Efficient assembly and release of SARS coronavirus-like particles by a heterologous expression system

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Abstract Virus-like particles (VLPs) produced by recombinant expression of the major viral structural proteins could be an attractive method for severe acute respiratory syndrome (SARS) control. In this study, using the baculovirus system, we generated recombinant viruses that expressed S, E, M and N structural proteins of SARS-CoV either individually or simultaneously. The expression level, size and authenticity of each recombinant SARS-CoV protein were determined. In addition, immunofluorescence and FACS analysis confirmed the cell surface expression of the S protein. Co-infections of insect cells with two recombinant viruses demonstrated that M and E could assemble readily to form smooth surfaced VLPs. On the other hand, simultaneous high level expression of S, E and M by a single recombinant virus allowed the very efficient assembly and release of VLPs. These data demonstrate that the VLPs are morphological mimics of virion particles. The high level expression of VLPs with correct S protein conformation by a single recombinant baculovirus offers a potential candidate vaccine for SARS.
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1. Introduction

The severe acute respiratory syndrome (SARS)-associated coronavirus (SARS-CoV), also named infectious atypical pneumonia, is a newly emergent member in the family *Coronaviridae* [1]. SARS-CoV causes a transmissible febrile respiratory disease for which an effective and safe vaccine is required [2]. SARS-CoV is phylogenetically distinct from other characterized coronaviruses [3]. The genome is composed of a relatively conserved region encoding an RNA-dependent RNA polymerase and a variable region containing open reading frames encoding sequences for viral structural proteins: spike (S), envelope (E), membrane (M), and nucleocapsid (N) [4,5]. Like some other coronaviruses, the variable region of SARS-CoV contains additional open reading frames that encode putative non-structural proteins whose function is largely unknown [6].

In the *Coronaviridae* family, the envelope M protein is a triple-spanning membrane glycoprotein that interacts with the nucleocapsid and S protein during assembly [7]. Another component essential in the assembly process is the small E protein. This protein is generally a minor virion constituent. It is largely embedded within the viral membrane and only its hydrophilic carboxy terminus protrudes inside the virion [8,9].

The spike (S) protein, which is the second most abundant envelope protein, is a large type-I transmembrane glycoprotein that is responsible for receptor binding and membrane fusion [10]. In coronaviruses infection, the S protein plays an important role in the immune responses against the virus as neutralizing antibody, passive antibody protection, and cellular immunity have all been related to this protein [11]. However, multi-protein structures such as virus-like particles (VLPs) composed of all major structural proteins that mimic the organizations and conformations of authentic native particles but lack the viral genome would be more likely to stimulate stronger cellular and humoral immune responses compared to a single protein vaccine containing only the S protein. Indeed, we have demonstrated for another animal disease, bluetongue disease of sheep, that while single or combined protein vaccines were protective in sheep against virulent BTV infection, BTV-VLPs were much better vaccines [12,13].

During coronavirus assembly, the N protein, which is located inside the virion complexed with the viral RNA [14], leads to the formation of the helical nucleocapsid. The M protein is associated with specialized intracellular membrane structures, and interactions between the M and E proteins and nucleocapsids result in budding through the membrane [6]. The S protein is incorporated into the viral envelope, again by interaction with M, and mature virions are released from the plasma membrane [15].

Assembly of coronavirus capsid proteins by a heterologous mammalian expression system has been reported for mouse hepatitis virus [16–18] and the transmissible gastroenteritis virus of swine [19]. However, assembly and expression of three coronavirus membrane-associated proteins such as M, E and S proteins of SARS at a high level by using the heterologous insect cell protein expression system that was used to generate BTV-VLPs could be a challenging task in comparison to non-envelope BTV-VLPs.

In this report, we used single and multigene baculovirus expression vectors to express all four structural proteins of SARS-CoV and to investigate the assembly of VLPs as a first step to the development of VLP vaccine for SARS. Our studies here present clear evidence of the expression and spontaneous assembly of the three structural proteins in appropriate molar ratios, as well as of their release from the cell surface as VLPs.

2. Materials and methods

2.1. Cloning and construction of recombinant baculoviruses

The plasmid genes encoding the small envelope protein (E), the membrane protein (M), the nucleocapsid protein (N) and the major structural spike glycoprotein (S) of SARS cloned into a pCR-blunt-TopoII vector (Invitrogen, Inc.), were obtained from Dr. Kirill Kalnin (Acambis Inc., MA). The full-length M, N and E genes were amplified by polymerase chain reaction (PCR) using primers which included recognition sites for *Bam*HI. The amplified genes were cloned into the *Bam*HI site of the pAcYM1 vector under the control of the polyhedrin promoter. The full-length gene encoding the S protein was excised from pBlunt vector with an *Eco*RI restriction enzyme digest, coupled to *Bgl*II linkers and inserted into a *Bgl*II site of the baculovirus transfer vector pTriEx-1. The correct orientation of the insertions was examined by PCR and restriction enzyme analysis. The constructs were co-transfected with triple cut, linearized *Autographa californica* (AcNPV) DNA to produce recombinant baculoviruses that individually expressed the M, N, E and S proteins.

In order to obtain a single recombinant baculovirus that expressed the M, E and S proteins, two new constructs were generated. The first contained the S and E genes cloned into the *Bam*HI and *Bgl*II sites, respectively, of the pAcVC3 vector under the control of the polyhedrin promoter. For the second construct, the M gene was inserted into the *Bgl*II site under the control of the p10 promoter of a pAcP102x vector, which also contains the GUS gene to allow easy selection of double recombinants when transfected with AcNPV DNA in *Spodoptera frugiperda* (*Sf*9) insect cells.

2.2. Purification of VLPs

Assembled VLPs were isolated either from the cytoplasm of infected cells or from the culture medium. Infected cells were pelleted by centrifugation at 4000 rpm for 15 min, resuspended in TEN buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 1 M NaCl) with 1% Triton X-100 and dounce-homogenized on ice. After centrifugation (3500 rpm for 30 min), the clarified supernatant was centrifuged on a linear 30–45% (w/w) sucrose gradient in TEN buffer at 27 000 rpm for 3 h. The opalescent band containing the particles was clearly visible in the middle of the gradients. To isolate VLPs from the culture media of the infected cells, the media were clarified by centrifugation at 3500 rpm for 30 min and then centrifuged at 27 000 rpm for 50 min on a 15% (w/w) sucrose cushion in TEN buffer at 4 °C. The pelleted particles were recovered in TNE buffer (10 mM Tris–HCl, pH 7.4, 1 mM EDTA, and 0.1 M NaCl) and stored for analysis. Alternatively, VLPs were further purified on a linear sucrose gradient (30–45%, w/w) as above and recovered in TNE buffer to a final concentration of 1 μg/μl.

2.3. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

The *Sf*9 cells were harvested, lysed and resolved by 10% or 15% SDS–PAGE [20]. The gels were either stained with Coomassie brilliant blue or subjected to Western blotting (WB) analysis using appropriate SARS-CoV antibodies [21].

2.4. Electron microscopy (EM)

For negative staining, aliquots of the VLP samples were placed on plastic carbon-coated grids and stained with 2% phosphotungstic acid (pH 5.2). Immunogold labeling was performed on VLP samples as described previously [22]. For thin sectioning, infected cells were fixed, agar embedded and cut into smaller cubes. The cubes were embedded in epoxy resin and ultrathin sections were cut and mounted onto copper grids. All the samples were examined on a Jeol 1200EX transmission microscope.

2.5. Immunofluorescence and fluorescence-activated cell sorter (FACS) analysis

The *Sf*9 cells were grown in chamber slides and infected with the recombinant baculovirus at an MOI of 4 for 48 h, immunofluorescence analysis was performed as described previously [23] and examined on a Zeiss LSH 510 microscope. For FACS analysis, *Sf*9 cells were infected as mentioned above and the analysis was performed as described previously [23], on a Becton–Dickinson FACSCalibur analyser running CellQuest software (Becton–Dickinson).

3. Results

3.1. Generation of recombinant baculoviruses expressing individual SARS-CoV proteins: inter-protein interactions in the formation of capsid structures

To determine whether each of the major SARS-CoV proteins could be expressed by single recombinant baculoviruses, each structural gene was used to generate a recombinant baculovirus that expressed the S, E, M or N proteins individually in *Sf*9 cells. Selected recombinant viruses were amplified and the expression of SARS-CoV protein was analyzed by SDS–PAGE in *Sf*9 infected cells. Each recombinant virus synthesized a protein of the expected size of the SARS-CoV proteins S, N, M or E (Fig. 1A). In addition, the specificity and authenticity of each recombinant protein were confirmed by WB analysis with mouse anti-SARS polyclonal antibody (Fig. 1B).

To determine if these recombinant proteins could interact with each other and assemble into particulate structures, *Sf*9
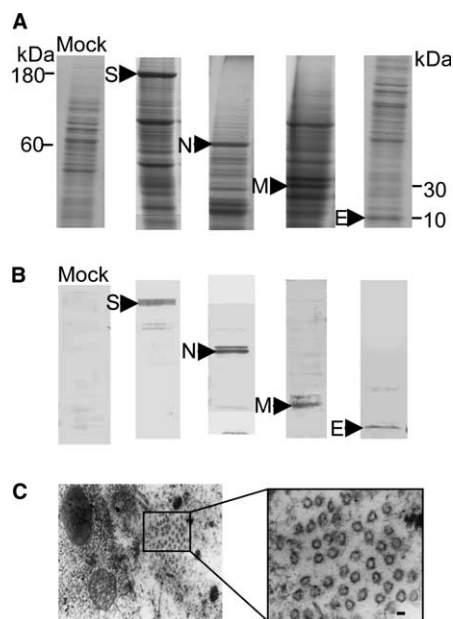

Fig. 1. Expression of S, N, M and E SARS proteins and detection of VLPs in *Sf*9 infected cells. *Sf*9 cells were infected with single recombinant baculoviruses at a MOI of 4 and analyzed by (A) SDS–PAGE followed by Coomassie blue staining and (B) WB analysis using mouse anti-SARS polyclonal antibody. The figure shows the clearly expressed SARS proteins when compared to the mock infected cells. It should be noted that the M protein appears as a double band in the gel and the larger form of the protein reacted poorly with our antibody. (C) EM analysis of negatively stained cell sections revealed the presence of VLPs. These particles were visible in the cytoplasm after 2 days co-infection with the M and E expressing recombinant baculoviruses. Bar = 100 nm.

cells were infected with recombinant baculoviruses expressing M, E or S alone or co-infected in combination of M and E; M and S or M, E and S. EM analysis of the negatively stained infected cell sections exhibited no particulate structures when each protein was expressed alone. However, cells infected with recombinant viruses expressing M and E had large numbers of particulate structures, with a diameter of approximately 100 nm, distributed throughout the cytoplasm (Fig. 1C). In contrast, no structures could be detected in the cells that were co-infected with recombinant viruses expressing M and S. The results demonstrate that the M and E proteins were sufficient for the assembly of particles but in the absence of the E protein, M and S could not assemble into VLPs. Cells co-infected with three different recombinant baculoviruses (M, E and S) resulted insufficient quantity of particles, assembled with three proteins, to be detectable by our assay system (data not shown).

To obtain purified VLPs containing M and E proteins, Sf9 cells were co-infected with two recombinant baculoviruses expressing M and E proteins, 48 h post-infection (PI) cells were lysed and VLPs were purified by sucrose gradient centrifugation. The assembled particles were isolated and the expression of SARS-CoV proteins was assessed by SDS–PAGE. Only two protein bands were visible, one of approximately 30 kDa in size, corresponding to the M protein and the other of 10 kDa in size identical to the E protein size. M protein runs slightly slower than the predicted size of 25 kDa in this analysis, probably due to the glycosylation of the protein on expression in the baculovirus system. The sucrose gradient purification revealed that the two SARS envelope proteins, M and E, could indeed self-assemble into particles (Fig. 2A). Importantly, WB analysis confirmed that the two visible bands were M and E proteins, which reacted with the mouse anti-SARS polyclonal antibody (Fig. 2B). To visualize the VLP morphology, the VLPs were examined by negative staining and EM. Smooth particles with approximate diameter of 100 nm were visualized, indicating that the VLPs were stable enough to tolerate the
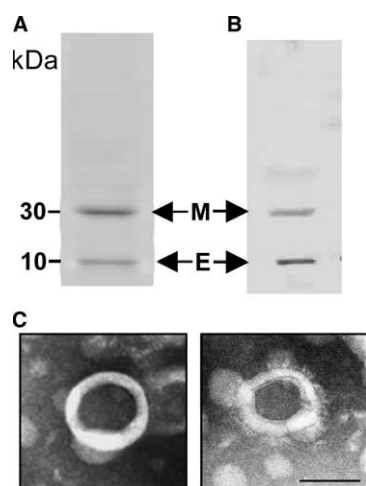
purification regime and the EM staining reagents (Fig. 2C). In the absence of S protein expression, there were no spike projections and this gave an even, smooth appearance to the spherical particles.

3.2. Generation of a recombinant baculovirus expressing three SARS proteins and demonstration of SARS-VLPs assembly and release

In order to enhance the VLP assembly, a triple recombinant baculovirus that would express S, M and E proteins was generated. When protein expression by this recombinant virus was assessed by SDS–PAGE, very high levels of expression of SARS proteins, particularly S and M proteins, were achieved approaching 500 μg recombinant protein per 1×10^6 infected cells (Fig. 3A). WB analysis of the infected cell lysate using a mouse anti-SARS polyclonal antibody revealed the presence of all three SARS-CoV proteins (Fig. 3B).

By immunofluorescence, we examined the SARS-CoV S protein for expression on the surface of the Sf9 cells when infected with the triple recombinant baculovirus. The S glycoprotein was observed at the periphery of the fixed infected cells, demonstrating the distribution of the S protein on the plasma membrane (Fig. 3C). Also, FACS analysis, using anti-S monoclonal antibody, clearly demonstrated the expression of the S protein on the live cell surface in comparison to the uninfected control cells (data not shown).

To discern if assembled VLPs, containing the S envelope glycoprotein, were indeed released from the infected cell, mimicking the coronavirus morphogenesis, Sf9 cells were infected with the triple recombinant baculovirus and were harvested at a time course of 48, 72 and 96 h PI. Both the cell lysates and culture medium were examined for the presence of VLPs. All three proteins were visible in both cell lysates and in
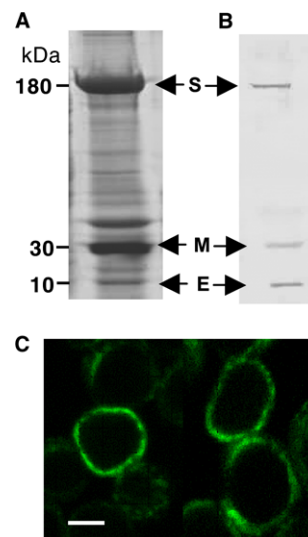
Fig. 2. Analysis of VLPs formed by Sf9 cells co-infected with M and E expressing recombinant baculoviruses. The VLPs were purified using a sucrose gradient and the protein composition was determined by (A) SDS–PAGE followed by Coomassie blue staining and (B) WB analysis using a mouse anti-SARS polyclonal antibody. (C) EM of negatively stained smooth SARS VLPs purified by sucrose gradient. Bar = 100 nm.

Fig. 3. Expression of S, M and E SARS proteins in Sf9 infected cells. To examine protein expression levels, the cells were infected with the triple recombinant baculovirus at a MOI of 4 and analyzed by (A) SDS–PAGE followed by Coomassie blue staining and (B) WB analysis using mouse anti-SARS polyclonal antibody. (C) Immunofluorescence analysis of fixed cells infected with the triple recombinant baculoviruses. Expression of the SARS S glycoprotein was detected with a primary anti-S monoclonal antibody and secondary anti-mouse FITC-conjugated antibody, on the plasma membrane of infected cells. Bar = 10 μm.

the media from 48 h onwards; particularly high level of S protein was present in the cells both at 48 and 72 h, but not in the medium. However, at 96 h larger amounts of proteins were synthesized and released into the medium (Fig. 4). Therefore, assembled VLPs released at 96 h PI were subjected to further investigation.

The SDS–PAGE analysis of putative VLPs purified from the supernatant showed three distinct protein bands of approximately 180, 30 and 10 kDa corresponding to the S, M and E SARS-CoV proteins, respectively (Fig. 5A). The expression of authentic proteins was subsequently confirmed by WB analysis, using a mouse anti-S polyclonal antibody (Fig. 5B).

In order to investigate whether the N protein could be incorporated within the VLPs, Sf9 cells were co-infected with the triple recombinant baculovirus together with the single recombinant virus expressing the N protein. Despite the fact that the N protein was expressed in the infected Sf9 cells, the examination of the purified VLPs released into the media 96 h PI still had only S, M and E proteins but did not exhibit the presence of the N protein (data not shown).

3.3. Morphology of released VLPs and the presence of S protein on the particles

We used EM analysis, to examine if the morphology of VLPs that consist of S protein in addition to the M and E proteins is different to the VLPs formed by only the M and E proteins. The negatively stained VLPs exhibited a very different morphology to that of the M and E. VLPs with the S protein had a spherical morphology with distinctive fine spike projections (Fig. 5C). Clearly, the three SARS-CoV proteins synthesized by a triple recombinant baculovirus in insect cells had interacted with each other and assembled into VLPs that were subsequently released from these cells. Thus, the VLPs not only closely resembled SARS-CoV virus in size and particle morphology but also the virus morphogenesis. The yield of VLP from infected cells was approximately 200 μg VLP per 1×10^9 infected cells.

The display of spikes ornamenting the surface of the VLPs was clearly visible as distinct projections and the presence of the S protein was confirmed by immunogold labeling (Fig. 5D). We were not able to identify the M protein by immunogold labeling and EM, perhaps due to low abundance of the M protein on the VLPs surface or inaccessibility of the M epitopes. However, the presence of the M protein was estab-
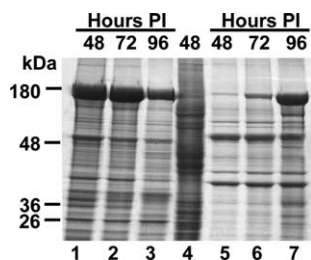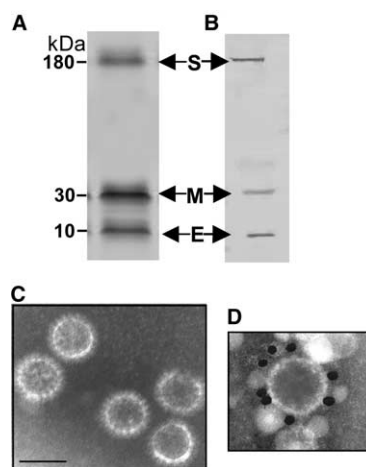

Fig. 5. Analysis of VLPs released from Sf9 cells infected with the triple recombinant baculovirus. Sucrose gradient purified VLPs from the supernatant of infected cells were analyzed by (A) SDS–PAGE followed by Coomassie blue staining and (B) WB analysis using a mouse anti-SARS polyclonal antibody. This figure shows the protein bands of the S, M and E SARS proteins. (C) EM of the negatively stained spiky SARS-VLPs purified by sucrose gradient from the culture medium of infected cells. Bar = 100 nm. (D) Electron micrograph of immunogold-labeled SARS-VLPs. The VLPs were probed using an anti-S monoclonal antibody counterstained with gold spheres coupled to anti-mouse IgG.

lished by the fact that the M protein is absolutely necessary in the organization of the viral envelope during assembly [7].

4. Discussion

In this paper we describe the production of SARS-CoV-like particles, by expression of the viral structural proteins in the baculovirus system. By co-expression of the M and E proteins we were able to produce well assembled, spikeless but stable VLPs. These smooth surfaced particles are probably an intermediate particle in virus assembly. The formation of VLPs by these two proteins has been reported for other coronaviruses [16–19], and a very recent report has been published regarding the production of SARS-VLPs by multiple co-infection with single baculoviruses expressing M, E and S proteins individually. In this report, the VLPs did not bud from the insect infected cells but instead they were isolated from cell lysates [24]. However, when we co-infected Sf9 cells with three recombinant viruses expressing M, E and S proteins, we were not able to generate stable VLPs with the spike protein on the surface that could be isolated from culture medium. For this reason and because of the inefficiency of the assembly process by co-infection with several recombinant baculoviruses, we generated a single recombinant baculovirus that expressed all three SARS proteins simultaneously. This, in turn, means that every infected cell in a culture receives all of the structural proteins of the virus and expresses them all at the same time. This feature is particularly important for the efficient assembly of multi-protein complexes where simultaneous infection with multiple baculoviruses is essential.

This is the first report describing the co-expression of the three SARS proteins (M, E and S) at very high level from a

Fig. 4. Time course of SARS-VLPs release from Sf9 infected cells. Sf9 cells were infected with the triple recombinant baculovirus at a MOI of 4 and at the indicated times cell lysates (lanes 1–3) and cell culture medium (lanes 5–7) were analyzed by SDS–PAGE followed by Coomassie blue staining. This figure shows the location of the VLPs for purification procedures. Lane 4, Sf9 mock infected control.

single recombinant baculovirus that allows spontaneous assembly of highly stable VLPs. Glycosylated S protein formed very distinct spikes on the surface of the VLPs as can be observed in Fig. 5C. VLPs budded from the plasma membrane of infected cells, similar to the authentic virions, allowing purification from the culture medium.

Previous studies on coronaviruses have indicated that the production of VLPs is dependent on the expression of the M and E proteins, but that the infectivity of VLPs is dependent on S protein [25]. The spike protein is the receptor binding protein, it has a crucial role during infection of cells and it is the main target for a protective humoral immune response [26]. In our study, the expression of the S protein was very high and the incorporation of S glycoprotein onto the surface of the VLPs makes these particles not only a powerful instrument for studies of the assembly and budding of SARS-CoV particles but also as a candidate vaccine and a tool for SARS diagnosis. Self-assembly of viral capsid proteins into VLPs for both RNA and DNA viruses has been reported and VLPs produced by this system usually retain the immunogenic and physiochemical properties of the native virions [27–32].

In our analysis, the E protein was only expressed in small amounts, although this protein was essential for the formation of VLPs. Thus, we can hypothesize that as with other coronaviruses [18,33,34], the protein component of these particles essentially consists of a matrix of laterally interacting M proteins, which in some way require the E protein for budding and, if available, the S protein is incorporated by specific interactions with M. We showed that the VLPs that were formed consisted of only the M, E and S proteins but not the N protein, indicating that N protein was not able to be incorporated. However, it was not unexpected that the SARS-CoV like particles were formed without the inclusion of nucleocapsid protein, as other coronaviruses also form VLPs in the absence of the N protein [16].

The approach presented here will allow us to further study the virus neutralization properties of SARS-CoV VLPs, as well as viral morphogenesis. Our data suggest that the VLPs hold promise for creating novel recombinant vaccine against SARS and possibly for other pathogens, in addition to understanding the protein–protein interactions in virus assembly.

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