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Baculovirus Surface Display of SARS Coronavirus (SARS-CoV) Spike Protein and Immunogenicity of the Displayed Protein in Mice Models

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ABSTRACT

The baculovirus surface display technique has provided an ideal tool to display foreign proteins with natural conformation, functions, and immunogenicity. In this work, we explored the application of this technique on SARS-associated coronavirus (SARS-CoV) spike (S) protein, and further analyzed the immunogenicity of displayed S protein. The entire ectodomain of S protein was fused between the gp64 signal peptide and the VSV-G membrane anchor and successfully displayed on the baculovirus surface. Subcutaneous injection with purified S-displayed baculoviruses without adjuvant elicited highly effective production of specific and neutralizing antibodies against S protein in mice. These results confirmed a successful surface display of S protein on baculoviruse, and suggested a potential role of S-displayed baculoviruses as a novel live virus-based vaccine candidate for SARS-CoV.

INTRODUCTION

THE SEVERE ACUTE RESPIRATORY SYNDROME (SARS), also named infectious atypical pneumonia, is a newly emergent transmissible febrile respiratory disease. In 2003, it caused a worldwide outbreak and infected more than 8000 people over 26 countries. The causative agent of SARS was soon identified as a new member in the family *Coronaviridae* (Ksiazek *et al*., 2003), and named severe acute respiratory syndrome-associated coronavirus (SARS-CoV) by the World Health Organization (WHO). The spike (S) protein is the major envelope glycoprotein of SARS-CoV that is responsible for cellular receptor binding (Gallagher and Buchmeier, 2001; Sui *et al*., 2004; Li *et al*., 2005) and membrane fusion (Sainz *et al*., 2005). It is also the main target for host immune defense during viral infection (Dai *et al*., 2004; Yi *et al*., 2005). Many works have also demonstrated the antigenic and immunogenic properties of the S protein, and recommended this protein as ideal immunogen in SARS-CoV vaccine design (Buchholz *et al*., 2004; Ernst *et al*., 2006).

Recombinant prototype baculovirus *Autographa californica* nucleopolyhedrovirus (AcMNPV) has been widely used as a vector to express heterologous proteins in insect cells and larvae (Kost *et al*., 2005). The baculovirus surface display system is a specialized baculovirus expressing system in which the target protein is incorporated into the envelope of the budded viruses and displayed on the surface of the virions (Boublik *et al*., 1995; Grabherr *et al*., 1997; Mottershead *et al*., 1997). This is accomplished by fusing the target protein either to the C-terminus of the gp64 glycoprotein of AcMNPV or between the signal peptide (SP) and the transmembrane domain (TM) of the gp64 (Grabherr *et al*., 1997). In alternative strategies, the gp64 TM was replaced with VSV-G TM (Chapple *et al*., 2002), or both the SP and TM of gp64 were replaced with the corresponding regions of the influenza virus neuraminidase (Borg *et al*., 2004). The baculovirus surface display was first used to raise monoclonal antibodies against the nuclear receptors LXRβ and FXR (Lindley *et al*., 2000). The displayed proteins have been proven to be very effective immunogens and successfully elicit antibody responses in mice. These include the hemagglutinin protein of Rinderpest virus (Rahman *et al*., 2003), *Theileria parva* p67 antigen (Kaba *et al*., 2003), and the major antigen site A of foot-and-mouth disease virus (Tami *et al*., 2004).

In this work, we displayed the entire ectodomain of SARS-

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associated coronavirus (SARS-CoV) spike (S) protein on the surface of the baculovirus AcMNPV virion. These S protein-incorporated baculoviruses were highly effective in eliciting specific and neutralizing antibodies against S protein in mice, implying a novel vaccine candidate for SARS-CoV.

MATERIALS AND METHODS

Cells and viruses

Sf9 *Spodoptera frugiperda* insect cells were maintained in TNM-FH Insect Medium (Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Gibco-BRL, Grand Island, NY) at 27°C. The DH10Bac *Escherichia coli* competent cells were produced by the $CaCl_2$ method and stored at −70°C. The SARS-CoV virus used in this work was isolated from Zhejing, China, in 2003.

Cloning of SARS-CoV spike protein gene

The extracted SARS-CoV RNA was reverse-transcripted using antisense primer Rs (5′-GCGGCCGC (*Not*I) TTATGTGTAA TGTAATTTGA CACCC-3′) and amplified by PCR using *Pfu* DNA polymerase and primer set Fs (5′-GGATCC (*Bam*HI) ATGTTTATTT TCTTATTATT TCTTACTC-3′)/Rs. After adding an adenine to each 3′-terminal of the PCR products by one cycle PCR using *Taq* polymerase, the 3′-adenine added PCR products was ligated directly to pGEM-T easy vector (Promega, Madison, WI). The sequence of the SARS-CoV spike protein gene was determined and submitted to NCBI (GenBank accession number: DQ231462).

Construction of display vector

Recombinant AcMNPVs were constructed using Bac-to-Bac Baculovirus Expression Systems (Invitrogen, Carlsbad, CA). The pFastBac DUAL vector was used as donor plasmid. The surface display vector was constructed by modifying pFastBac DUAL (Fig. 1). First, *Bgl*II/*Bam*HI-digested gp64 signal peptide (SP, codon 1–34) was introduced into the single *Bam*HI site. Then, the transmembrane (TM) and the cytoplasmic terminal domain (CTD) of VSV-G (codon 441–511) were introduced into the *Not*I/*Xba*I sites. Finally, the ectodomain of spike protein (Sec, codon 12–1190) was introduced into the *Bam*HI/*Not*I sites to construct S-vsvG–Dual vector.

Generation of recombinant viruses

Recombinant pFastBac DUAL plasmid was transformed and transposition in DH10Bac *E. coli* competent cells and the recombinant bacmid DNA were used to transfect Sf9 cells. Recombinant viruses S-vsvG were harvested from the supernatant of the transfected cells and amplified by serial infections. An unrecombinant bacmid was also used to transfect Sf9 cells, and the resultant unrecombinant viruses (Bac) were used as controls in further experiments.

Purification of recombinant AcMNPVs

Supernatant of infected Sf9 cells at 48 h.p.i. (hours postinfection) was centrifuged at 5000 × *g* for 30 min at 4°C (Sigma, 3K15, Germany). The supernatant was centrifuged at 100,000 × *g* for 2 h at 4°C (Beckman, Ti70, Palo Alto, CA), then the pellet was resuspended and overlayed on a 5-ml 25–56% discontinuous sucrose gradient, and centrifuged at 100,000 × *g* for 2 h at 4°C (Hitachi, S52ST, Tokyo, Japan). The virus band was collected and dialyzed overnight at 4°C against PBS (pH 7.2).

Western blot

Samples were analyzed by SDS-PAGE gel and electroblotted to nitrocellulose membrane (Osmonics, Pittsburgh, PA). The membrane was blocked in TBS (pH 7.4) containing 5% milk for 30 min at 37°C, and then incubated with a rabbit anti-SARS-CoV S protein antibody (diluted 1:200) for 1 h at 37°C. After washing three times in TTBS (TBS containing 0.625% Tween 20), the membrane was incubated with an alkaline phosphatase (AP)-conjugated goat antirabbit IgG (Vector, Burlingame, CA, diluted 1:200) for 1 h at 37°C. After washing an additional three times in TTBS, the blots were developed with BCIP/NBT substrates (Promega, Madison, WI) at room temperature (RT).

Immunofluorescence

Infected Sf9 cells were washed three times in phosphate-buffered saline (PBS) (pH 7.2) and fixed in 4% paraformaldehyde for 15 min at RT. The cells were blocked in 3% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) for 30 min at 37°C, and then incubated with a rabbit anti-SARS-CoV spike protein antibody (diluted 1:100) for 1 h at 37°C.

After washing six times in PBS, the cells were incubated with a TRITC-conjugated goat antirabbit antibody (Vector, diluted 1:100) for 1 h at 37°C. After six additional washes, the cells were viewed under a fluorescence microscope (Leica, Wetzlar, Germany).

Vaccination and neutralization assays

Six-week-old Balb/c female mice were subcutaneously inoculated with ~5 × 10^8 p.f.u. purified S-vsvG or Bac viruses without adjuvant (three mice in each vaccine group). Mice were

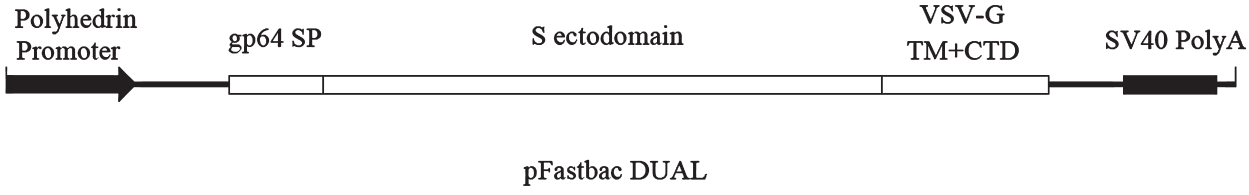

FIG. 1. The construction of baculovirus display vector.

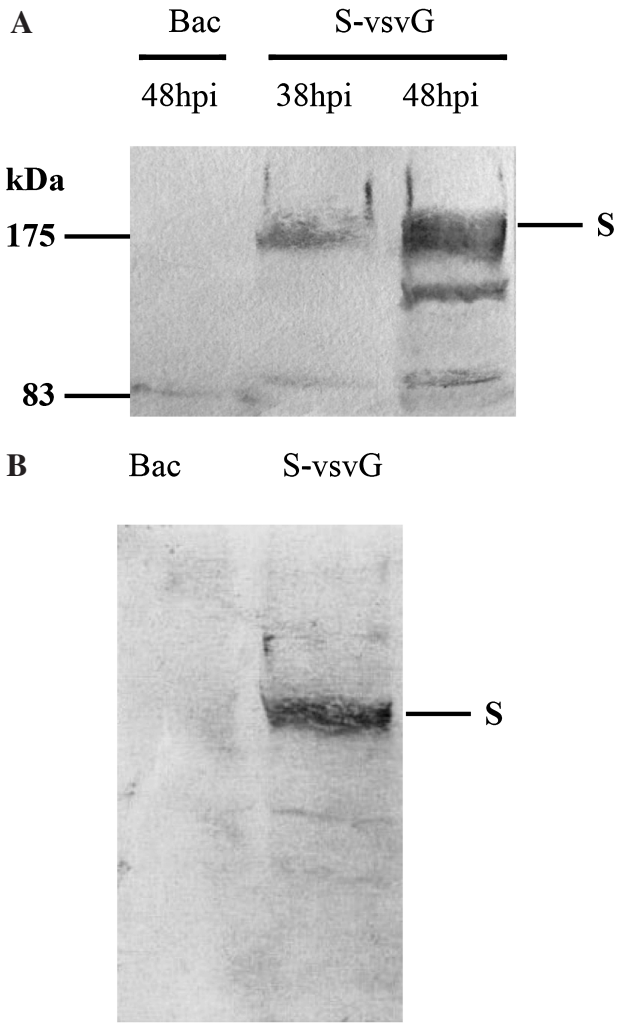

FIG. 2. Western blot detection of S protein in infected Sf9 cells and purified viruses. (**A**) Expression of S protein (~175 kDa bands) was detected in S-vsvG-infected Sf9 cells. Bac: unrecombinant baculovirus. (**B**) S protein was detected in purified S-vsvG viruses.

boosted once at day 10 after first inoculation and killed at day 28. Sera were heated at 56°C for 30 min prior to test in neutralization assays. The neutralizing activity of the sera were determined using a SARS-CoV S protein pseudovirus (SARS/HIV) carrying a luciferase gene as previously described (Zhang *et al*., 2004). The infectivity of the pseudovirus was calculated as a percentage in the average amount of luciferase activity from the test culture relative to that of untreated controls, which reflected the neutralizing activity of serum samples. Each serum sample was tested twice at each dilution, and the average luciferase activity for each serum sample was used to calculate the relative infectivity of the pseudovirus.

ELISA

Purified S protein or purified Bac was used as antigens in enzyme-linked immunoabsorbent assay (ELISA). Antigens were coated on 96-well plates at 4°C overnight. After washing three times, the plates were blocked in 3% milk at 37°C for 30 min. Each serum sample was serially two-fold diluted from 1:80 to 1:10,240 in 3% milk and incubated at 37°C for 1 h. After washing four times, Peroxidase(HRP)-conjugated goat anti-mouse IgG or HRP-conjugated goat antirabbit IgG (Vector) was diluted at 1:10,000 in 3% milk and incubated at 37°C for 1 h. After additional four times of washing, the plates were developed with H_2O_2 and *o*-phenyldiamino at RT, and reaction was terminated by 2 N H_2SO_4. OD450 was read under a Sunrise plate reader (Tecan, Mannedorf, Switzerland).

RESULTS

Construction of recombinant baculoviruses displaying S protein

The baculovirus surface display vectors was constructed by inserting the gp64 signal peptide (SP), the entire ectodomain of SARS S protein, and the transmembrane (TM) and cytoplasmic terminal domain (CTD) of VSV-G protein into the pFastBac DUAL plasmid (Fig. 1). The TM&CTD of VSV-G protein was previously confirmed as successful membrane anchors led to

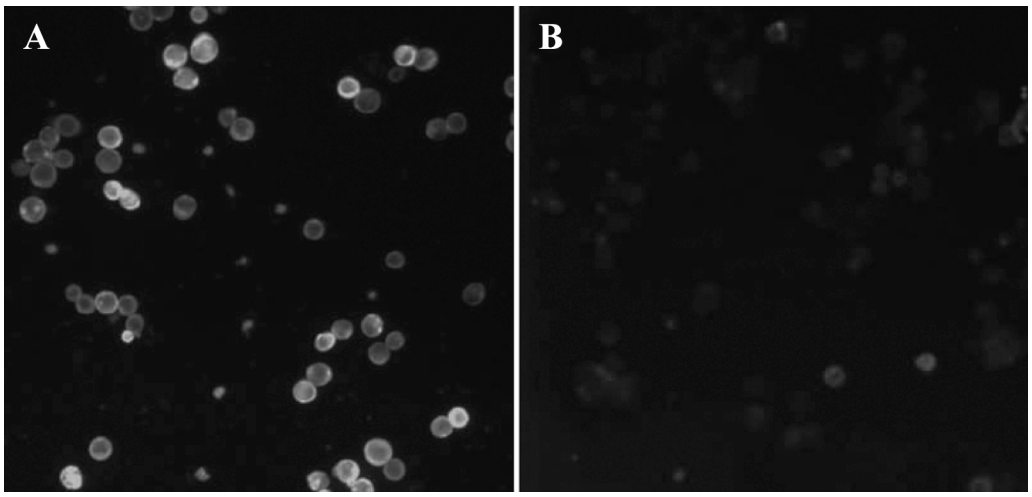

FIG. 3. Immunofluorescence microscopy of Sf9 cells infected with recombinant baculovirus. The S protein was shown to be located on the cytoplasmic membrane of Sf9 cells. (**A**) S-vsvG infected Sf9 cells. (**B**) Uninfected Sf9 cells.

nonpolar incorporation of heterologous proteins, and it was also reported to out-perform gp64 TM&CTD in higher efficiency of incorporating hetorologous proteins (Chapple and Jones, 2002).

To confirm the expression of S fusion protein in S-recombinant viruses (S-vsvG) infected Sf9 cells, the infected cells were analyzed by Western blot and immunofluorescence at 38 or 48 h.p.i. Obvious expression of S fusion protein of ~175 kDa weight was detected at 38 h.p.i. and the expression level was abundant at 48 h.p.i. (Fig. 2A). The detected S fusion protein was much larger than its predicted MW of 143 kDa, suggesting posttranslational modifications such as glycosylation. The images of immunofluorescence microscopy showed that the expressed S fusion protein was located on the cytoplasmic membrane of Sf9 cells (Fig. 3).

To investigate whether our displayed vector could successfully incorporate S protein into the envelope of the virus particle, viruses were purified by ultracentrifugation and discontinuous sucrose gradient ultracentrifugation from supernatants of infected Sf9 cells, and the presence of S protein was identified by Western blot. The S protein of ~175 kDa was detected only in purified S-vsvG viruses (Fig. 2B).

Immunogenicity of baculovirus displayed S protein

To evaluate the immunogenicity of S protein displayed on the surface of baculoviruses, mice were immunized subcutaneously with purified S-vsvG or unrecombinant viruses (Bac) without adjuvant. At day 28, the sera were collected and analyzed by ELISA (Table 1). High titers of S-specific antibodies were detected in S-vsvG immunized mice sera, with end-point titers of 2560 to 5120, which is about 10-fold higher than that of Bac immunized mice sera (with end-point titers of 320). However, equivalent levels of baculovirus-specific antibodies were detected both in S-vsvG and in Bac immunized mice sera, with end-point titers of 2560 to 5120. The SARS patient serum showed an end-point titer of 1280 for S-specific antibodies, and 160 for baculovirus-specific antibodies.

We further used a SARS-CoV S protein pseudovirus (SARS/HIV)-Huh 7 cell infection system (Zhang *et al*., 2004) to determine the levels of neutralizing antibodies against SARS-CoV in these mice sera. The inhibitory effect of sera on SARS/HIV infectivity reflected its neutralizing activity. The S-vsvG viruses immunized mice sera showed significant neutralizing activity to SARS/HIV and could inhibit 50% of the pseudovirus infectivity up to 1:320 dilution (Fig. 4). No neutralizing activity was observed in mice sera immunized with unrecombinant baculoviruses.

TABLE 1. IMMUNOGENICITY OF S-DISPLAYED BACULOVIRUSES IN ELISA

	S-specific Ab titer	*Bac-specific Ab titer*
S-vsvG	2560, 2560, 5120	2560, 5120, 5120
Bac	320, 320, 320	2560, 2560, 5120
Positive	1280	160

The end-point titers of each S-vsvG and unrecombiant baculoviruses (Bac) immunized serum against purified S protein or purified Bac were shown. SARS patient serume (positive) was used as a positive control for S-specific antibodies.

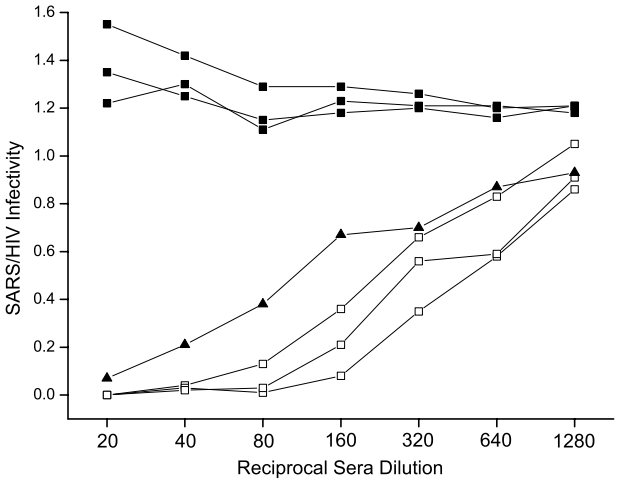

FIG. 4. The neutralizing activity of S-vsvG immunized mice sera for the SARS/HIV pseudovirus. The S-vsvG immunized mice sera (blank square) showed high neutralizing activity and efficiently inhibited SARS/HIV infectivity. Unrecombinant baculovirus (Bac) immunized mice serum (filled square) showed no inhibition effect. SARS patient serum (filled triangle) was used as a positive control.

DISCUSSION

Baculovirus expressing system had become widely used to express heterologous genes in cultured insect cells and insect larvae. As a protein expressing system, it had unique advantages such as modifications of the target protein, high level of expression, and limited host range, which made it safer to work with than most mammalian virus vectors. As a specialized baculovirus expressing system, the baculovirus display technology made it possible to produce recombinant baculoviruses incorporated with well-modified, correctly folded, and functionally active heterologous proteins on the surface. The displayed proteins were either used as effective immunogens, or used as specific ligands to enhance the transduction efficiency of mammalian cells (Raty *et al*., 2004; Kitagawa *et al*., 2005).

Here, we reported the successful baculovirus display of the SARS-CoV S protein. So far, this is the largest protein that reported to be displayed on baculovirus surface. Mice immunized with purified S-displayed baculoviruses exhibited effective production of specific antibody against S protein, and the antisera showed neutralizing activity to a SARS-CoV pseudovirus. Notably, the neutralizing titer level (1:320) of our S-displayed baculoviruses was similar to that of several reported candidate SARS-CoV vaccines (Yang *et al*., 2004; Faber *et al*., 2005), suggesting that the S-displayed baculovirus could be an effective live virus vaccine candidate for SARS-CoV. Considering the limited host range of baculovirus, the risk carried by S-displayed baculovirus vaccines were much lower than for most other mammalian virus vector vaccines and most widely used inactivated virus vaccines. In addition, by applying the similar display strategy to other baculoviruses, the recombinant viruses could be amplified in insect larvae such as silkworms to largely reduce the production cost of baculovirus vaccines. The intrinsic ability of baculoviruses to efficiently activate both primary and secondary immune responses would confer additional pro-

tection for vaccinees. In one report, the wild-type baculovirus induced strong innate immune response that helped the vaccinated mice survived from a lethal influenza challenge (Abe *et al*., 2003). Finally, as other virus-based vaccines, no adjuvant would be needed which simplified the vaccination procedure.

In the last few years, outbreaks of infectious animal diseases such as avian influenza and SARS have had huge impacts on pharmaceutical and biotechnology industries, and become a burden on the healthcare systems and economies of several nations. Dealing with outbreaks requires proper diagnosis, drug administration, and vaccination. Of these, only vaccination is preventive, and critical for the avoidance of pandemics of these diseases. Our work implied the baculovirus display system as a useful and safe method in novel vaccine research and development, a key point in minimizing the impact of similar viral infectious disease on human lives and the economy.

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