C-terminal repetitive motifs in Vp130 present at the unique vertex of the *Chlorovirus* capsid are essential for binding to the host *Chlorella* cell wall.

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Abstract

Previously, Vp130, a chloroviral structural protein was found to have host cell wall-binding activity for NC64A-viruses (PBCV-1 and CVK2). In this study, we have isolated and characterized the corresponding protein from chlorovirus CVGW1, one of Pbi-viruses that have a different host range. In NC64A-viruses, Vp130 consists of a highly conserved N-terminal domain, internal repeats of 70-73 aa motifs and a C-terminal domain occupied by 23-26 tandem repeats of a PAPK motif. However, CVGW1 was found to have a slightly different Vp130 construction where the PAPK repeats were not in the C-terminus but internal. Immunofluorescence microscopy with a specific antibody revealed that the C-terminal region containing the Vp130 repetitive motifs from PBCV-1 and CVK2 was responsible for binding to *Chlorella* cell walls. Furthermore, by immunoelectron microscopy and immunofluorescence microscopy, Vp130 was localized at a unique vertex of the chlorovirus particle and was found to be masked through binding to the host cells. These results suggested that Vp130 is localized at a unique vertex on the virion, with the C-terminal repetitive units outside for cell wall binding.

Introduction

Chlorovirus or *Chlorella* viruses are large icosahedral, double-stranded DNA- containing viruses that infect certain strains of the unicellular green alga *Chlorella* (Van Etten et al., 1991; Van Etten, 2003; Yamada et al. 2006). These viruses belong to the family *Phycodnaviridae* and are ubiquitous in the natural environment (Van Etten et al., 1991; Yamada et al., 1991). The virions of chloroviruses are ~190 nm in diameter and have a lipid bilayer membrane inside the outer glycoprotein capsid. In a typical lytic cycle, a vertex of an icosahedral virus particle attaches to the surface of the host and then rapidly degrades the cell wall at the point of attachment. Then, the viral core is released into the host cytoplasm through this digestion point, leaving an empty capsid on the cell wall (Van Etten et al., 1991).

Attachment of the virion to the host cell wall probably alters the virion structure to some degree, allowing exposure of cell wall digesting enzymes stored inside the capsid. Following host cell wall degradation, the internal viral membrane probably fuses with the host membrane (Mehmel et al., 2003). Several proteins having polysaccharide-degrading activity, which may be involved in cell wall digestion, have been found in viral particles (Yamada et al., 1997; Hiramatsu et al., 1999; Sun et al., 1999). Digestion of the cell wall is rapidly followed by depolarization of the host membrane which is probably caused by a virus-encoded K^+ channel (called Kcv) located in the internal membrane (Mehmel et al., 2003; Frohns et al., 2006). However, the arrangement of these proteins involved in the initial events on the virion as well as the molecular mechanisms of interaction between those proteins and the host cell components are still unclear.

The chloroviruses infecting *Chlorella* strain NC64A (NC64A-viruses) or *Chlorella* strain Pbi (Pbi-viruses) have very strict host specificity: NC64A-viruses do not infect strain Pbi and NC64A does not serve as a host for Pbi-viruses (Van Etten et al., 1991). Such a strict host selection may be mainly based on a specific interaction between host receptors and viral receptor-binding proteins. In a previous work, Onimatsu et al. (2004) detected Vp130 from CVK2, a chlorovirus isolated in Kyoto, Japan, as a structural protein having cell wall-binding activity. The gene for Vp130 was identified in the genome of PBCV-1, the prototype of *Chlorovirus*, corresponding to an ORF (open reading frame) combining A140R and A145R. This protein was shown to be well conserved among various NC64A-viruses. A recombinant Vp130 protein formed in *Escherichia coli* specifically bound to the host cells and competed with CVK2 to bind to host cells. Furthermore, anti-Vp130 antibody raised in mice almost completely inhibited CVK2 infection. These results indicate that Vp130 is most likely a host-recognizing protein on the virion.

In the present study, we identified and characterized the Vp130 gene of CVGW1, one of the Pbi-viruses isolated in Galway, Ireland. With various recombinant portions of CVK2/CVGW1-Vp130 expressed in *E. coli* and antibodies against Vp130, we have

characterized Vp130 in respect to its binding activity and location on the virions.

Results

Isolation of the CVGW1-Vp130 gene

In our previous work (Onimatsu et al. 2004), anti-Vp130 antibody raised in mice detected an approximately 130 kDa band in two Pbi-viruses (CVGW1 and CVSN1) as well as in various NC64A-viruses. To isolate the gene for the Vp130-like protein from CVGW1, polymerase chain reaction (PCR) was performed with a set of primers derived from the sequence of the CVK2-Vp130 gene. An amplified fragment, approximately 2.9 kb, was cloned and sequenced. The nucleotide sequence showed that this fragment contained a large open reading frame having 48% amino acid identity with CVK2-Vp130. However, this ORF was smaller by 147 aa than that of CVK2 and no stop codon was seen in the 3' end, so this 2.9 kb fragment was considered to be the 3'-truncated portion of the CVGW1-Vp130 gene. When *Hin*dIII fragments of CVGW1 genomic DNA was hybridized to this 2.9 kb fragment as a probe, a 7.7 kb hybridizing band was detected (data not shown). Using inverse-PCR with the 7.7 kb band as template and a set of primers correspond to the ends of the 2.9 kb fragment, a 4.8 kb outer region was amplified. After sequencing this fragment, we identified the complete nucleotide sequence of the gene for CVGW1-Vp130 (DDBJ accession No. AB219450). The deduced 1073 amino acid sequence of CVGW1-Vp130 is compared with CVK2-Vp130 in Fig. 1. In CVK2, Vp130 consists of an N-terminal domain, internal repeats of 70~73 aa motifs alternating with 1-2 copies of PAPK and a C-terminal domain occupied by 23 tandem repeats of the PAPK motif. Although the N-terminal regions of both proteins were similar to each other (61% identical), CVGW1 had some differences in the C-terminal region: the characteristic repetitive units consisted of seven 60~74-aa sequence motifs, the first and second elements of which were connected not by PAPK but by a SIAKR or SIKAR sequence. Between the sixth and seventh repetitive units, there were 15 tandem repetitions of the PAPK

unit, which were at the C-terminus in CVK2 or PBCV-1 (Fig. 1). The amplification of the truncated fragment (2.9 kb) using PCR was caused by this shift of the long PAPK repetition sequence in CVGW1.

Recently, the genome sequence of Chlorovirus MT325, a Pbi-virus, was determined and made available on the internet (http://greengene.uml.edu.). ORF 188 showed 98% amino acid identity with CVGW1-Vp130, suggesting that this ORF corresponds to the Vp130 equivalent in MT325. All structural features seen in CVGW1-Vp130 were also well conserved in MT325-Vp130 (Fig. 1).

Expression of three recombinant portions of Vp130 protein in E. coli BL21

For further functional characterization of Vp130 and confirmation of cell wall-binding activity of CVGW1-Vp130, three different portions of CVK2-Vp130 and CVGW1-Vp130 were generated in *E. coli* as recombinant proteins. Glutathione- S-transferase (GST) -fused constructs were prepared as described in the Materials and Methods. As shown in Fig. 2A, the construct K2-NC, prepared previously (Onimatsu et al., 2004) contains the N-terminal 919-aa fragment of CVK2-Vp130 and is missing the 208-aa C-terminal portion. The construct K2-N contained the N-terminal 500-aa portion of CVK2-Vp130 and lacked the entire C-terminal PAPK repetitive domain. The construct K2-C had the C-terminal 400-aa portion of K2-NC consisting of five repetitive units of the large repeat. Of the CVGW1-Vp130 derivatives, the construct GW1-NC contained CVGW1-Vp130 without the C-terminal 140-aa portion. The construct GW1-N contained the N-terminal 500-aa portion of CVGW1-Vp130, and the construct GW1-C contained the C-terminal 420-aa portion of the construct GW1-NC, consisting of six repetitive units. All of these constructs were successfully expressed in *E. coli* BL21, and the recombinant proteins were recovered by affinity chromatography on a glutathione Sepharose 4B column (Fig. 2B). As previously described (Onimatsu et al., 2004), the entire Vp130 protein did not accumulate in *E. coli* cells, probably because of inhibitory effects of the long tandem repeats of PAPK.

Adsorption of recombinant constructs to the host Chlorella cells

We examined direct binding of the Vp130 derivatives to the surface of *Chlorella* cells. Cells were treated with the recombinant proteins and their adsorption was detected by immunofluorescence microscopy after treatment with anti-Vp130 antibody and FITC-conjugated anti-mouse IgG antibody as previously described (Onimatsu et al. 2004). The recombinant Vp130 proteins formed in *E. coli*, especially the constructs containing the repetitive units, were very aggregative and aggregation was enhanced by removal of the GST portion at the N-terminus. Therefore, all of these constructs were used in the GST-fused form in this study. When the cells were mixed with recombinant constructs containing the C-terminal repetitive domain (K2-C, K2-NC, GW1-C, and GW1-NC), strong FITC signals were detected on the cell surface (Fig. 3). Whereas, such signals were not observed when mixing with proteins having the N-terminal domain but not the C-terminal region (K2-N and GW1-N). Interestingly, CVK2-constructs and CVGW1-constructs bound equally to cells of both strains NC64A and SAG241-80. In the previous work, recombinant CVK2-Vp130 specifically bound to NC64A cells and not to SAG241-80 cells (Onimatsu et al., 2004). This discrepancy may be due to conformational changes of recombinant Vp130 protein expressed in *E. coli* cells. In fact, removal of the GST portion reproduced the selective recognition of both K2-NC as well as GW1-NC; namely, K2-NC bound to cells of *Chlorella* strain NC64A but not to those of strain SAG241-80 as reported previously and GW-NC bound to cells of strain SAG241-80 but not to those of strain NC64A as shown in Fig. 3. However, because of aggregation of GST-removed forms of K2-C and GW1-C, they could not be tested for the host specificity. To confirm the cell wall binding activity of the C-terminal region of Vp130, its competitive effects on CVK2 adsorption was tested by the method described previously (Onimatsu et al., 2004). When cells of *Chlorella* strain NC64A (2×10^6 cells) were treated with various amounts of K2-C and then added with CVK2, virus adsorption was inhibited to a certain extent, as shown in Table 1. At 160 μg/2×10^6 cells, K2-C inhibited the adsorption

by approximately 52%. The value was comparable with the previous data (Onimatsu et al., 2004). These results suggested that the C-terminal repetitive region of Vp130 was important in binding to the host cells.

Adsorption of C-terminal constructs to the various Chlorella strains

To characterize binding activity of the C-terminal constructs, eight other *Chlorella* strains including non-host strains were subjected to the adsorption assay as above. The results are summarized in Table 2. In addition to virus-host strains (NC64A, 211-6, and SAG241-80), four strains that belong to *C. vulgaris* (C-135, 211-1e, 211-11b, and C-27) showed binding to the K2-C and GW1-C constructs. On the other hand, these constructs did not show any adsorption to cells of *C. saccharophila* C-211, 211-9a and C-169. No FITC signals were observed as expected with the N-terminal constructs for either *Chlorella* strains (data not shown). These results raised the question whether the C-terminal region of Vp130 can distinguish some specific strains from a wide range of *Chlorella* species. In our previous work, we observed that strains belonging to *C. vulgaris* were sensitive to cell wall-digesting enzymes encoded by chloroviruses in spite of their virus resistance (Chuchird et al., 2002). Those strains and several other strains were tested for affinity to Vp130. As shown in Table 2, all strains of *C. vulgaris* tested in this experiment bound to K2-C and GW1-C, whereas other strains (some are not listed in the table) did not. As described previously (Chuchird et al., 2002), the strains to which Vp130 bound always belonged to the group having a "rigid wall" (Takeda 1991) which contained glucosamine (Meints et al., 1988; Kapaun et al., 1992; Takeda 1995). The C-terminal region may have binding activity to cell wall structures containing glucosamine.

The role of the N-terminal domain

We have shown that the C-terminal repetitive units of Vp130 are essential for its binding activity above. Then, we characterized the N-terminal domain with respect to its role in viral

infection and positioning on the virus particle. Using affinity columns immobilized with K2-N or K2-C constructs, specific antibodies against each of these constructs were purified from rabbit's anti-Vp130 antiserum and used to see their inhibitory effect in virus infection as described previously (Onimatsu et al. 2004). CVK2 particles were treated with various amounts of these antibodies and the host cell infectivity was evaluated by the number of plaques that appeared on the assay plates with strain NC64A as a host. As shown in Table 3, CVK2 infection was inhibited by the treatment with either antibody in a dose-dependent manner, with anti-K2-N antibody being more effective (Table 3). When CVK2 particles were first treated with each of the antibodies and then with FITC-conjugated secondary antibody, strong fluorescence was observed using fluorescence microscopy with anti-K2-C antibody, while less fluorescence was seen with anti-K2-N antibody (data not shown). These results suggested that the C-terminal repetitive units of Vp130 were exposed to the outer surface of the virion, which makes it easier to catch the receptor, and that the N-terminal region may have an important role (structurally or/and functionally) in the subsequent events triggered by receptor binding.

Localization of Vp130 on the virus particle

Vp130 on the particle of CVK2 was detected by immunoelectron microscopy using the anti-K2-C antibody and a 10-nm gold-coupled anti-rabbit IgG antibody. We observed more than 2,000 virus particles, 10% of which were labeled with gold-coupled antibody. As shown in Fig. 4A, gold particles were observed to be attached exclusively to one of 12 vertices of the icosahedron. Virus particles with gold labels at multiple sites were never observed. To confirm this, Vp130 protein was visualized by immunofluorescence microscopy after attachment of CVK2 particles to the host cells. The cells of strain NC64A attached to CVK2 particles were fixed with 4% paraformaldehyde, followed by treatment with anti-K2-C rabbit antibody and FITC-conjugated anti-rabbit IgG antibody. As a positive

control, a sample of the virus-adsorbed cells was treated with anti-CVK2 antiserum raised in mouse against to CVK2 particles and FITC-conjugated anti-mouse IgG antibody. The strength of the FITC signals almost corresponded with the signals from DAPI which stained the virus DNA in the positive control. However, no FITC signals were obtained with anti-K2-C antibody (Fig. 4B). As shown in Fig. 4C, free CVK2 particles treated with anti-K2-C antibody showed strong FITC signals. Fixation with 4% paraformaldehyde did not affect the labeling efficiency. These results indicated that Vp130 was located at a unique vertex on the virus icosahedral particle and after attachment to the host cell wall at the vertex, Vp130 was sterically masked. The interaction between Vp130 and the host receptor might cause a drastic structural alteration in Vp130, so that antibody could not bind to Vp130. We concluded that Vp130 is located at one of the vertices and chloroviruses possibly attach to the hosts by the unique vertex where Vp130 is located.

Discussion

Interaction between Vp130 and receptor

In this study, comparison of the Vp130 genes of two kinds of chloroviruses (CVK2 and CVGW1) provided us with an interesting structural feature of this protein. Each Vp130 protein consisted of a highly conserved N-terminal domain and the remaining variable region containing the repetitive units connected by a PAPK sequence and the long tandem PAPK repetition. Repetitive units varied characteristically depending on the type of viruses and the long PAPK repetition was located at the C-terminus of the NC64A-viruses but in an internal region for Pbi-viruses (Fig. 1). Using recombinant portions of Vp130 protein, the repetitive region was shown to be involved in the binding activity to host receptors (Fig. 3). The binding was not essentially affected by the removal of the long PAPKs or removal of a few of repetitive units. Probably, each unit can bind to receptor molecules scattered over the whole

surface of the host cell wall and seven repetitions may make the interaction stronger. Though the highly conserved N-terminal domain was not necessary to the adsorption of Vp130 to the host cells, anti-K2-N antibody caused drastic inhibition in CVK2 infection (Table 3). In the usual infection process, the adsorption of virus particles is immediately followed by degradation of the cell wall at the attachment point. Several proteins with polysaccharide-degrading activity, possibly responsible for the cell wall digestion, have been found in viral particles (Hiramatsu et al., 1999; Sun et al., 1999; Yamada et al., 1997). Therefore, the N-terminal region of Vp130 may be anchored to the particle to interact with these enzymes and/or participate in drastic structural alteration after binding of C-terminus to the virus receptor.

In this study, it is unclear whether Vp130 is the sole agent that interacts with the host receptor and is responsible for the host specificity. Meints et al. (1988) predicted that the receptor molecules of chloroviruses is carbohydrate that is unaffected by treatment with cellulase and pectinase but is destroyed by an enzyme preparation from chlorovirus lysates. In our previous work, Chuchird et al. (2001) revealed that treatment of host cells with CVK2-encoded polysaccharide-degrading enzymes reduced attachment of CVK2 particles to the host cells and that various *Chlorella* strains including virus host and non-host strains showed sensitivity to the enzymes. The enzyme-sensitive strains also showed binding affinity to the Vp130 C-terminal recombinant constructs in this study (Table 2). According to a classification based on components of the cell wall, those strains share glucosamine as the rigid-wall component (Kapaun et al 1992; Meints et al. 1988; Takeda 1995). In actual virus infection, some minor structural differences in the cell wall of these strains may be recognized by viral proteins, likely Vp130. For selective adsorption, Vp130 itself may need to be full length and/or to be folded intact for assembly into the virion. The molecular basis for specific interaction of chloroviruses and the host cells will be obtained by structural characterization of intact Vp130 isolated from virus particles and by identification of the virus receptor molecules from the host cell wall.

Position of Vp130 on the virion

According to the ultrastructural study by cryo-electron microscopy and three-dimensional image reconstruction of PBCV-1 particles (Yan et al., 2000), twelve pentamer capsomers exist at the five-fold vertices. Each pentamer has a cone-shaped axial channel at its base. One or more proteins appear below the axial channel and outside the inner membrane, which may be responsible for digesting the host cell wall during infection. This structure was complemented and extended by fitting the structure of the major capsid protein Vp54 (Simpson et al., 2003). Recently, by atomic force microscopy investigation, Kuznetov et al. (2005) reported that each pentagonal vertex (approximately 10 nm in diameter) consists of a single apical globular protein and five other unique globular proteins surrounding the apical protein. At least some of these vertex-proteins may be involved in the viral attachment to the host cell wall. Although these structural studies did not reveal a unique vertex on the chlorovirus icosahedral particles, immunoelectron microscopy and immunofluorescence microscopy localized Vp130 to a unique vertex on the virus particles in this study (Fig. 4). We believe that Vp130 should be one of the two proteins at a unique vertex and that the other 11 five-fold vertices may consist of other protein instead of Vp130.

The presence of a unique vertex was previously suggested by a few works: Becker et al. (1993) reported a unique vertex (empty vertex) on particles of CVG-1, a member of Pbi-viruses. Using electron microscopy of ultrathin sections and negatively stained CVG-1 particles, they observed a distinct space between the outer and inner layers of the particles at a unique vertex. Another example of a unique vertex was suggested by an electron micrograph showing a distinctive 20- to 25-nm spike structure extended from one vertex of the PBCV-1 particle (Van Etten et al., 1991). The particle appeared to be packed asymmetrically and the interior membrane appeared to be located inside the capsid which extended to the unique vertex.

In the course of chlorovirus replication, complete capsids are assembled prior to DNA accumulation, so that genomic DNA should be incorporated through an opening on the capsid

(Van Etten et al., 1991). By electron microscopy of CVK2-infected *Chlorella* cells, we observed possible DNA packaging-intermediates where DNA was invaginated into the virus particles through one vertex of the capsid (data not shown). This observation suggests the presence of a unique vertex with specific functions which may play important roles in DNA packaging as well as ejection of DNA into the host cytoplasm in infection. In fact, such a unique vertex has been reported for the bacteriophage PRD1 (Gowen et al., 2003; Stromsten ett al., 2003; Abrescia et al., 2004). The unique vertex extends to the virus internal membrane via two integral membrane proteins which are necessary for the binding to the putative packaging ATPase.

Recent studies suggested that the structural homology shared between the viral capsid proteins and structures of chlorovirus (Yan et al., 2000; Simpson et al., 2003), adenovirus, archaeral virus STIV (Khayat et al., 2005; Rice et al., 2004) and bacteriophage PRD1 (Abresia et al., 2004) are evolutionally related and have evolved from a common ancestor. Therefore, fundamental structural aspects of the virus including the genome packaging and ejection machineries might have been inherited from a viral ancestor.

Materials and methods

Cells and viruses

Cells of *Chlorella* strain NC64A (Muscatine et al., 1967) were cultured in modified Bold's basal medium (MBBM) as described previously (Van Etten et al., 1983). *C. vulgaris* C-27, C-135, and C-150, *C. saccharophila* C-211 and C-169 were obtained from the algal culture collection of the Institute of Molecular and Cellular Biosciences, The University Tokyo. *C. vulgaris* 211-1e and 211-11b, *C. saccharophila* 211-9a, *Chlorella* sp. 211-6, and *Chlorella* sp. SAG241-80 were from the Algal Culture Collection, Plant Physiology Institute, University of Goettingen. All of these strains were cultured in MBBM at 25 ℃ in light except for strain SAG241-80. Strain SAG 241-80 was cultured in Jaworski's medium

supplemented with 0.2% Lab-lemco powder (Oxoid, Hampshire, UK). Chlorovirus CVK2 was isolated from natural water in Japan using a plaque-forming assay with *Chlorella* strain NC64A as the host (Yamada et al., 1991). CVGW1 was isolated from natural water in Galway, Ireland with *Chlorella* sp. SAG 241-80 as the host. For electron microscopy analyses, CVK2 particles were purified on a 10-40% zonal sucrose gradient. After centrifugation (20000×g, 30 min, 4 ℃), the virus zone was collected.

Preparation and Southern hybridization analysis of viral DNA

DNA was isolated from purified chloroviral particles by phenol extraction as described previously (Yamada et al, 1991). DNA restriction fragments separated by agarose gel electrophoresis were transferred to nylon filters (Biodyne, Pall BioSupport), hybridized with probes labeled with fluorescein (Gene Images Kit, Amersham Bioscience), and detected with a CDP-Star detection module (Amersham Bioscience).

DNA cloning and sequencing

To isolate the gene for CVGW1-Vp130, initially a 2.9 kb fragment was amplified by PCR with CVGW1 genomic DNA as the template and a set of oligonucleotide primers which corresponded to the sequence of CVK2-Vp130 as follows: 5'-ATAAAGAGCT-CATGTCATCTGAAGAAACTGC-3' corresponding to the 5' end of the CVK2 Vp130 gene (forward primer) and 5'-ACGAAGCTTTCATTTATTTTTTAGGTGCGG-3' corresponding to the 3' end of the CVK2-Vp130 (reverse primer). As a template for inverse-PCR, a 7.7 kb *Hin*dIII band of CVGW1 genomic DNA which hybridized with the 2.9 kb PCR product was recovered from the gel and self-ligated with T4 DNA-ligase. Inverse-PCR was performed with the circularized 7.7 kb band and a set of primers as follows: 5'-GGCTAAGCACGGA-ATTCAACTGAATGAAGT-3' corresponding to the 3' region of the 2.9 kb fragment (forward primer) and 5'-AGCTCAGCGAATTCGAAAGGGGTATACTCA-3' corresponding to the 5'

region of the 2.9 kb fragment (reverse primer). The amplified 4.8 kb fragment was ligated into a pGEM-T Easy vector (Promega) and cloned into *E. coli* JM109. DNA sequencing by the dideoxy method was performed using an Automated Laser Fluorescence (ALF) DNA sequencer (Amersham Bioscience). The nucleotide sequence determined for the 3222-bp CVGW1-Vp130 gene has been deposited with the DDBJ database under accession No. AB219450.

Expression and purification of the recombinant proteins

To isolate and characterize Vp130 and its derivatives, GST-fusion constructs were generated in *E. coli* using the pGEX4T-3 plasmid vector (Amersham Bioscience) as follows. In our previous work, the entire coding region except for the 3'-region containing the PAPK repeats was fused to GST in the recombinant plasmid pVp130N (Onimatsu et al., 2004) and its product has been designated as K2-NC in this work. Two other CVK2-Vp130 derivatives were obtained as follows: to make the construct K2-N, the N-terminal 1.5 kb DNA fragment was amplified from CVK2 genomic DNA using PCR with the synthetic primers 5'-T*CCCGGG*GAATGTCATCTGAAGAAACTGCT-3' (forward primer) and 5'-GGTTTCA-*GCGGCCGC*TTACCAGCACCTCTC-3' (reverse primer), and for the construct CVK2-C, the C-terminal 1.2 kb fragment was similarly amplified with the primers 5'-AAGAGA-G*CCCGGG*GTAAACCCACGGTGAAA-3' (forward primer) and 5'-CCAATT*GCGG-CCGC*CAGACGGTCTGTTACC-3'; the forward and reverse primers were designed with *Sma*I and *Not*I sites, respectively indicated in italics. The resultant PCR products were digested with *Sma*I and *Not*I and ligated into the corresponding sites of pGEX4T-3. For CVGW1-Vp130, the recombinant plasmids GW1-NC and GW1-C were constructed in similar ways: a 2.7 kb fragment for GW1-NC, lacking the 347 bp 3'-region of CVGW1-Vp130 and a 1.3 kb fragment for GW1-C containing six units of repetitive elements were amplified. For the amplification, the primer sets 5'-T*CCCGGG*GAATGTCATCTGAAGAAACTGCT-3' (forward primer) and 5'-GTG*GCGGCCGC*GGAGCTGTTTTCTTAGAGA-3' (reverse

primer), 5'-GGC*CCCGGG*CATCTACTAAGACACGGGTTG-3' (forward primer) and the same reverse primer as used for GW1-NC were used for GW1-NC and GW1-C, respectively. The fragments obtained were ligated in the same way into pGEX4T-3 at the *Sma*I and *Not*I sites. The GW1-N construct was obtained by removing a 1.3 kb C-terminal region from the CVGW1-NC construct: after digestion of the recombinant plasmid for CVGW1-NC with *Cla*I and *Not*I, the resulting plasmid was ligated with T4 DNA ligase after being filled-in with T4 DNA polymerase. *E. coli* BL21 transformed with each of the constructs was cultured for 2-3 h at 37 ℃. Protein expression was induced by the addition of 1 mM IPTG for 2 h. The bacterial cells were lysed by sonication in PBS (phosphate buffered saline), pH 7.3 containing 1% Triton X-100. The hybrid protein was purified by affinity chromatography on a glutathione-Sepharose 4B column (Pharmacia Biotech, USA).

Production and purification of anti-Vp130 antibodies

Anti-Vp130 antibody was raised in mice as described previously (Onimatsu et al., 2004). For the anti-Vp130 rabbit antibody, the purified K2-NC protein (200 μg) treated with thrombin to remove the GST portion was emulsified in Imject Alum adjuvant (PIERCE Biotechnology) and injected subcutaneously into female rabbits (Std:NZW, 13-week old). Subsequent boosts with antigen in incomplete adjuvant were given at 14-day intervals. Rabbit serum was collected 4 days after each boost. To purify the antibodies, affinity columns immobilized with K2-N or K2-C, from which GST were removed, were prepared with an UltraLink Immobilization Kit (PIERCE Biotechnology). According to the manufacturer's instruction, 1 mg of the protein was used for the immobilizing reaction. Two ml of rabbit's anti-serum was applied to the columns and washed with 20 gel-bed volumes of 0.1% Tween 20 in PBS. Then, antibodies were eluted with 0.1 M glycine-HCl (pH 2.2) and neutralized dialytically with PBS (pH 7.3). Protein concentration of antibodies was estimated by the Bradford method using the Bio-Rad protein assay regent and BSA (Boehringer Mannheim) as

the standard.

Immunofluorescence microscopy and immunoelectron microscopy

Direct binding of Vp130 derivatives to the *Chlorella* cell wall was detected in a similar way using specific antibody as described previously (Onimatsu et al. 2004). In the present study, 100 μg of each protein fused to GST was mixed with a 100 μl sample of *Chlorella* cells at mid-log phase (2.4×10^7 cells/ml). For labeling the CVK2 particles attached to the host cells, a 2 μl (10^9 pfu/ml) and a 100 μl suspension of NC64A cells (2.4×10^7 cells/ml) were mixed for 2 minutes at room temperature. After centrifugation, virus-attached cells were fixed with 4% paraformaldehyde for 10 min at room temperature and then washed twice with 0.1% Tween 20 in PBS. The samples were treated with anti-K2-C antibody and fluorescein isothiocyanate (FITC)-conjugated secondary antibody (Goat $F(ab')_2$ Anti-rabbit IgGs, Biosourse, Camarillo, CA). As a positive control, anti-Vp54 antiserum raised in mice was used instead of anti-K2 antibody and FITC-conjugated anti-mouse IgG (Goat $F(ab')_2$ Anti-mouse IgGs, Biosource) was used for detection. Attached viruses were detected as DAPI or FITC signals under an Olympus BX60 fluorescence microscope. For immuno-gold-labeling, a 100 μl sample of CVK2 (10^{10} pfu/ml) suspended in 0.1% Tween 20 in PBS containing 1% BSA was treated with anti-K2-C antibody at 37℃ for 1 h. After primary antibody treatment, virus particles were fixed in 4% paraformaldehyde for 20 min and subjected to a purification procedure by centrifugation in a 10-40% zonal sucrose gradient at 20,000×g for 30 min at 4℃. Then, the purified particles were settled on 300-mesh nickel grids coated with formbar. All subsequent steps were performed by floating the grid with the specimen side down on the solutions with Parafilm (Pechiney P. P., Menasha, WI) at the base. The virus-settled grids were treated with anti-rabbit IgG conjugated with 10-nm colloidal gold (Amersham Biosciences) at 37 ℃ for 1

h. After two times of 10 min wash with 0.1% Tween 20 in PBS, the grids were fixed in 4 % glutaraldehyde for 15 min. After one times of wash with distilled water, the grids were negatively stained with 2% phosphotungstic acid. The labeled samples were viewed on a transmission electron microscope (JEOL-100CX).

Virus adsorption assay

To confirm the cell wall binding activity of the C-terminal portion of CVK2 Vp130 (K2-C), virus adsorption assay was performed as desceibed previously (Onimatsu et al., 2004). Cells of *Chlorella* sp. NC64A growing exponentially were harvested and pretreated with K2-C: 200 µl of cell suspension (2 x 10^6 cells/ml) was added with various amounts of K2-C protein. After standing at 25˚C for 10 min, the cells were washed and suspended in 200 µl of MBBM. The adsorption of CVK2 to the K2-C-treated *Chlorella* cells was assayed by addition of 50 µl of virus preparation containing approximately 1,400 pfu of CVK2 to the cell suspension. The samples were incubated for 30 min at 25˚C and centrifuged at 5,000 x g for 10 min at 4˚C. Unadsorbed virus particles were titered by plaque assay with strain NC64A as a host (Van Etten et al., 1983).

Acknowledgments

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Figure Legends

Fig. 1. Comparison of the amino acid sequences of Vp130 among chloroviruses. PBCV-1 and CVK2 are NC64A-viruses and CVGW1 and MT325 are Pbi-viruses. Repeated units are shown in green (PBCV-1 and CVK2) and blue (CVGW1 and MT325) boxes, and PAPK, SIKAR/SIAKR sequences are indicated in pink and orange boxes, respectively.

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Fig. 2. Production of three recombinant portions of CVK2 and CVGW1 Vp130 proteins in *E.coli* cells. (A) Schematic representation of deletion constructs of the CVK2 and CVGW1 Vp130 proteins. Under the full-length sequence, each construct is shown. PAPK repeats are indicated by black boxes. The repetitive units of CVK2 and CVGW1 Vp130 are indicated by gray and hatched boxes, respectively. (B) After induction with IPTG, GST-fusion proteins were separated by SDS-PAGE and stained with Coomassie Brilliant Blue. Derivatives CVK2 Vp130 and CVGW1 Vp130 are shown in the upper and lower panels, respectively. Lanes: 1, total protein from *E. coli* cells BL21 with each construct after induction with IPTG; 2, soluble fraction after centrifugation; 3, pellet fraction; 4, protein purified by a glutathione-Sepharose

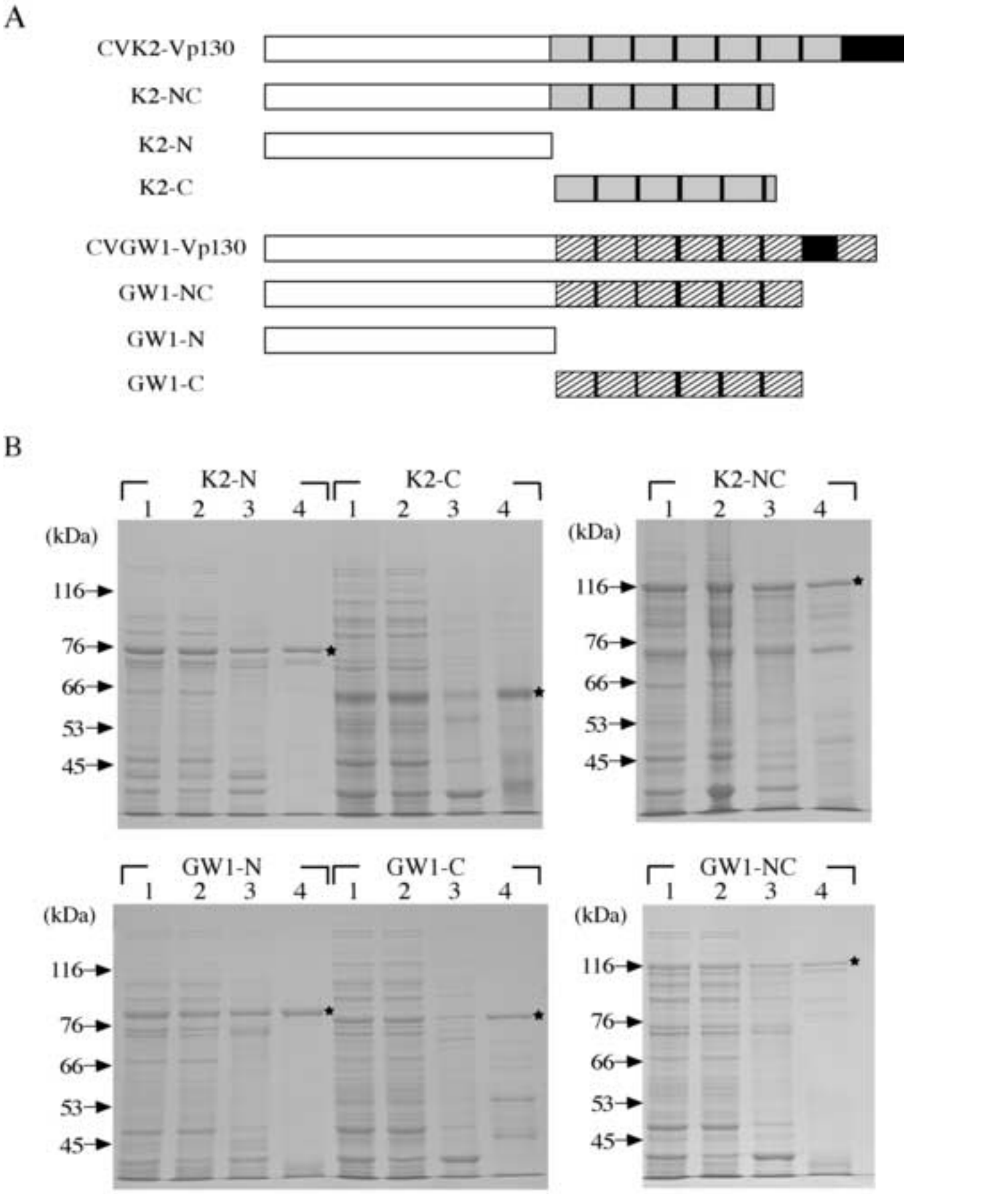
4B affinity column. The positions of the size markers are marked on the left. Asterisks indicate major protein bands of the expected size.

Fig. 3. Adsorption of recombinant portions of Vp130 protein to the cell surface of *Chlorella* sp. NC64A (upper panels) and *Chlorella* sp. SAG 241-80 (lower panels). Cells of both *Chlorella* strains were observed under a fluorescence microscope after treatment with recombinant proteins, anti-Vp130 antibody and FITC-conjugated anti-mouse antibody. Binding of recombinant proteins was detected as green-yellow spots around the cells. Each construct used for the treatment is indicated on the top. Recombinant proteins are as indicated in Fig. 2A. K2-NCR and GW1-NCR are K2-NC and GW1-NC from which GST-tag was removed, respectively.

Fig. 4. Visualization of Vp130 on the virion. (A) Immunoelectron micrographs of CVK2 particles. CVK2 particles were treated with anti-K2-C antibody and 10-nm gold conjugated secondary antibody. Arrows indicate the gold particles. Bars indicate 100 nm. (B) Detection of Vp130 on CVK2 particles adsorbed to NC64A cells. Viruses adsorbed to host cells were treated with anti-CVK2 antiserum (upper panels) as a positive control and with anti-K2-C antibody (lower panels). (C) Detection of Vp130 on CVK2 particles by antibody treatment similar to (B). In (B) and (C), the CVK2 particles were visualized by treatment with DAPI and FITC-conjugated secondary antibodies. DAPI and FITC signals were detected as blue and green spots, respectively. Bars indicate 10 μm.

Figure 1

Figure 2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K2-C
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Figure 3

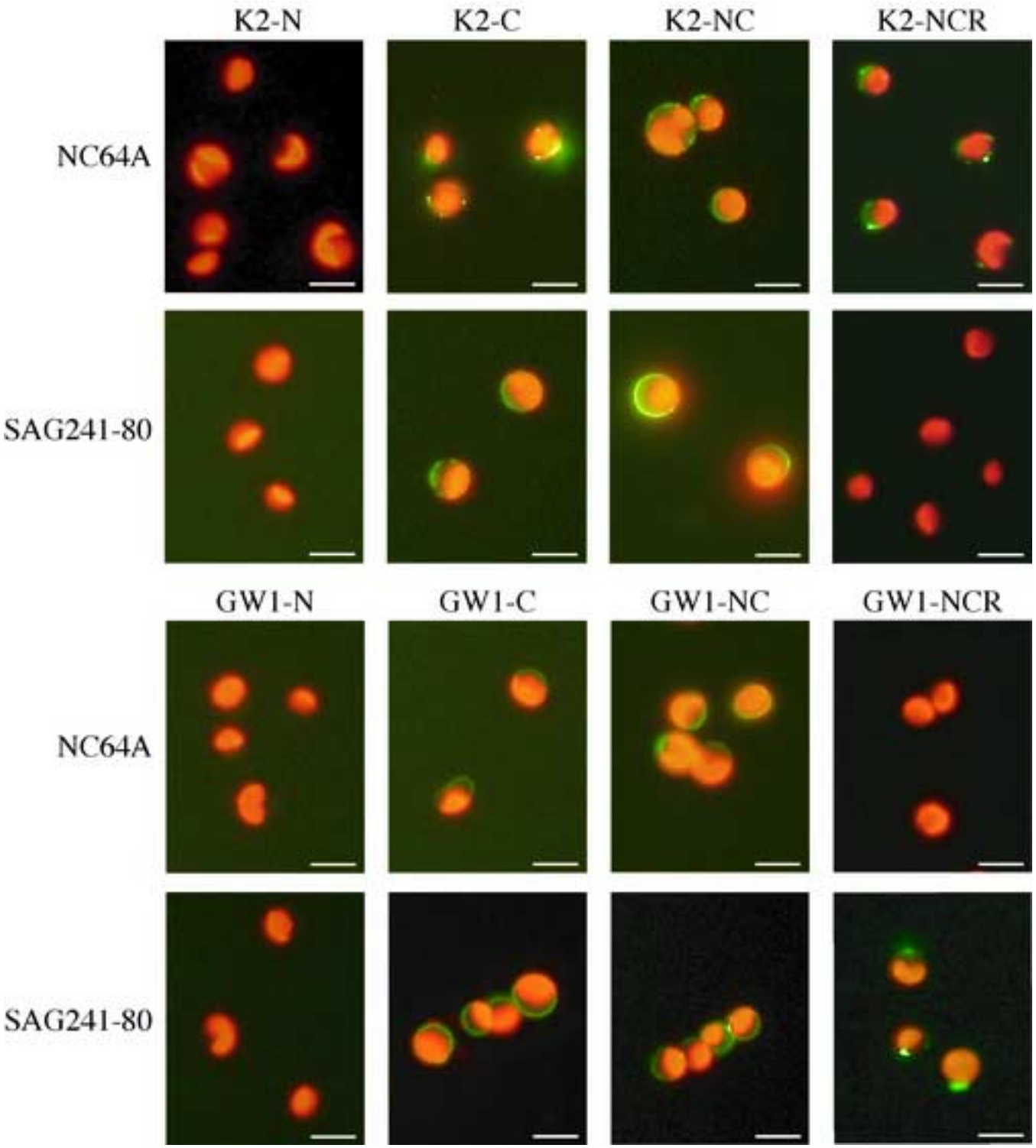
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Figure 4

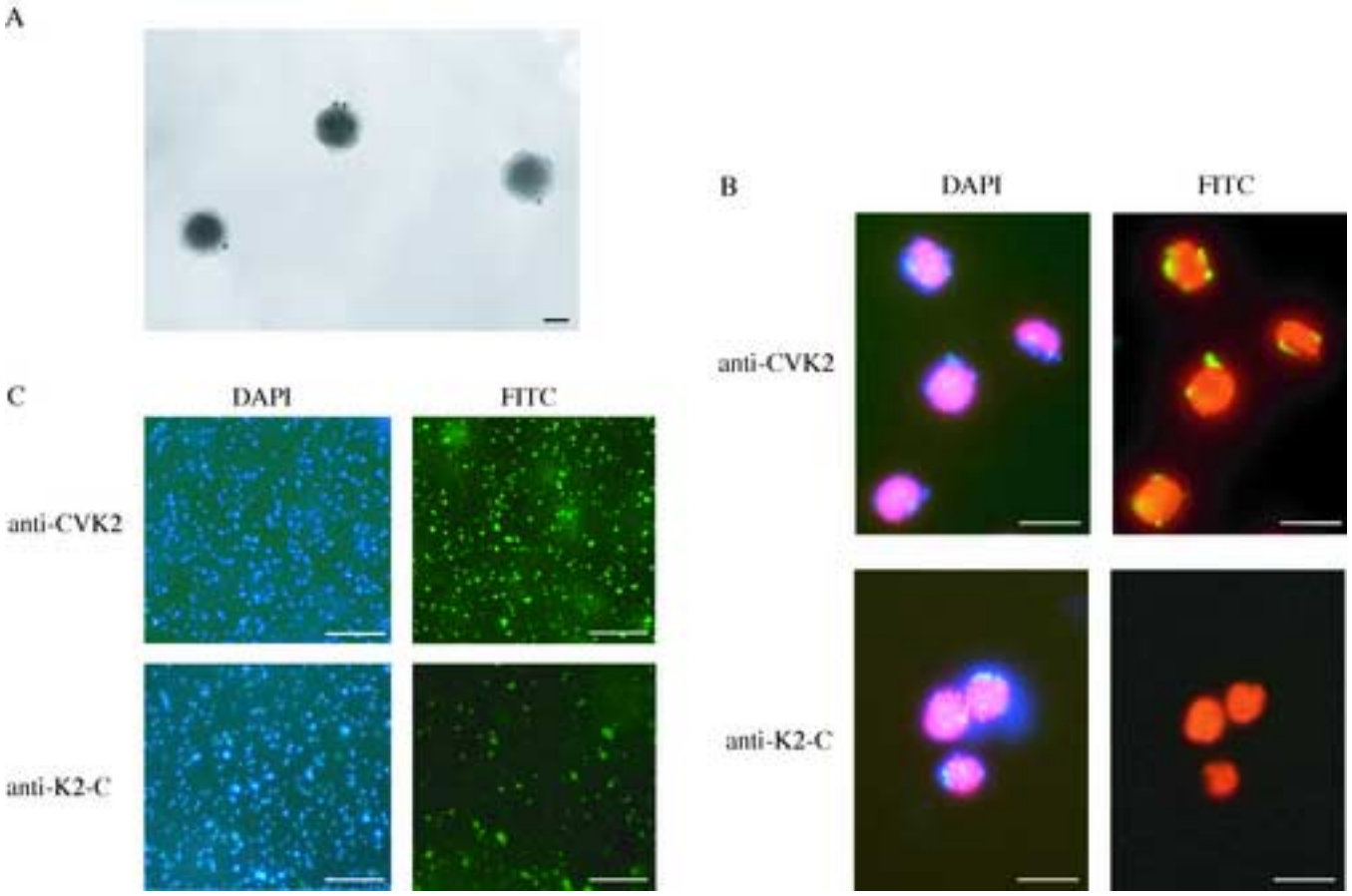

Table 1

Table 1 Inhibition of viral adsorption to host cells by K2-C protein

Amount (μg/ $2x10^6$ cells)	Plaque number appeared	Inhibition[a] (% of control)
0	330	25
40	352	27
80	583	44
160	682	52
Control[b]	1320	(100)[c]

Cells of *Chlorella* strain NC64A ($2x10^6$ cells) were treated with various amounts of K2-C protein and then added with CVK2 (approximately 1400 pfu). After precipitating the cells, unbound viruses were assayed by plaque formation.

[a] Plaques that appeared on the assay plate were compared with the control.

[b] The viruses was assayed without adsorption experiment.

[c] With 100% inhibition, 1320 plaques (control) should appear.

Table 2

Table 2 Adsorption of the C-terminal constructs to various *Chlorella* strains

	K2-C	GW1-C
Chlorella sp. NC64A[1]	+	+
Chlorella sp. 211-6[1]	+	+
Chlorella sp. SAG241-80[2]	+	+
Chlorella saccharophila C-211	-	-
Chlorella saccharophila 211-9a	-	-
Chlorella saccharophila C-169	-	-
Chlorella vulgaris C-135	+	+
Chlorella vulgaris C-27	+	+
Chlorella vulgaris 211-1e	+	+
Chlorella vulgaris 211-11b	+	+

[1]Host of NC64A-viurses [2]Host of Pbi-viurses

Table 3

Table 3 Inhibition of CVK2 infection by anti-K2-N and anti-K2-C antibodies

	anti-K2-N antibody			anti-K2-C antibody		
Amount (ng)	50	100	200	50	100	200
Plaque number appeared	948	727	312	1122	930	703
Inhibition (%)	32	48	78	20	34	50

uninhibited:1404

CVK2 particles were treated with various amounts of anti-K2-N antibody, anti-K2-C antibody and subjected to plaque assay with *Chlorella* strain NC64A as the host.