inhibited (Fig. 20A).

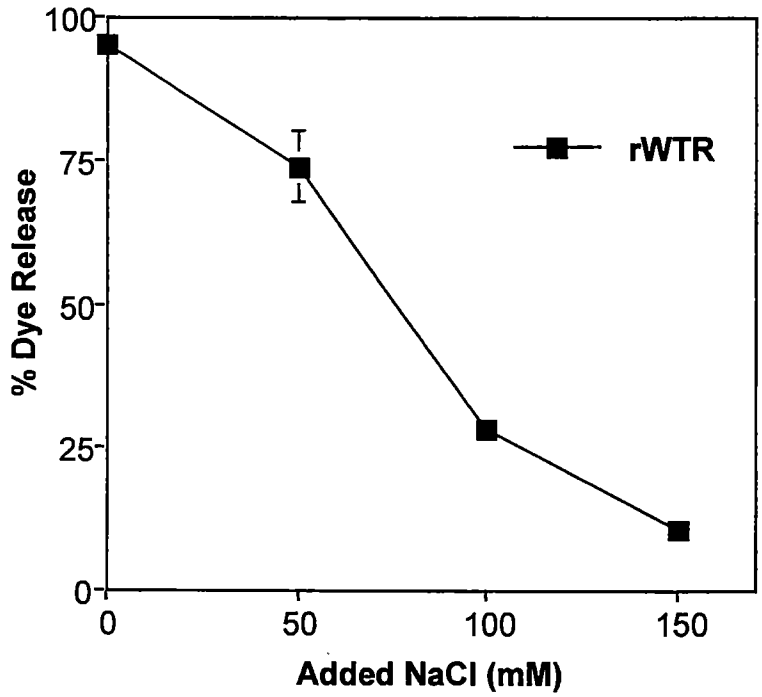
The effect of pH on the interaction between rWTR and PC/PA liposomes was also investigated. rWTR (0.2 μM) was pre-equilibrated in PBS at the stated pH values and then incubated with PC/PA liposomes. As shown in Fig. 20B, acidic pH conditions reduced the level of dye release caused by the presence of rWTR. In contrast, no effect of basic pH on dye release was observed.

Effect of secondary structure of the R domain on the interaction with liposomes

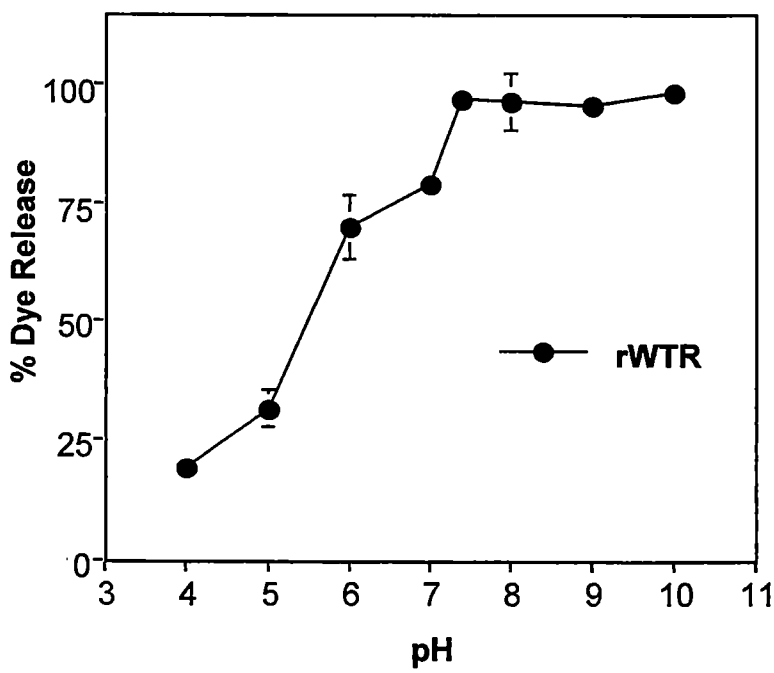
To determine if the secondary structure of the R domain contributes to its interaction with PC/PA liposomes, three R domain mutants, each with a single proline substitution, were analyzed by the dye release assay. rP1, rP2, and rP3 contain proline substitutions for Arg 3, Lys 7, or Ala 27, respectively (Fig. 15C).

Figure 20. Effects of NaCl concentration and pH on dye release by rWTR. (A) PC/PA liposomes were incubated with 0.2 μM of rWTR in the presence of 50-150 mM of supplementary NaCl. (B) PC/PA liposomes were incubated with 0.2 μM of rWTR at differing pH.

A


100
75
50
25
0
% Dye Release
0
50
100
150
Added NaCl (mM)
rWTR

B


100
75
50
25
0
% Dye Release
3
4
5
6
7
8
9
10
11
pH
rWTR

In addition, a fourth mutant (rP4) having alanines substituted for the proline residues at positions 18, 19, and 21 (Fig. 15C) was also analyzed. Interestingly, the proline substitutions within the N-terminal 16 residues (rP1 and rP2) had a negative effect on the interaction of the R domain with the liposomes (Fig. 21A). The proline substitution within the arginine-rich region (rP3) had no effect on dye release (Fig. 21A). Interestingly, the substitution of alanines for three prolines near the arginine-rich region measurably enhanced the rate of dye release as compared to that of rWTR (Fig. 21A).

Residues 6-14 of the R domain were predicted by the AGADIR program to fold into an α-helix (Munoz and Serrano, 1977). To determine if there is a correlation between α-helicity of the R domain and the ability to induce dye release, the α-helical content of rWTR, rP2, and rP4 was measured by CD in the presence of 20% TFE. The degree of α-helical conformation was greatest for rP4 and that of rP2 was slightly less than that of rWTR (Fig. 21B). By comparison, Fig. 21C shows the percentage of α-helix of rWTR, rP2, and rP4 measured by CD in the presence of 0-20% TFE. The α-helix content of rWTR, rP2, and rP4 in the absence of TFE was <19%, however, the α-helix content of the proteins were increased in the presence of TFE (Fig. 21C). At the highest concentration of TFE (20%), the α-helix content of rWTR, rP2, and rP4 was 29%, 25%, and 38%, respectively. The results in Fig. 21 suggest that the formation of an α-helix within the N-terminal 16 aa of the R domain is involved in the interaction between the R domain and PC/PA liposomes.

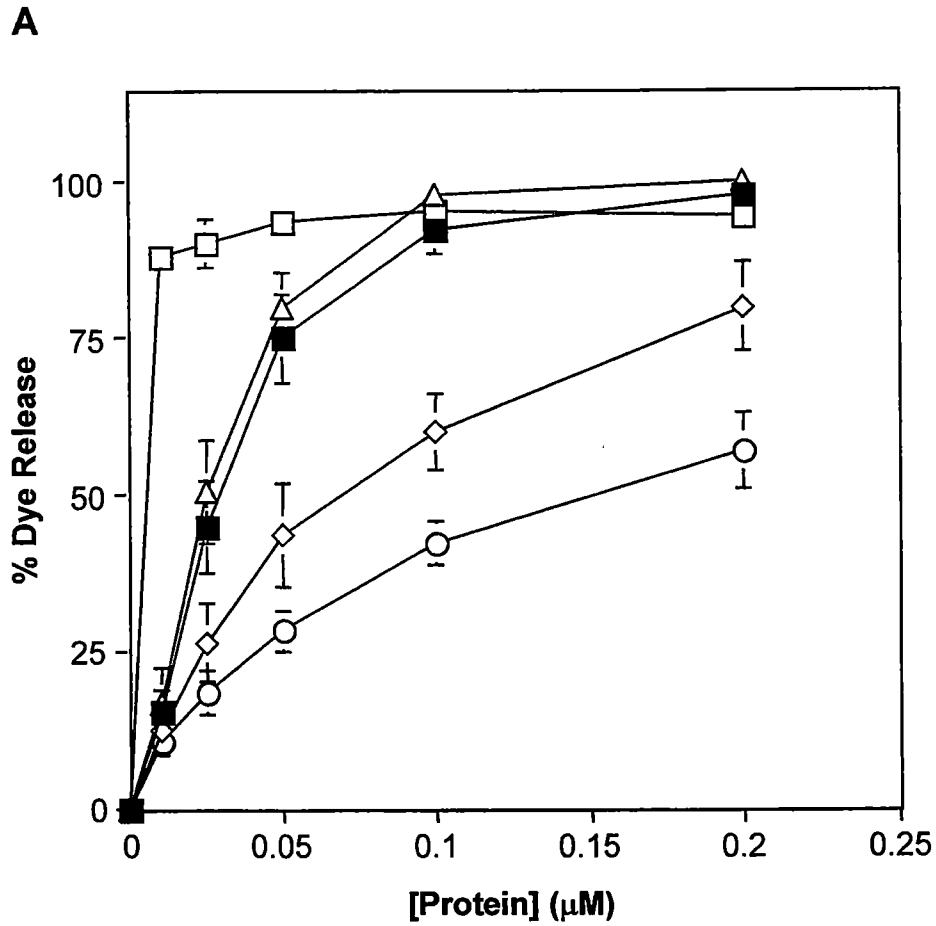
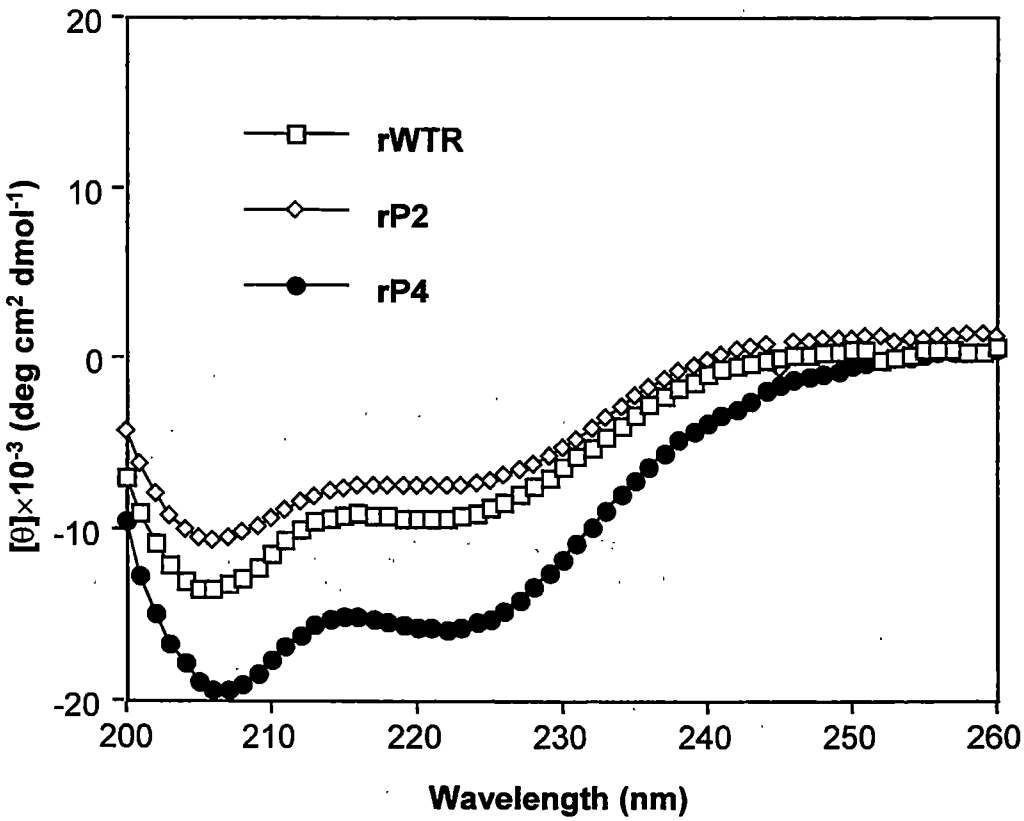


Figure 21. Effect of secondary structure of the R domain on liposome lysis. (A) PC/PA (8:2) liposomes were incubated with varying concentrations (0-0.2 μM) of rWTR (—■—), rP1 (—◇—), rP2 (—○—), rP3 (—△—), and rP4 (—□—). Percent calcein release was determined as described in Materials and Methods.

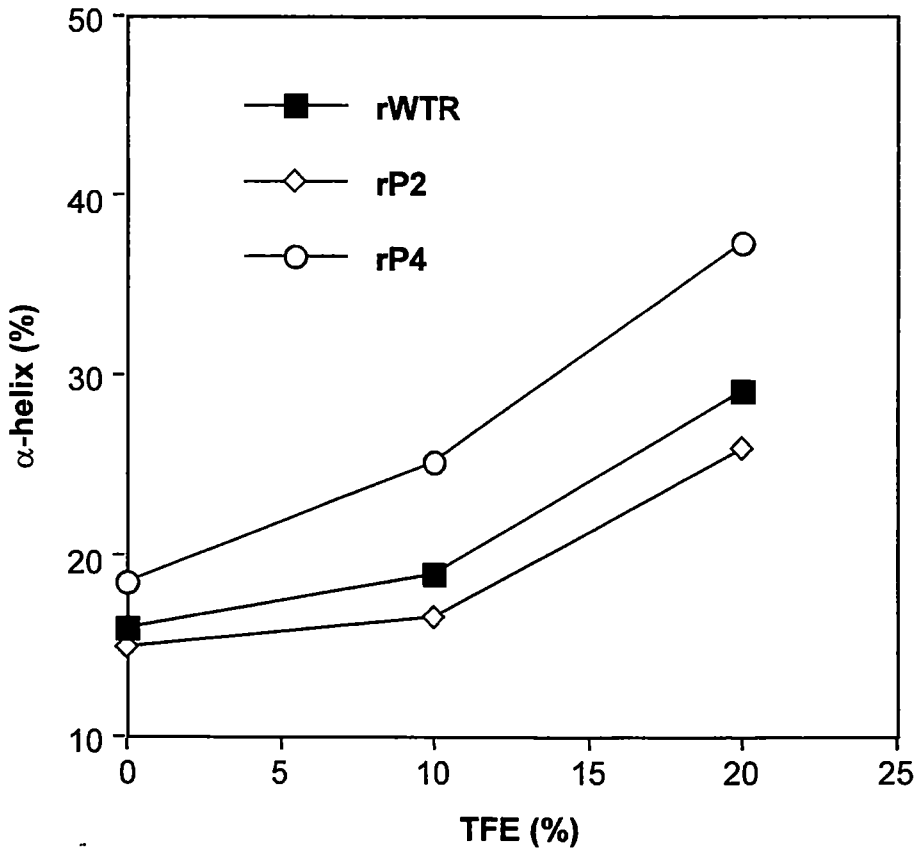
B



(B) CD spectra of rWTR, rP2, and rP4 in the presence of 20% TFE. The proteins (5 μM) were incubated at room temperature for 30 min in 20 mM potassium phosphate buffer containing 20% TFE prior to CD analysis.

Figure 21 (continued)

C



(C) α-helicity of rWTR, rP2, and rP4 in 0%, 10%, and 20% TFE. The proteins (5 μM) were incubated at room temperature for 30 min in 20 mM potassium phosphate buffer containing varying concentrations (0-20%) of TFE prior to CD analysis.

Figure 21 (continued)

Lipid composition dependence of the liposome-rWTR interaction

To determine if the dye release mediated by rWTR is dependent upon the lipid composition, liposomes with different lipid compositions were tested. PG, PE, and PS were included in the experiment. PE is the only one among these three that is a non-bilayer forming lipid. The incubation of rWTR with PC/PE (8:2) liposomes resulted in a release of 53% of the dye while 40% of the dye was released following incubation with PC/PE/PG (7:2:1) liposomes (Fig. 22). Little dye release was observed when rWTR was incubated with PC/PS (8:2), PC/PG (9:1), or PC/PE/PS (2:5:3) liposomes (Fig. 22). These results suggest that the rWTR-liposome interaction may be dependent upon PA or PE lipids.

The interaction between liposome and CP peptide$_{1-30}$

The experiments described above implicated the N-terminal 30 aa of the SCPMV CP function in its interaction with liposomes. To determine if these 30 residues are sufficient for the interaction, a peptide corresponding to aa 1-30 of the CP (CP peptide$_{1-30}$) was tested for its ability to interact with PC/PA liposomes. As shown in Fig. 23A, incubation of varying amounts of the peptide with liposomes resulted in release of nearly 100% of the dye with 0.1 μM peptide, results similar to those observed for rWTR (cf. Fig. 17B). To determine if the peptide has the same lipid requirements for binding as rWTR, CP peptide$_{1-30}$ was incubated with PC, PC/PA (8:2), PC/PE (8:2), or PC/PE/PG (7:2:1) liposomes. The results shown in Fig. 23B indicate that the peptide also interacts strongly with anionic (PA) or non-bilayer forming (PE) lipids.

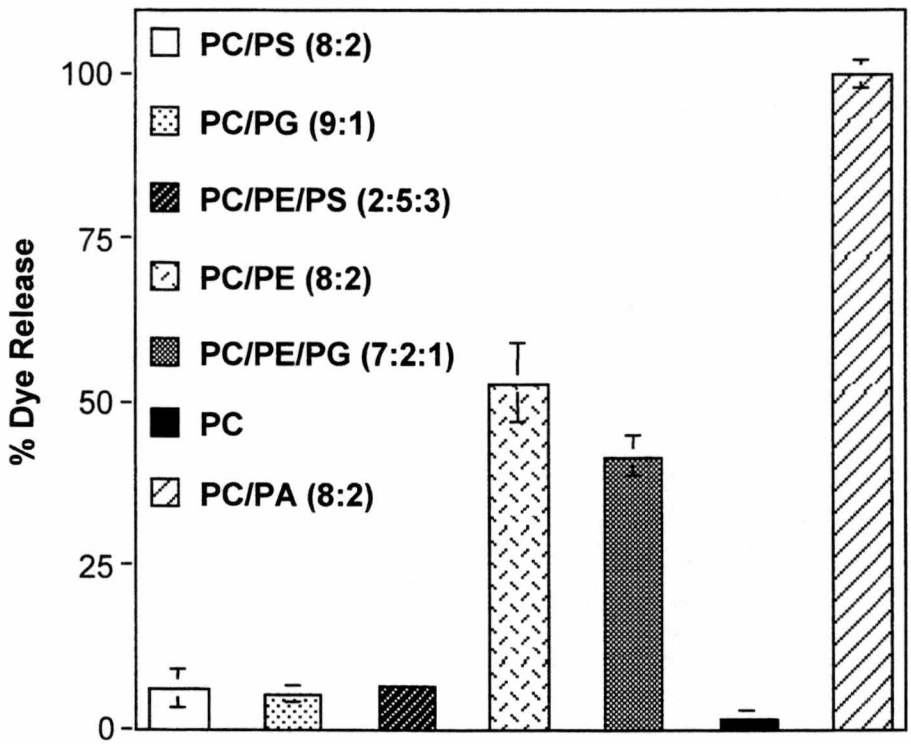
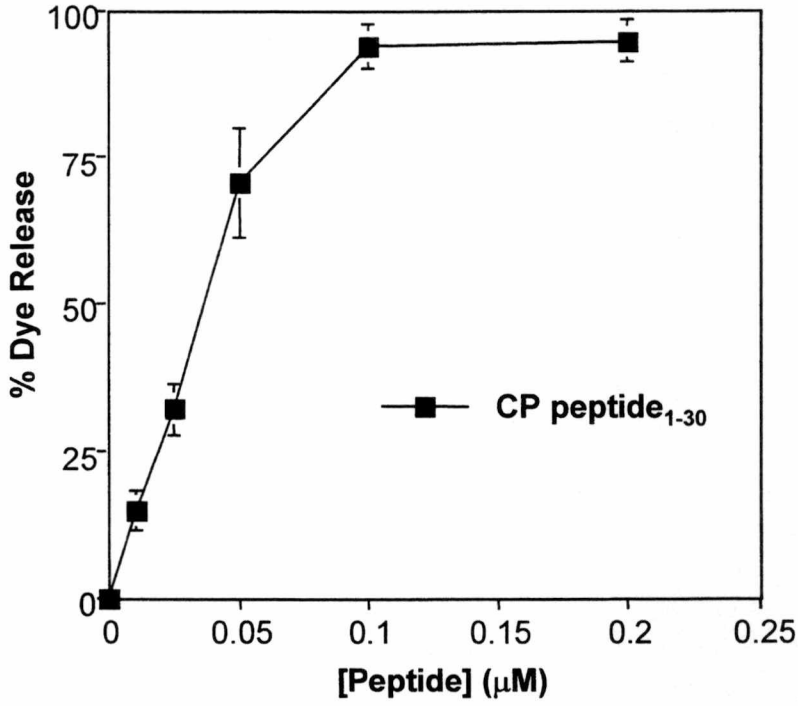


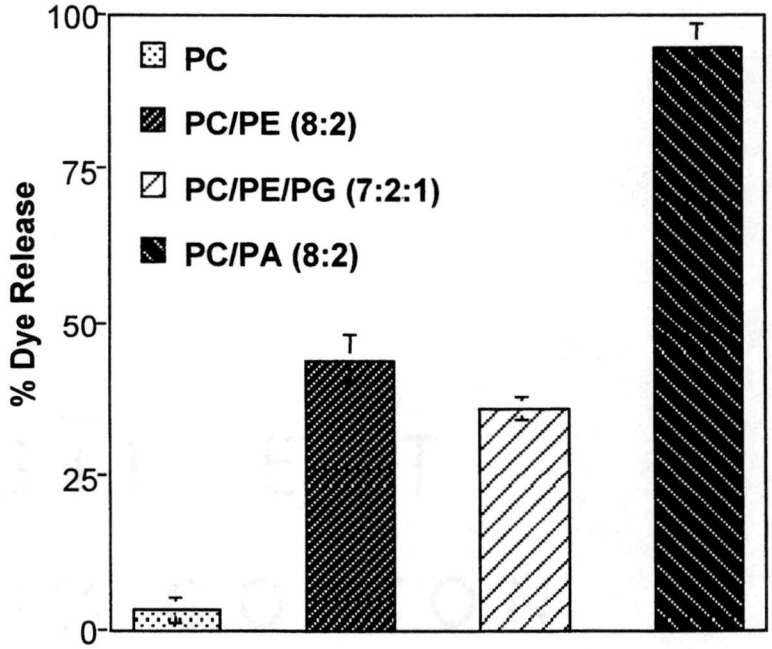
Figure 22. Lipid composition dependence of the rWTR-liposome interaction. Calcein release from liposomes composed of PC/PS (8:2), PC/PG (9:1), PC/PE/PS (2:5:3), PC/PE (8:2), or PC/PE/PG (7:2:1) was measured in the presence of 0.2 μM of rWTR. Calcein release from PC and PC/PA (8:2) is shown for comparison.

Figure 23. Liposome lysis by CP peptide$_{1-30}$. (A) PC/PA (8:2) liposomes were incubated at room temperature for 2 hr with varying concentrations (0-0.2 μM) of CP peptide$_{1-30}$. The percent calcein release was determined as described in Materials and Methods. (B) CP peptide$_{1-30}$ (0.2 μM) was incubated with PC, PC/PE (8:2), PC/PE/PG (7:2:1), or PC/PA (8:2) liposomes at room temperature for 2 hr and the percent calcein release was measured as described in Materials and Methods.

A


100
75
50
25
0
% Dye Release
CP peptide1-30
0
0.05
0.1
0.15
0.2
0.25
[Peptide] (μM)

B


100
75
50
25
0
% Dye Release
PC
PC/PE (8:2)
PC/PE/PG (7:2:1)
PC/PA (8:2)

The CD data reported in Fig. 21 suggest that the N-terminus of the SCPMV CP may fold into an α-helix under certain conditions. In chapter II, CP peptide$_{1-30}$ has been shown to be a random coil conformation in 20 mM potassium phosphate (pH 7.4). Therefore, it is possible that the secondary structure of this region of the CP changes from a random coil to an α-helix upon membrane binding. To determine if this is the case, CD spectra of the CP peptide$_{1-30}$ in the presence and absence of PC/PA liposomes was determined. As shown in Fig. 24, CP peptide$_{1-30}$ was shown to be a random coil in 20 mM potassium phosphate (pH 7.4). When the peptide was incubated in the presence of a saturating level of PC/PA liposomes, the α-helical content of the peptide was increased to about 25% (Fig. 24). This result demonstrated that the N-terminal 30 aa of the CP has a tendency to form an α-helix upon membrane binding.

DISCUSSION

In this study we investigated the interaction between the SCPMV CP and artificial membranes. Consistent with the previous results of Datema *et al.* (1987) we have demonstrated that the interaction between the SCPMV CP and liposomes is mediated by the N-terminal 30 residues of the CP. Residues 1-16 in particular were shown to play a critical role in the liposome interaction. This region of the R domain is predicted to fold into an α-helix, and it was demonstrated that the N-terminal 30 residues of the R domain undergo a

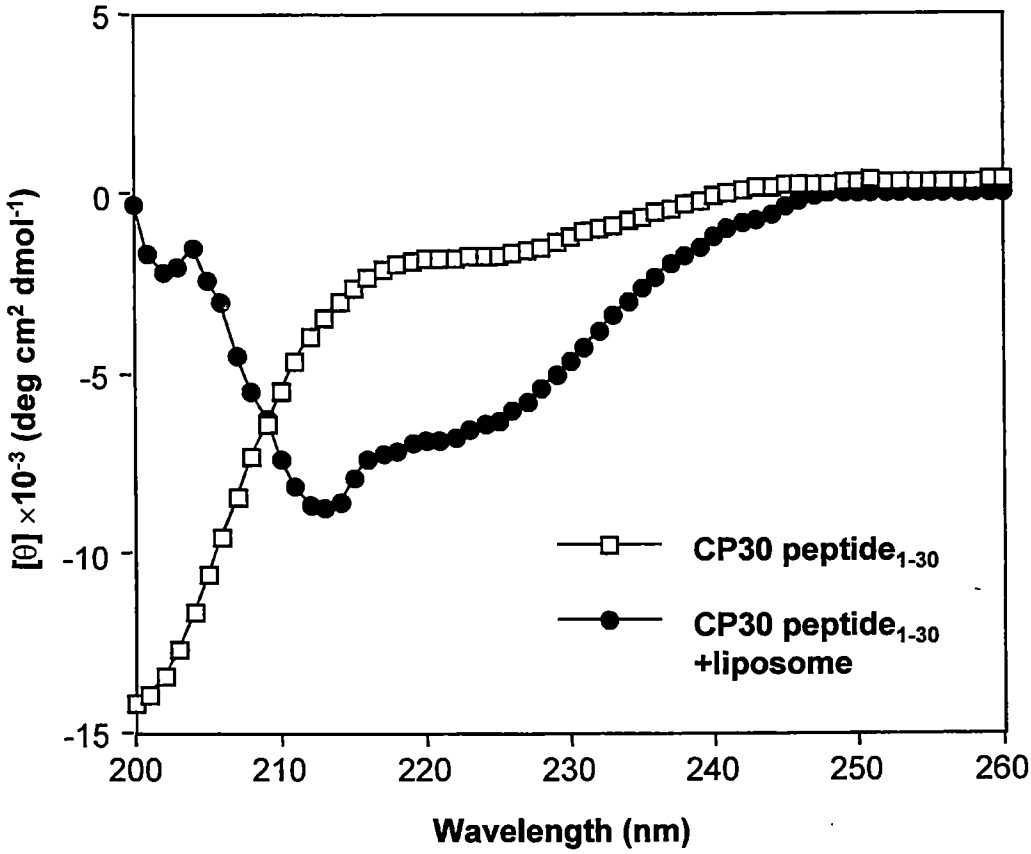


Figure 24. CD spectra of CP peptide$_{1-30}$ in the absence or presence of PC/PA (8:2) liposomes.

conformational change from random coil to α-helix upon interaction with the liposome. The interaction was also shown to be strongly dependent on the presence of PA or PE lipids, and it was shown to be sensitive to high salt concentration and acidic pH.

The involvement of the arginine-rich region in the membrane-R domain interaction, the sensitivity of the interaction to high salt, and the requirement for anionic lipids suggest that electrostatic interactions significantly contribute to the ability of the CP to bind liposomes. Thus, the sensitivity of the interaction in high salt or acidic pH may be due to a shielding effect by Na^+ or H^+. However, factors other than electrostatic interactions are likely to be involved because elimination of the arginine-rich region in rΔ17-30 or rA1 did not completely abolish the ability of the R domain to interact with liposomes. In addition, 28% dye release was observed in the presence of 100 mM supplemental NaCl.

It is clear from the results that the N-terminal 16 aa of the R domain are also a major contributor to the interaction with liposomes. This region contains only three basic residues, and they were shown to be largely dispensable for the liposome interaction. Residues 6-14 of the R domain were predicted by the AGADIR program to fold into an α-helix (Munoz and Serrano, 1977). It was shown that CP $peptide_{1-30}$ is able to adopt a significant percentage of α-helical structure in 50% TFE (Chapter II) and in the presence of liposomes. Furthermore, a substitution mutation which increased the α-helical content of the R domain increased the rate of dye release from liposomes, and substitution

mutations which decreased the α-helical content of the R domain decreased the level of dye release. These results strongly suggest that the ability of the 16 N-terminal residues to interact with membranes is due to its potential to form an α-helix. By analogy, it has been shown that the γ peptide of the FHV CP induces structural change from a random coil to an α-helix upon addition of liposomes (Bong *et al.*, 2000).

Based on the results we propose that the N-terminal 30 aa of the CP interact with membranes in a two step process. First, the basic residues in the arginine-rich region are expected to interact with negatively charged groups on the membrane electrostatically, causing structural change in the N-terminus of the R domain from a random coil to an α-helical conformation. This α-helical conformation may locate proper amino acids into a correct position so that they induce membrane disruption.

The interaction of the R domain with membranes was shown to be dependent on the presence of an anionic lipid (PA) or a non-bilayer forming lipid (PE). Non-bilayer lipids adopt the molecular shape of a cone or wedge and prefer to pack in an inverted phase with the polar head groups facing inward and the hydrophobic acyl chains projecting toward the exterior (Bruce, 1998). These lipids form a reversed hexagonal array known as H_{II}-phase organization (Bruce, 1998). Both PA and PE are found in the plant plasma membrane with PE being the most abundant. In etiolated seedlings of the legume *Vigna radiata* (mung bean) PA and PE constitute 16% and 38%, respectively, of the total phospholipid

in the plasma membrane (Yoshida and Uemura, 1986). Thus, these phospholipids may play an important role in the infection cycle of SCPMV in its host.

The biological relevance of the membrane-R domain interaction is not known, but there are several points in the life cycle of the virus in which a CP-membrane interaction may be involved.

1.) The interaction may be necessary to dock the virus particle to a cellular membrane where viral RNA synthesis can occur. Positive-sense RNA virus RNA replication usually occurs in association with a cellular membrane (Chen and Ahlquist, 2000; Schaad *et al.*, 1997). After SCPMV entry it is expected that the virus particle will expand under physiological conditions resulting in the externalization of the R domain. The exposed R domain may then allow the particle to dock to a membrane that is the site of viral RNA replication. Subsequent translation of the viral RNA would result in the production of the viral RNA polymerase near its site of function.

2.) The interaction may be necessary for SCPMV cell-to-cell movement. It has been demonstrated that local movement of SCPMV requires the CP (Sivakumaran *et al.*, 1998). This means that either the virus particle or an RNA-CP complex moves from cell to cell. The mechanism of SCPMV cell-to-cell movement is not yet known. One possibility is that the expanded SCPMV particle docks to the plasma membrane within or near the plasmodesmata and that this step is necessary to allow transport of SCPMV particles through the plasmadesmata.

3.) The interaction may be important for beetle transmission of SCPMV. It has been demonstrated that following ingestion of SCPMV by the vector the virus enters the hemocoel through the peritrophic membrane-lined midgut but not through the cuticle-lined foregut or hindgut. These observations suggest that a specific interaction between the virus and some component of the midgut occurs and that this interaction is necessary for the passage of ingested viruses from the midgut to the hemocoel (Wang *et al.*, 1994).

4.) The CP or the virus particle may form an ion channel in a cellular membrane to alter the ion flux into or out of the cytoplasm. It has been observed that early after infection with PV, the plasma membrane becomes permeable to ions and the cytoplasmic level of Na^+ increases while that of K^+ decreases (Fernández-Puentes and Carrasco, 1980). It is not known if the PV capsid is responsible for these changes in membrane permeability. The alteration of ionic conditions in the infected cell may be favorable for virus infection particularly for enhanced translation of the viral RNA (Carrasco, 1995). It has been demonstrated that SCPMV translation is insenstive to high salt *in vitro* (Sivakumaran and Hacker, unpublished data).

Further research on the SCPMV life cycle will be necessary in order to determine if one of these proposed functions for the CP-membrane interaction is correct.

CHAPTER IV

Future directions

UNDERSTANDING THE MOLECULAR MECHANISMS INVOLVED IN SCPMV ASSEMBLY

1. To determine the role of the interaction between the R domain and the viral RNA in SCPMV assembly *in vivo*. Site-specific mutagenesis of the full-length infectious SCPMV cDNA clone will be performed to generate R domain mutants. The ability of the mutant viruses to replicate and assemble in plants and plant protoplasts will be determined.
2. To determine the role of protein secondary structure in the RNA binding activity of the R domain. The conformation of CP peptide$_{1\text{-}30}$ in the presence and absence of RNA or oligophosphate will be determined by CD.

UNDERSTANDING THE MEMBRANE-INTERACTION ACTIVITY OF THE SCPMV CP

1. To determine if the R domain alone has ion channel activity. The ability of rWTR or CP peptide$_{1\text{-}30}$ to cause ion flux across a planar lipid bilayer will be determined in collaboration with Dr. Carl Helrich in the Department of Physics at Goshen College.

2. To determine if the SCPMV CP or the R domain alone interacts with membranes *in vivo*. Tagged CP or R domain will be expressed exogenously in plant cells, and the subcellular localization of the protein will be determined using immunohistochemical and biochemical approaches.

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APPENDIX

ACRONYMS FOR SELECTED VIRUSES

BMV	Brome mosaic virus
BSSV	Blueberry shoestring virus
CCMV	Cowpea chlorotic mottle virus
CfMV	Cocksfoot mottle virus
CMV	Cucumber mosaic virus
FHV	Flock house virus
HIV-1	Human immunodeficiency virus 1
LTSV	Lucerne transient streak virus
PV	Poliovirus
RYMV	Rice yellow mottle virus
SBMV	Southern bean mosaic virus
SCMoV	Subterranean clover mottle virus
SCPMV	Southern cowpea mosaic virus
SNMoV	Solanum nodiflorim mottle virus
SoMV	Sowbane mosaic virus
STNV	Satellite tobacco necrosis virus
TBSV	Tomato bushy stunt virus
TCV	Turnip crinkle virus
TRoV	Turnip rosette virus
VTMoV	Velvet tobacco mottle virus

VITA

Sook-Kyung Lee was born in Kimjae, Korea on August 3, 1966. She received her Bachelor of Science degree in Microbiology in August 1992 from Chungnam National University, Taejon, Korea. After working at a hospital for two years as a technician, she entered the Master's program in Microbiology at the University of Tennessee, Knoxville in August of 1994. She is presently working on her Ph. D. in Microbiology at the University of Tennessee, Knoxville, and will receive her Ph. D. degree in August of 2001.