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Short communication

VP1 protein of *Foot-and-mouth disease virus* (FMDV) impairs baculovirus surface display

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ABSTRACT

The *Foot-and-mouth disease virus* (FMDV) causes important economical losses in livestock farming. In order to develop a novel subunit vaccine against FMDV, we constructed recombinant baculoviruses that display the protein VP1 of FMDV on their surface, with either polar (fused to gp64) or nonpolar (fused to anchor membrane from VSV-G protein) distribution. Insect cells infected with the different recombinant baculoviruses expressed VP1 fusion protein to high levels. However, the recombinant VP1 protein was not carried by budded virions. Subcellular localization of VP1 revealed that the trafficking of the fusion protein to the cell plasma membrane was impaired. Our results suggest that VP1 contains cryptic domains that interfere with protein secretion and subsequent incorporation into budded baculoviruses.

Foot-and-mouth disease (FMD) is the most economically important disease of cattle and other farm animals. Its etiological agent is the *Foot-and-mouth disease virus* (FMDV) that belongs to the *Aphtovirus* genus of the *Picornaviridae* family (Rueckert, 1985). This disease is enzootic in many areas of the world and its control is based on the slaughter of infected and contacted animals, and on regular vaccination with inactivated virus-based vaccines (Pereira, 1981). There are a number of concerns about the biological safety of such vaccines that have encouraged efforts to develop alternative vaccines using genetic engineering technology.

Viral particles and virus-like particles are particularly effective carriers of antigens, whereby numerous identical copies of the antigen are attached to a particulate carrier. In this way, several viruses from animal (Ferstandig et al., 1994; Kit et al., 1991; Kushnir et al., 2012; Stebbings et al., 2012; Zhang, 2012) and plant (Fernandez-Fernandez et al., 1998; Joelson et al., 1997; Porta et al., 1996; Turpen et al., 1995) have been used as carriers to develop candidate subunit vaccines that express multiple antigenic sites or complex proteins in particles.

The baculovirus display strategy has several advantages as a vaccine vehicle. The use of purified virions as immunogens alleviates the need for additional adjuvants in the vaccine formulation due to the intrinsic immunostimulatory effect often associated with baculoviral immunogens (Abe et al., 2003, 2005; Molinari et al., 2010). Additionally, the presentation of the antigen in its native conformation on the baculovirus surface makes it readily accessible for interactions with cellular components of the immune system. In fact, several pathogen antigens have been successfully displayed using this system and some of them induced high titers of antigen-specific antibodies (Boublik et al., 1995; Kaba et al., 2003; Lin et al., 2008; Peralta et al., 2007; Syed et al., 2009; Tami et al., 2000, 2004).

The classical baculovirus surface display method is based on the expression of foreign proteins fused to the baculovirus gp64 envelope glycoprotein (Whitford et al., 1989). The foreign peptide is inserted between the signal peptide and mature domain of gp64. As a result, the fusion protein is translocated to the plasma membrane and incorporated into the baculovirus envelope, along with native gp64 peptides, in a pole of the virion (Oker-Blom et al., 2003). Another strategy developed to express a foreign protein on baculovirus surface consists in the expression of the foreign peptide inserted between signal peptide of gp64 and the membrane anchor sequence of *Vesicular stomatitis virus* (VSV) G protein (Chapple and Jones, 2002). In this way, the fusion protein acquires a non-polar distribution on the surface of baculovirus (Oker-Blom et al., 2003).

We have previously reported the construction and characterization of two recombinant baculovirus which carry antigens derived from FMDV serotype C: AcSupA which exposes the peptide SiteA (a highly antigenic portion of VP1 that elicits neutralizing antibodies against FMDV) on its surface, and AcSupP1 which exposes the 73 kDa polyprotein P1 of FMDV (that contains a full VP1 copy) on its surface (Tami et al., 2000, 2004). Until now, 5 antigenic sites have been identified in P1 (Crowther et al., 1993; Kitson et al., 1990; Thomas et al., 1988) that contribute to the induction of an immune response against FMDV. Because of the poor efficiency of incorporation of P1 on AcSupP1 surface, and since VP1 protein comprises 3 (including site A) of the 5 antigenic sites in P1, we decided

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to develop a new generation vaccine for FMDV using the full VP1 protein.

We took advantage of our previous experience in the baculovirus technology (Peralta et al., 2007; Tami et al., 2000, 2004) and constructed two recombinant baculoviruses, AcSupVP1 and AcSDVP1, designed to expose the full VP1 protein on the virions surface. First, the coding sequence of VP1 protein of FMDV serotype O1-Campos was amplified using primers SupVP1For (5′agaattctgaccactgcgggc3′) and SupVP1Rev (5′agaattccaaagtctgtttcacagg3′). This sequence was cloned into pVLSup vector (Tami et al., 2000) between the signal sequence and the mature protein domain of gp64. The resulting plasmid was used to obtain the recombinant baculovirus AcSupVP1 following the methodology described by Tami et al. (2000). In addition, a recombinant baculovirus AcSupA that carries the dominant immune epitope (antigenic site A) of FMDV (comprised between amino acids 135 and 160 of VP1 protein) was similarly constructed.

Sopodoptera frugiperda Sf9 cells were infected with each recombinant baculovirus or an AcNPV wild type virus (wt) and subsequently harvested 4 dpi. The presence of FMDV-gp64 fusion proteins was analyzed by Western blot with a monoclonal antibody (MAb) directed against the site A of FMDV VP1 serotype O1campos (Fig. 1A, upper left panel). A MAb AcV5 was used to detect wild type and recombinant gp64 proteins. As seen in the upper-left and upper-right panels of Fig. 1A, the recombinant proteins gp64-A and gp64-VP1 were properly expressed and had the expected molecular weights (66 kDa and 86 kDa, respectively).

To determine if the fusion proteins were incorporated on the surface of recombinant baculoviruses, the supernatants of the infected cells were harvested, clarified and concentrated by ultracentrifugation (82,000 × *g*, Beckman). The pellets were resuspended in PBS and analyzed by Western blot. The results showed that both recombinant baculoviruses contained wt gp64 protein (Fig. 1A, lower right panel). However, gp64-VP1 fusion protein was not incorporated into AcSupVP1 virions even when gp64-A fusion protein, that contains VP1 epitope A, was effectively incorporated into the AcSupA recombinant baculovirus (Fig. 1A, lower left panel). This unexpected result can not be explained by the size of the VP1 insert because larger proteins have been successfully expressed and incorporated into recombinant baculoviruses surface (Peralta et al., 2007; Tami et al., 2000; Yoshida et al., 2003). Furthermore, Meng et al. (2011) were able to express the VP1 protein from Enterovirus 71 (which also belongs to the *Picornaviridae* family) on the baculovirus surface by fusion to gp64, although Enterovirus71 and

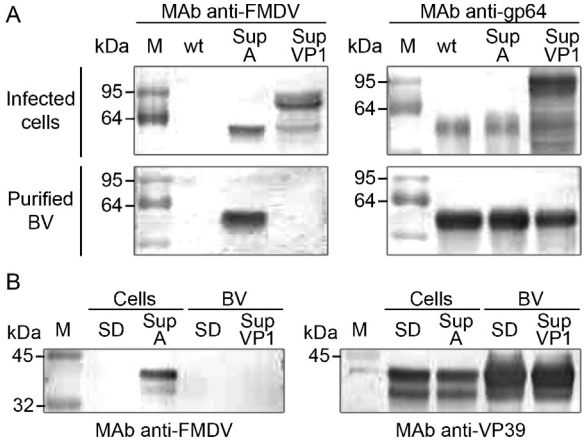

Fig. 1. Detection of recombinant proteins in infected cells and purified baculovirus by Western blot. (A) Reaction of fusion proteins gp64-VP1, gp64-siteA and wild type gp64 using a MAb anti-FMDV and the MAb AcV5 (anti-gp64). (B) Detection of fusion protein SD-VP1 by the MAb anti-FMDV and the baculoviral protein vp39 using a specific MAb.

FMDV VP1 proteins share less than 20% amino acid identity (data not shown).

This fact led us to hypothesize that perhaps there is an intramolecular interaction between gp64 and FMDV-VP1 which may prevent the correct processing of the fusion protein. Therefore, pVLSup vector was modified to express recombinant antigens in a non-polar way on baculovirus surface. The coding sequence of the mature protein domain of gp64 was replaced by the transmembrane and cytoplasmic terminal domain of VSV G protein coded in the position 4401–4613 bp of VSV genome (Rose and Gallione, 1981). The resulting plasmid pVL-SDVP1 was used to obtain the recombinant baculovirus AcSDVP1 by the methodology described above (Tami et al., 2000). As a control, AcSD recombinant baculovirus that lacks the VP1 insert was constructed. Next, the expression of the fusion protein and its localization into the virus was analyzed by Western blot. The right panel of Fig. 1B shows the presence of viral proteins in all samples analyzed as revealed with an anti-vp39 antibody. However, as seen in the left panel of Fig. 1B, the VP1-VSV fusion protein (36 kDa) was only present in cells infected with the AcSDVP1 baculovirus and was not incorporated into the virion. In sum, neither VP1-gp64 nor VP1-VSV fusion proteins were able to reach the virion of recombinant baculoviruses. These results indicate that the inability of recombinant baculoviruses to incorporate VP1 fusion proteins is due to an intrinsic property of this protein rather than an incompatibility with the fusion partner.

To discard the possibility that the impediment to incorporate the fusion protein into the virion was due to a particular characteristic of VP1 derived from O1-Campos serotype, we also performed baculovirus display for VP1 proteins derived from serotypes C3-Argentina85 and A-Argentina2001. In those cases, we obtained similar results (data not show).

In silico analysis of gp64VP1 and SD-VP1 sequences with different subcellular localization predictors (e.g. TargetP from http://www.cbs.dtu.dk/services) failed to identify potential cryptic signal for subcellular retention. In fact, the programs predicted that all fusions are targeted to the secretory pathway, resulting in plasma membrane exposure. This led us to investigate if the fusion proteins reached the plasma membrane but, for some reason, failed to incorporate into the virion at the budding moment. Cells infected with the baculovirus AcSupVP1 or AcSupA were subjected to inmmunofluorescence according to Alfonso et al. (2012) using a primary mouse monoclonal antibody anti-FMDV (1/50 dilution) followed by an anti-mouse secondary antibody conjugated to Alexxa488 (Invitrogen) (1/800 dilution). The samples were then analyzed by confocal microscopy (TCS-SP5, Leica) to determine the subcellular location of the fusion proteins. Both fusion proteins were detected at high levels in the perinuclear region of infected cells, but only gp64-A protein was observed on the cell plasma membrane (Fig. 2A, upper panels). To rule out the possibility that gp64-VP1 would not be observed in the plasma membrane due to interference of the high level of fluorescence in the interior of the cell, we performed an additional immunofluorescence experiment without permeabilizing the cells prior to staining. Consistent with the previous experiment, it was possible to detect gp64-A protein on the surface of infected cells. In contrast gp64-VP1 expressing cells were only non-specifically stained in the cytoplasm. The results indicate that gp64-VP1 was absent in the budded virions due to an inability to reach the cell plasma membrane rather than an impediment to incorporate into de virions.

Finally, we analyzed the subcellular localization of retained gp64-VP1 protein. For this, we performed co-localization experiments using different plasmids encoding for insect cells organelle markers fused to mCherry red fluorescent protein that has been

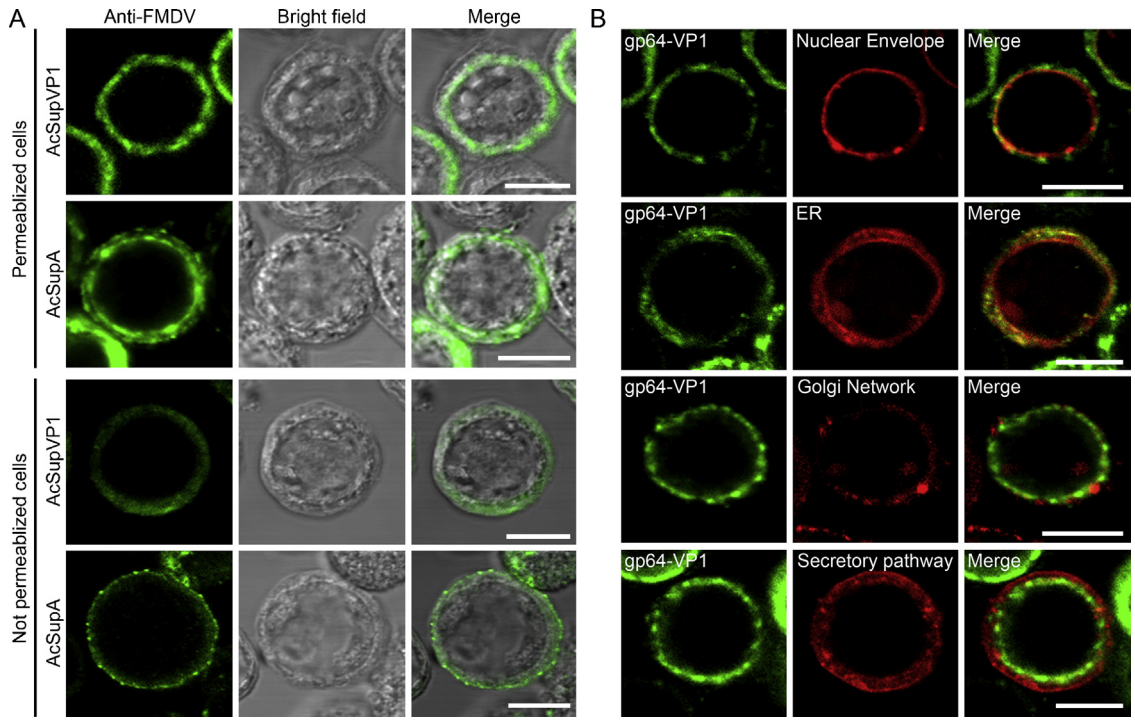

Fig. 2. Subcellular localization of the fusion protein gp64-VP1 in Sf9 infected cells. (A) Sf9 insect cells were infected with recombinant baculoviruses AcSupVP1 or AcSupA, and 60 h later they were fixed, permeabilized or not as indicated and subjected to immunofluorescence with an anti-FMDV MAb and an Alexa FluorH 488-conjugated anti-mouse secondary antibody. Samples were then analyzed by confocal microscopy (B) Co-localization of gp64-VP1 with cellular markers. Sf9 cells were transfected with mCherry-based nuclear envelope, endoplasmic reticulum (ER), Golgi network or secretory pathway cellular marker plasmids (Maroniche et al., 2011) and infected with AcSupVP1 24 h after transfection. After 60 h, the subcellular localization of the fusion protein gp64-VP1 was revealed by inmmunofluorescence as explained before. Samples were then analyzed by confocal microscopy. White bars represent 10 μm.

previously developed by Maroniche et al. (2011). Sf9 cells were transfected with the organelle marker constructs using Cellfectin II (Invitrogen). At 24 h post-transfection, cells were infected with the recombinant virus AcSupVP1 or AcSupA at moi = 1. At 48 hpi, the cells were processed for immunofluorescence as explained above. Upon confocal microscopy analysis, we observed that gp64-VP1 is located outside the nuclear envelope in membranous-like structures that do not co-localize with the Golgi network or the secretory pathway markers (Fig. 2B). Unexpectedly, the subcellular localization of the endoplasmic reticulum (ER) marker resulted altered during late baculovirus infection in comparison with uninfected cells (Supplementary Fig. 1); however, at earlier infection times we observed some cells where the gp64-VP1 fusion protein partially co-localized with the ER marker. Although these experiments were not conclusive, we speculate that gp64-VP1, as well as gp64-A, is forced to accumulate in ER-derived membranous structures due to its over-expression, because immunofluorescence of these structures is similar in both cases. However, unlike gp64-A, gp64-VP1 is unable to continue trafficking to the plasma membrane due to a yet unknown property of the VP1 protein that interferes with the correct secretion of the fusion protein. Moreover, the interference made by VP1 seems to arise in its mature conformational state since it does not affect trafficking when forming part of the FMDV P1 polyprotein (Tami et al., 2000, 2004).

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.03.018.

The results obtained in this study lead us to conclude that, although we were able to construct a recombinant version of VP1 with appropriate signal peptide and cell membrane anchor intended for subsequent incorporation on the surface of baculovirus, it is necessary to characterize potential cryptic regions within VP1 that interfere with secretion and subsequent incorporation into budded baculoviruses before developing a VP1 based vaccine against FMDV using the baculovirus display method.

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