Papaya Ringspot Virus Coat Protein Gene for Antigen Presentation in Escherichia coli

Supawat Chatchen1, Mila Jurícek2, Paloma Rueda3 and Sunee Kertbundit1,2,*

1Institute of Molecular Biology and Genetics, Mahidol University, Salaya Campus, Nakon Pathom 73170, Thailand
2Institute of Experimental Botany, Czech Academy of Sciences, Na Perníkarc 15, 160 00 Prague 6, Czech Republic
3Ingenasa, Hermanos Garcia Noblejas 41-2, 28037 Madrid, Spain, Immunologia y Genetica Apl. S.A. (INGENASA), Hnos. Noblejas 41, 4 degrees, 28037, Madrid, Spain

Received 18 May 2005, Accepted 19 August 2005

The coat protein (CP) of Papaya ringspot virus (PRSV) was analyzed for presentation of the antigenic peptide of animal virus, Canine parvovirus (CPV), in Escherichia coli (E. coli). The 45 nucleotides fragment coding for the 15-aa peptide epitope of the CPV-VP2 protein was either inserted into the PRSV-cp gene at the 5', 3' ends, both 5' and 3' ends or substituted into the 3' end of the PRSV cp gene. Each of the chimeric PRSV cp genes was cloned into the pRSET B vector under the control of the T7 promoter and transformed into E. coli. The recombinant coat proteins expressed from different chimeric PRSV cp genes were purified and intraperitoneally injected into mice. All of the recombinant coat proteins showed strong immunogenicity and stimulate mice immune response. The recombinant coat proteins containing the CPV epitope insertion at the C terminus and at both N and C termini elicited ten times higher specific antisera in immunized mice compared with the other two recombinant coat proteins which contain the CPV epitope insertion at the N terminus and substitution at the C terminus.

Keywords: Antigen presentation, Canine parvovirus, Epitope, Papaya ringspot virus

Introduction

Several plant viruses have been used as efficient presentation systems for expressing antigenic peptides or epitopes (Usha et al., 1993; Fitchen et al., 1995; Turpen et al., 1995; Joelson et al., 1997; Johnson et al., 1997; Sugiyama et al., 1997; Yusibov et al., 1997; Koprowski and Yusibov, 2001). Epitopes are short peptides that contain antigenic determinants and can stimulate the immune response against the pathogen from which they are derived. The fusion of an epitope to a large carrier molecule such as the coat protein (CP) of plant virus can enhance the immunogenicity and stability of the epitope (Fitchen et al., 1995). The CP of potyvirus has been demonstrated as a good candidate of carrier molecule for presenting epitope (Jagadish et al., 1993; Fernandez-Fernandez et al., 1998, 2001). Experiments with Johnson grass mosaic potyvirus by Jagadish et al. (1996) revealed that hybrid CPs containing either short peptides or large target antigens fused at the N- or C-terminals retained the ability to assemble into chimeric potyvirus-like particles (PVLPs). The chimeric PVLPs were highly immunogenic in mice and rabbits (Jagadish et al., 1996).

Papaya ringspot virus (PRSV) is a member of the genus Potyvirus. It is grouped into two types; type P (for papaya) infects both papaya and cucurbits (e.g. squash, pumpkin, cucumber, watermelon) while type W infects only cucurbits. (Tennant et al., 1994; Gonsalves, 1998). The complete nucleotide sequences of several strains of PRSV have been determined (Yeh et al., 1992; Attasart et al., 2002; Charoenasilp et al., 2003). Similar to other potyviruses, the genome of PRSV consists of positive polarity single stranded RNA of about 10 kb surrounded by ~2,000 subunits of CP (Shukla et al., 1989). The N and C termini of the potyvirus CP are exposed at the surface of virions and are reported to be highly immunogenic (Shukla et al., 1988).

Canine parvovirus (CPV) is a member of the genus Parovirus (Siegel et al., 1985). It caused a sudden outbreak of severe acute enetic disease in many species of dogs around the world (Apple et al., 1979, Burtonboy et al., 1979). The VP2 protein is the major structural protein of CPV (Tsao et al., 1991). The peptide 6L15 epitope at the N-terminal domain of the VP2 protein has been used to induce the CPV specific neutralizing antibodies against CPV (Casal et al., 1995) and

*To whom correspondence should be addressed.
Tel: 66-2-800-3624 ext 1376; Fax: 66-2-441-9906
E-mail: kertbundit@ueb.cas.cz
had been expressed in plum pox potyvirus (Fernandez-Fernandez, et al., 1998).

In this present study we analyzed the ability of using the PRSV CP as a carrier molecule for CPV epitope expression in E. coli. The expression plasmids containing the cp gene of the PRSV with the insertion and substitution of the 6L15 epitope sequence at different positions of the cp gene were constructed. The recombinant coat proteins expressed in the E. coli were purified and used to induce immune response in mice. The immunogenicity of the expressed epitope was reported.

Materials and Methods

**Bacterial strain and plasmids.** *Escherichia coli* strain BL21(DE3) pLysS (Studier 1991) was used for plasmid transformation. Plasmid pRSET B expression vector (Invitrogen) was used for cloning and expression of PRSV cp gene and recombinant cp genes.

**Construction of CP and recombinant CP-CPV expression vectors.** The intact cp gene was amplified from the cDNA of PRSV type P by PCR reaction using CP-F/CP-R primers (Table 1). The recombinant cp genes which contains the 45 nucleotides encoding the CPV 6L15 epitope inserted either at the 5', 3' or both 5' and 3' ends were obtained by PCR amplification using CP-F/CP-R, CP-F/CPV-R or CP-V/CP-R primers (Table 1). The amplified DNA fragments of the intact cp gene and the recombinant cp genes were cloned into the pRSET B expression vector at the NeoI/HindIII positions after the T7 promoter. Plasmid CP contains the DNA fragments of the intact cp gene and recombinant cp-R/CPV-R primers (Table 1). The amplified cp gene was inserted at the 5', 3' and both 5' and 3' ends, respectively. These plasmids were transformed into E. coli. BL21 (DE3) pLysS.

In order to substitute the 6L15 epitope sequence for the sequence located at the 3' end of the cp gene (nt 9,986 to 10,030 in the PRSV type P RNA sequence), the amplified intact cp gene was blunt digested with HindIII and inserted into the pUC19 vector at the Smal/HindIII sites. The recombinant plasmid cp gene with the 6L15 epitope sequence substitution at the 3' end was obtained by PCR amplification of the cp gene using the CP-CPV/CP-R primers. The amplified fragment was used to replace the PstI/HindIII fragment of the intact cp gene in the pUC19 vector. The recombinant cp gene was digested with NcoI/HindIII and cloned into the pRSETB expression vector to yield plasmid CP4. This plasmid was transformed into E. coli. BL21(DE3) pLysS.

**Sodium Dodecyl Sulfate Polyacrylamide gel electrophoresis.** The SDS-PAGE was performed to determine the expressed protein from recombinant clones according to the procedure previously described by Laemmli (Laemmli, 1970). Gels were stained with Coomassie brilliant blue R-250 solution and destained with 10% acetic acid and 10% methanol.

**Purification of fusion CP-CPV proteins from E. coli.** The recombinant clone was grown at 37°C in 50 ml LB broth containing 100 µg/ml of ampicillin and 34 µg/ml of chloramphenicol. When the culture reached OD₆₀₀ of 0.4-0.6, the protein expression was induced by adding 0.1 mM IPTG and the culture was grown at 30°C for 4 h. Bacterial cells were collected by centrifugation at 6,000 × g for 5 min at 4°C and resuspended in 5 ml of start buffer (20 mM sodium phosphate, 0.5 M NaCl pH 9.0). The cell suspensions were kept in ice bath and disrupted by a French Press. The cell lysates were centrifuged at 12,000 × g for 1.5 min at 4°C. The fusion protein was separated from the supernatant by HiTrap affinity column (Amersham Pharmacia Biotech) according to the manufacturer’s instruction. The supernatant was applied into the column prepared with Ni²⁺ ions. The loaded column was washed with wash buffer containing 25 mM imidazole. The bound protein was eluted by 2 ml of elution buffer containing 150 mM imidazole. The protein concentration was measured by Bradford Protein assay (Bio-Rad).

**Western blot analysis.** The purified fusion proteins were analyzed by SDS-PAGE and blot transferred onto a nitrocellulose membrane (PROTRAN®) by semi-dry method using Trans-Blot SD Electrophoretic Transfer Cell (BIO-RAD). The nitrocellulose membrane was rinsed for 3 times with 100 ml of phosphate-buffered saline (PBS) and incubated with 5% skim milk and 0.2% Tween 20 in PBS) for 1 h. The blocked membrane was subject to incubation with blocking solution

---

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Nucleotide sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP-F</td>
<td>5'-CATGCCATGGAGCCAGTCCAAATTGAAGCTGTTGG-3'</td>
</tr>
<tr>
<td>CP-R</td>
<td>5'-CCCAAGCTTATCCGCATACCCAGGAGAGAG TGC-3'</td>
</tr>
<tr>
<td>CPV-F</td>
<td>5'-CATGCCATAGTGTCAACCCAGACGCTGTTGCAACCGTCTGCTTACAGAATGAAAGAAGCTCAGTCCAAATTGAAGCTGTTGG-3'</td>
</tr>
<tr>
<td>CPV-R</td>
<td>5'-CCCAAGCTTATCCGCATACCCAGGAGAGAG TGC-3'</td>
</tr>
<tr>
<td>CP-CPV</td>
<td>5'-AAAGCTGCAATTCACCAAGGAGGGTGTCAACCGTCTGCTTACAGAATGAAAGAAGCTCAGTCCAAATTGAAGCTGTTGG-3'</td>
</tr>
</tbody>
</table>
containing 1:1,000 dilution of primary antibody for 1 h. The anti-CPV monoclonal antibody 3C9 (Ingenasa) was used as primary antibody for detection of the 6L15 epitope of CPV VP2 antigen. After one hour incubation with the primary antibody, the membrane was incubated in the blocking solution containing the 1:5,000 dilution of secondary antibody (anti-mouse IgG conjugated with horseradish peroxidase) for 1 h. The peroxidase reaction was developed using the ECL plus western blotting detection reagent (Amersham Pharmacia Biotech) according to the manufacturer’s instruction.

Immunization of mice. Groups of three 5-week-old mice strain BALB/c were immunized intraperitoneally. Eighty microgram of either PRSV CP or purified fusion proteins was emulsified with Freud’s complete adjuvant (Sigma) and used for the primary immunization on the day 0. The booster immunization was performed on day 21 using the same amount of purified fusion protein emulsified with incomplete adjuvant. Mice were tail bled on day 0 before immunization and on days 35 and 42. The sera from immunized mice in the same group were pooled and used for NCM-ELISA.

Analysis of antibodies by NCM-ELISA. Sera recovered from immunized mice were analyzed by NCM-ELISA for reactivity against the VP2 antigen of CPV. The 1-1000 ng of VP2 antigen was loaded onto nitrocellulose membranes. The membrane was soaked in blocking solution (5% skim milk and 0.2% Tween 20 in PBS) for 1 h. The membrane was incubated in blocking solution containing 1:500 dilution of the primary polyclonal antibody obtained from each group of immunized mice at room temperature for 1 h. The 1:500 dilution of anti-CPV monoclonal antibody 3C9 (Ingenasa) was used as positive control to detect the VP2 peptide. The membrane was washed 3 times with PBS-T and incubated with 1:5,000 dilution of secondary antibody (anti-mouse IgG conjugated with horseradish peroxidase) for 1 h. The detection reagent (ECL plus western blotting detection reagent, Amersham Pharmacia Biotech) was used to develop the peroxidase reaction.

Results

Recombinant CP:CPV expression vectors. In order to evaluate the positions on the cp gene of PRSV which are suitable for foreign protein expression, the 6L15 epitope sequence of the CPV was inserted and substituted into the cp gene. Five E. coli BL21 clones containing the native cp gene and recombinant cp genes on the expression plasmids were generated (Fig. 1). Clone CP contains the native cp gene while clone CP1 contains the epitope sequence insertion at the 5’ end after the start codon of the cp gene. Clone CP2 contains the epitope sequence insertion at the 5’ end before the stop codon of the cp gene and clone CP3 contains the epitope sequence insertion at both 5’ and 3’ ends. Clone C4 contains the epitope substitution at the 3’ end of the cp gene.

Expression of PRSV:CPV recombinant protein in E. coli. After induction with IPTG, E. coli BL21 clones harboring the pRSETB based recombinant plasmids efficiently produced recombinant proteins. The relative molecular weights of the native CP and the CP-4 are 38 kDa; the CP-1 and the CP-2 are about 40 kDa and the CP-3 is 42 kDa, as estimated by visual inspection of SDS-PAGE, which were consistent with size of native CP and the inserted size of epitope in each constructs. (Fig. 2A and B). Both of the native and recombinant CPs were present in the pellet and were insoluble. The protein aggregates were dissociated into the soluble fraction by dissolving with sodium-phosphate buffer pH 9 and purified by Ni²⁺ ions affinity column. The yield of the native and recombinant CPs after affinity column purification was approximately 80-200 µg/l of bacterial cultures. The purified recombinant CPs were specifically detected in western blot analysis using the anti-CPV monoclonal antibody 3C9 (Fig.
Immunogenicity of chimeric CP-CPV. The immunogenicity of recombinant proteins CP-1, CP-2, CP-3 and CP-4 were tested by BALB/c mice immunization. The purified native CP was used in control mice. The specificity and sensitivity of the antisera obtained from the immunized mice responded to the CPV VP2 antigen were evaluated in NCM-ELISA analysis (Fig. 4) and compared with the anti-CPV monoclonal antibody 3C9. There is no cross-reaction between the antiserum to the native CP with the CPV VP2 antigen. The antisera CP-2 and CP-3 showed the same sensitivity to detect as low as 10 ng of CPV VP2 antigen which is about ten times less sensitive than the monoclonal antibody 3C9. The antisera CP-1 and CP-4 exhibited about ten times lower sensitivity than the antisera to the CP-2 and CP-3 as they can not detect the CPV VP2 protein at concentration lower than 100 ng. These results demonstrated that recombinants CPs are immunogenic when inoculated intraperitoneally into mice and there is no common antigenic part between the CPV VP2 antigen and the PRSV coat protein.

NCM-ELISA analysis of the reaction between the antisera and the partially purified PRSV particles indicated that all antisera can detect the partially purified PRSV particles at concentration of 1 ng (Fig. 5). This result indicated that the PRSV CP part in all of the recombinant proteins can stimulate mouse immune response at the same level as the native CP. The monoclonal antibody 3C9 cannot react with the partially purified PRSV particles at concentration of 1000 ng which indicated that there is no common antigenic part between the CPV VP2 antigen and the PRSV viral particle.

Discussion

Potyvirus is a group of single stranded RNA viruses with rod-shaped, self-assemble in an ordered manner, and accumulates to high levels in infected tissues. The CP of potyvirus is a good candidate carrier molecule for epitope presentation. The potyvirus CP can express in heterologous host expression systems including bacterial, yeast or insect cell (Jagadish et al., 1996).

In this study, the effectiveness of using the CP of PRSV as an antigen presentation system in E. coli is reponed. The recombinant cp genes containing the 6L15 epitope sequence at several positions were constructed and expressed in E. coli. The reactivity of the anti CPV monoclonal antibody 3C39 to
all recombinant CPs in western blot analysis revealed the presence and accessibility of CPV epitope on the recombinant CPs. The epitope placed on N- or C- terminus or on both N- and C- termini can effectively induce the mouse immune response although the sensitivity of the antisera raised against the recombinant CP containing the epitope on the N-terminus is about 10 times lower than the recombinant CPs containing the epitope on the C-terminus or on both N- and C- termini. Recombinant CP with the substitution of the epitope on the C-terminus showed the same ability to raise the mouse immune response as the recombinant CP containing the epitope on the N-terminus. These results are in agreement with the result of poxvirus JGMV (Jagadish et al., 1993). The CP of JGMV can be fused with the epitopes either on the N- and/or C-terminus without abolishing the ability of the protein to polymerize into virus-like particles when expressed in E. coli and can stimulate the mouse immune response. The decrease of the immunogenicity of the CPV epitope when inserted at the N-terminus of the PRSV CP or replacement with the amino acids on the C-terminus of the PRSV CP revealed that the position of the epitope presentation affects the immunogenicity. The identification of immunogenic hot spots within plum pox virus (PPV) coat protein by peptide scan showed that the N-terminal domain of the PPV CP is not uniformly immunogenic and some regions are preferentially recognized by sera (Fernandez-Fernandez et al., 2002). The result from our experiment indicated that epitope inserted at the position on the C-terminus of the PRSV CP can expose to the surface of the recombinant protein and trigger the mouse immune response more efficient than the epitope inserted at the N-terminus or the epitope substitution at the C-terminus. It is important to verify the appropriate position of the epitope insertion on the CP to get the high immunogenicity of the epitope.

Therefore, we have demonstrated here that the PRSV CP can be effectively used for antigen presentation to produce sufficient quantities of an animal viral antigen in E. coli. The recombinants PRSV CPs are success fully used to stimulate an animal immune response to the pathogen. The epitope production can easily amplify in sterile bacterial culture system. It is also possible to develop the PRSV infectious particle containing the chimeric CP to express epitope on the surface of the viral particle. The advantage of developing the infectious virus particle is the exclusion of sterile culture system. However there are some restrictions on the expression of foreign peptides on the cp gene of some viral particles (Fernandez-Fernandez et al., 2002; Porta et al., 2003). Effect of the chimeric cp on virus assembly, disassembly and stability will be important considerations for engineering the chimeric PRSV infectious virus.

Acknowledgment We thank Nuanwan Pungtanom and Aurathai Tanwanchat for assistance. This work was supported by the grant from BIOTEC, NASA, Thailand, project code BT-B-06-02-14-4503 and by the grant from Thai Research Fund, project code TRF-RDG0020016.

References


