Mutagenesis of the palmitoylation site in vaccinia virus envelope glycoprotein B5

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The outer envelope of vaccinia virus extracellular virions is derived from intracellular membranes that, at late times in infection, are enriched in several virus-encoded proteins. Although palmitoylation is common in vaccinia virus envelope proteins, little is known about the role of palmitoylation in the biogenesis of the enveloped virus. We have studied the palmitoylation of B5, a 42 kDa type I transmembrane glycoprotein comprising a large ectodomain and a short (17 aa) cytoplasmic tail. Mutation of two cysteine residues located in the cytoplasmic tail in close proximity to the transmembrane domain abrogated palmitoylation of the protein. Virus mutants expressing non-palmitoylated versions of B5 and/or lacking most of the cytoplasmic tail were isolated and characterized. Cell-to-cell virus transmission and extracellular virus formation were only slightly affected by those mutations. Notably, B5 versions lacking palmitate showed decreased interactions with proteins A33 and F13, but were still incorporated into the virus envelope. Expression of mutated B5 by transfection into uninfected cells showed that both the cytoplasmic tail and palmitate have a role in the intracellular transport of B5. These results indicate that the C-terminal portion of protein B5, while involved in protein transport and in protein–protein interactions, is broadly dispensable for the formation and egress of infectious extracellular virus and for virus transmission.

INTRODUCTION

The Poxviridae is a family of complex, dsDNA-containing viruses whose replication cycle takes place in the cell cytoplasm (Moss, 2007). Poxviruses are assembled and released by a complex process involving several stages, including the non-infectious immature virus (IV), the intracellular mature virus (IMV), the intracellular enveloped virus (IEV), the cell-associated enveloped virus (CEV) and the extracellular enveloped virus (EEV) (Moss, 2007; Roberts & Smith, 2008; Smith & Law, 2004).

IMV particles are cytosolic and remain intracellular until cells are lysed. The final virus envelopment occurs after some IMV are transported from the assembly areas and become wrapped by a double layer of intracellular membranes derived from the early endosomes (Tooze et al., 1993; van Eijl et al., 2002) or trans-Golgi network (Schmelz et al., 1994). The resulting IEV is transported to the cell periphery, where the outer membrane fuses with the plasma membrane. This process leads to the export of enveloped virions to the extracellular space, where virus particles may remain attached to the plasma membrane as CEV or be released from the cell as EEV (Blasco & Moss, 1992). In cell culture, enveloped virions (CEV plus EEV) are responsible for virus transmission, as mutants that form normal amounts of IMV but are blocked in virus wrapping are impaired in transmission (Blasco & Moss, 1991). Specific roles have been assigned to CEV and EEV in virus transmission. CEV particles can induce the formation of actin tails that are important for efficient cell-to-cell spread. Conversely, EEV mediate long-range dissemination of virus (Blasco & Moss, 1992; Boulter & Appleyard, 1973; Payne, 1980).

At least nine virus-encoded proteins are present in the EEV or IEV envelope. Of these, A33 (Roper et al., 1996), A34 (Duncan & Smith, 1992), B5 (Engelstad et al., 1992; Isacs et al., 2002), F13 (Hirt et al., 1986) and A56 (Shida, 1986) proteins are present in IEV, CEV and EEV. Protein A56, the virus haemagglutinin (and probably K2, which forms a complex with A56), is an atypical EEV membrane protein, as it is not enriched in enveloped virions with respect to plasma-membrane levels and is absent in approximately one-third of EEV particles (Ichihashi & Dales, 1971; Krauss et al., 2002; Lorenzo et al., 2000; Payne, 1979). In addition to the above, some proteins are present in IEV but absent from CEV and EEV, like proteins A36 and a complex between F12 and E2 (Dodding et al., 2009; Parkinson & Smith, 1994; van Eijl et al., 2000, 2002; Zhang et al., 2000).

The function of the IEV and EEV envelope proteins in the morphogenesis and spread of vaccinia virus has been studied in detail using genetic approaches (Smith et al., 2002). For instance, proteins B5 and F13 are required for efficient and complete wrapping of IMV. Other proteins, such as F12 and A36R, are key mediators of IEV transport to the cell surface or the induction of actin tails.
B5 is a 42 kDa type I glycoprotein with a large extracellular domain mostly composed of four short consensus repeats (SCRs) typical of complement-control proteins (Engelstad et al., 1992; Isaacs et al., 1992; Takahashi-Nishimaki et al., 1991). Over the years, the list of functions of B5 has expanded, including roles in virus wrapping (Engelstad & Smith, 1993; Wolfe et al., 1993), CEV retention (Herrera et al., 1998; Katz et al., 2002), actin-tail formation (Herrera et al., 1998; Mathew et al., 1998; Newsome et al., 2004) and in the dissolution of the EEV membrane prior to infection (Law et al., 2006; Roberts et al., 2009). In addition, B5 has been shown to be the primary target of EV-neutralizing antibodies (Bell et al., 2004) and to be important for virus neutralization and in vivo protection (Bell et al., 2004; Benhnia et al., 2009; Chen et al., 2006; Putz et al., 2006). B5 has been studied in detail regarding its interaction with other envelope proteins and its intracellular transport (Lorenzo et al., 2000; Mathew et al., 1999, 2001; Perdiguer & Blasco, 2006; Perdiguer et al., 2008; Rodger & Smith, 2002; Ward & Moss, 2000).

At least four of the vaccinia envelope proteins (F13, B5, A33 and A36) are modified by palmitoylation (Grosenbach et al., 2000). Protein S-palmitoylation, the most common form of protein S-acylation, is the post-translational addition of a 16-carbon fatty acid to the side chain of cysteine, forming a thioester linkage. This modification is reversible and in many instances acts as a dynamic mechanism to control protein function, protein transport, protein–protein or protein–membrane interactions. The observation that palmitoylation is so widespread in envelope proteins suggests that this modification might have a role in the biogenesis of the envelope. In this work, we have carried out a mutagenesis study of the potential palmitoylation sites in protein B5.

RESULTS

Prediction of palmitoylation sites in the B5 sequence

In transmembrane proteins, palmitoylation usually takes place in cysteines located in the vicinity of the cytosolic face of the membrane. Inspection of the sequence of protein B5 indicated that residues Cys 301 and Cys 303 lie close to the boundary between the transmembrane domain and the cytoplasmic tail, in a position optimal for palmitoylation. Previously, it has been suggested that these cysteine residues could be palmitoylated, although individual mutation of either cysteine residue did not block palmitate incorporation (Grosenbach et al., 2000).

Palmitoylation sites can be predicted based on sequence analysis with a high success rate using several algorithms. Among these, we have used the programs CSS-Palm (Ren et al., 2008) and CKSAAP-Palm (Wang et al., 2009) to predict potential palmitoylation sites in the B5 protein sequence. Using either algorithm, Cys 301 and 303 were predicted as the preferred palmitoylation sites, being the residues with highest scores (not shown). Additionally, the programs identified other two cysteine residues (Cys 11 and 21) as potential secondary palmitoylation sites. However, we consider it unlikely that residues 11 and 21 are palmitoylated, as they are part of the signal peptide and the ectodomain of B5, respectively.

Construction of B5 mutant versions

To confirm experimentally the two palmitoylation sites predicted in the B5 sequence and to study the functional implications of B5 palmitoylation, we sought to generate a non-palmitoylated (np) version of B5. With this aim, the two candidate cysteine residues (positions 301 and 303) in the B5 cytoplasmic tail were mutated to serine. The mutations were introduced into the complete B5 and into a version of B5 lacking most of the cytoplasmic tail (residues 306–317). The mutated versions were named by specifying the domains of B5 maintained, adding np to versions with Cys 301, 303 mutated to Ser. Domains are denoted R (SCR repeats domain), S (stalk region), T (transmembrane domain) and C (cytoplasmic domain). Thus, RSTC is the complete version, RST is the B5 version lacking the cytoplasmic tail, and RSTCnp and RSTnp are the respective versions with the 301, 303 Cys-to-Ser mutations. In addition to the complete coding sequence of the gene and to facilitate detection, a V5 epitope tag was appended to the position corresponding to the N terminus of the mature protein, i.e. immediately adjacent to the signal-peptide cleavage site. The different B5 versions were inserted in the normal B5R locus by recombination into the genome of a B5R deletion mutant (v-ΔB5). Thus, they are expressed from the authentic B5 promoter, merely being modified by the inclusion of the V5 epitope in the extracellular domain. A schematic view of the different B5 versions and their sequence around the transmembrane domain are shown in Fig. 1.

Palmitoylation is known to modulate stability of a number of proteins. Because of this, we checked the lack of effect of the mutations on B5 accumulation by comparing B5 levels at late times post-infection (p.i.) by Western blotting (not shown).

Incorporation of palmitate into mutated B5

To test whether cysteine residues 301 and 303 are required for the addition of palmitate to B5, we carried out metabolic-labelling experiments in cells infected with the mutant viruses. Infection was carried out in culture medium containing [3H]palmitate and, at 9.5 h p.i., cell extracts were prepared and analysed by SDS-PAGE (Fig. 2). In agreement with previous reports (Child & Hruby, 1992; Grosenbach et al., 2000), the most intensely labelled proteins corresponded to the 37–42 kDa region. These bands probably reflected incorporation of [3H]palmitate into the B5 and F13 proteins, as one of the two bands was
absent in cells infected with the corresponding deletion mutant (Fig. 2). The most intense band, with an apparent molecular mass of 38 kDa, was present in cell extracts from v-ΔB5R-infected cells, but was absent in those from v-ΔF13L-infected cells, and thus presumably corresponds to palmitoylated F13 protein. A labelled protein with a slightly larger apparent molecular mass was also detected in v-ΔF13L-infected cell extracts. This protein is probably protein B5, as it was absent in cells infected with v-ΔB5R virus. Further identification of \[^{3}H\]palmitate-labelled proteins was obtained after immunoprecipitation with mAbs to F13 or B5 (Fig. 2, lower panels). We detected a minor, 23–28 kDa protein that incorporated \[^{3}H\]palmitate and co-immunoprecipitated with B5. This protein could be A33, as it is known to be palmitoylated and forms a complex with B5 (Grosenbach et al., 2000; Perdiguer & Blasco, 2006).

Palmitate was incorporated in B5 version RSTC, indicating that the V5 epitope did not interfere with the incorporation process. Also, B5 appeared to be labelled efficiently in cells infected with the v-ΔF13L virus, indicating that F13 is not necessary for palmitoylation of B5. Notably, complete absence of palmitate incorporation was evident in the B5 version with Cys 301 and 303 mutated to serine (versions RSTCnp and RSTnp), providing direct evidence that, collectively, Cys residues 301 and 303 are necessary for palmitoylation of B5.

**Virus transmission**

To assess the effect of the above mutations on virus transmissibility, we measured the formation of extracellular virus and the plaque phenotype of the different virus mutants (Fig. 3). Viruses expressing palmitoylated versions of B5 (RSTC and RST) formed large virus plaques in a standard 2 day plaque assay. These were approximately 7-fold larger than plaques formed by v-ΔB5R, and similar in size to those of the parental Western Reserve (WR) strain. Non-palmitoylated versions RSTCnp and RSTnp also formed large plaques. However, quantification of the...
plaque area showed that a slight decrease in plaque size was derived from the mutations of the two cysteines (Fig. 3b). This small effect was significant both in the complete B5 version RSTC ($n=67$, $P<0.05$) and in the version lacking the cytoplasmic tail, RST ($n=69$, $P<0.01$). These results correlated with measurements of extracellular virus formation by the different viruses (Fig. 3c). Virus titres in the medium of BSC-1-infected cells decreased by about 2-fold in the non-palmitoylated versions with respect to their palmitoylated counterparts.

**Interaction of mutant B5 with other envelope proteins**

B5 is known to interact with other envelope proteins, such as F13, A34 and A33. To test whether palmitoylation of B5 is involved in the establishment of those protein–protein interactions, extracts of cells infected with the virus mutants were subjected to immunoprecipitation with anti-B5 mAb, followed by Western blotting with different antibodies (Fig. 4). Clearly, palmitoylation of B5 was not required for its interaction with A34, as RSTCnp co-immunoprecipitated with A34 as efficiently as the complete version. Also, the cytoplasmic tail of B5 could be removed completely without affecting the B5–A34 interaction.

In contrast, a decrease in the immunoprecipitation of A33 and F13 was noted in cells infected with non-palmitoylated versions of B5 (Fig. 4, panels $\Delta$A33 and $\Delta$F13). This was not due to decreased amounts of A33 or F13, as levels of these two proteins were unchanged in Western blots of cells infected at late times (not shown). The decrease in the amount of immunoprecipitated A33 and F13 was more evident in the RSTnp version, suggesting that both the cytosolic domain and the palmitate are involved in the interaction of B5 with proteins A33 and F13. We conclude that the palmitate portion of B5 is involved in, although not absolutely required for, the interaction with A33 and F13, whereas it is dispensable for the interaction with A34.

**Localization of B5 versions by immunofluorescence**

We next investigated whether palmitoylation of B5 is involved in determining the intracellular distribution of the protein. Immunofluorescence experiments were carried out on cells infected with the mutant viruses, where proteins B5 and F13 were stained with antibodies to the V5 epitope and anti-F13 mAb, respectively (Fig. 5). In cells infected with virus vRSTC or vRSTCnp, B5 maintained the characteristic distribution of envelope proteins, showing an intense juxtanuclear labelling revealing the accumulation of the protein in the Golgi complex, and a peripheral punctate staining corresponding to enveloped virions. In cells infected with virus vRST and vRSTnp, slight differences with respect to the normal staining pattern were noted, as B5 showed, in addition to Golgi complex and virion staining, a more diffuse cytoplasmic distribution, as well as nuclear-membrane staining. A slight decrease in the

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Fig. 3. Plaque phenotype and extracellular virus production of the virus mutants. (a) Photographs of the virus plaques formed in a standard 2 day plaque assay by the viruses indicated. (b) The area of individual plaques (in pixels) and the mean ± SD are represented for the different viruses. A representative experiment out of three independent experiments is shown. (c) Cell-associated and extracellular virus production. To measure virus production, BSC-1 cells were infected at an m.o.i. of 3. At 24 h p.i., extracellular (grey bars) and cell-associated (hatched bars) viruses were harvested and titrated by a standard plaque assay.
number of labelled virions in the cell periphery was noted in vRSTnp-infected cells. The distribution of the RST version of B5 confirms previous work demonstrating a role of the cytoplasmic tail in the endoplasmic reticulum (ER) to Golgi transport of B5 (Mathew et al., 2001).

Incorporation of the proteins into extracellular virus

To analyse a possible role of palmitoylation in the acquisition of the envelope and in virus egress, we analysed the presence of envelope proteins in the medium of cells infected with the different mutants. As the virus mutants were derived from the WR virus strain, which implies a low production of EEV (see Fig. 3c), total particulate matter was used for this experiment, and thus may represent extracellular virus and other sedimentable structures present in the culture medium at late times p.i. Media from infected cells were collected by high-speed centrifugation and analysed by Western blotting with antibodies to different envelope proteins (Fig. 6). F13 and A33 were present in the medium irrespective of the B5 version expressed, and even in the medium of cells infected with B5-deficient virus vAB5. A34 was also present in the medium regardless of the B5 version expressed, but was completely absent in vAB5-infected cells. We were unable to consistently detect variations in the amount of the proteins analysed when comparing the medium from cells infected with viruses expressing palmitoylated versus non-palmitoylated versions of B5.

Localization of mutant B5 in the absence of infection

The immunofluorescence pattern of the RST constructs suggested a partial modification of the normal intracellular distribution of B5. Specifically, labelling of the nuclear membrane suggested retention of the protein in a pre-Golgi compartment (see arrows in Fig. 5). To investigate the consequences of the mutations in the cytoplasmic tail on B5 transport, we determined the subcellular distribution of the different B5 versions in the absence of infection. To this aim, mutant B5 genes were cloned in plasmids, transfected into cells and stained by immunofluorescence 24 h after transfection. Double-labelling experiments were used to determine co-localization with the ER marker calnexin or the Golgi marker wheat germ agglutinin (WGA) (Fig. 7).

As expected, B5 version RSTC produced a staining pattern coincident with the Golgi area (Fig. 7b). Version RSTCnp had a similar distribution pattern, indicating that palmitate had no major effect on the transport of the complete version. In contrast, versions RST and RSTnp were localized in the nuclear membrane and reticular cytoplasmic structures that could represent the ER. This was confirmed by colocalization with the ER protein calnexin (Fig. 7a). Notably, this ER pattern was more pronounced in the RST version, while the RSTnp version showed, together with some ER labelling, an evident Golgi staining. This result confirms a role of the cytoplasmic tail in the transport of the B5 protein in the absence of infection. Amino acids 306–317 in the cytoplasmic tail act as a positive factor for the export of B5 from the ER, whereas palmitate seems to act as a negative factor for the ER-to-Golgi transport of B5.

Effect of A33 on the distribution of non-palmitoylated B5 in transfected cells

Because B5 palmitoylation is involved in the interaction with A33, as revealed by coimmunoprecipitation experiments, we studied the distribution of the different mutations of B5 when coexpressed with A33 (Fig. 8). Protein A33 was localized to the plasma membrane and to a central area, coincident with the Golgi complex (not shown). The distribution of A33 was not modified substantially by coexpression of B5.

In contrast, when coexpressed with A33, differences were noted when comparing the different versions of B5 under study. Version RSTC was present in a juxtanuclear region

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\text{Fig. 4. Interaction of B5 with envelope proteins. BSC-1 cells were infected with the viruses indicated at an m.o.i. of 3. After 24 h, cell extracts were prepared, immunoprecipitated with anti-B5 mAb and separated by SDS-PAGE. The interaction of the different B5 versions used in this study with envelope proteins was revealed by Western blotting with different antibodies, indicated on the right. The positions of molecular mass markers are indicated on the left of each panel. Note that the gel used to detect A33 was run under non-reducing conditions to preserve recognition by the mAb and, as a consequence, the A33 dimer was detected. The upper band in the zB5 and zF13 panels corresponds to the antibody used to immunoprecipitate B5.}
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(Golgi complex) and in the plasma membrane. As RSTC expressed alone was retained intracellularly (see Fig. 8, left column), we conclude that A33 facilitates the transport of RSTC to the plasma membrane. Non-palmitoylated version RSTCnp was similarly present in the Golgi complex, but the plasma-membrane staining was significantly lower. Therefore, palmitate is involved in the A33-induced transport of RSTC to the plasma membrane. Version RST, which in the absence of A33 labelled the ER/nuclear membrane, was partially transported to the Golgi complex and the plasma membrane when coexpressed with A33. In contrast, lower plasma-membrane labelling was detected for the non-palmitoylated version RSTnp. These results indicate that the palmitate enhances transport of B5 along the exocytic pathway to the plasma membrane in the presence of A33.

**DISCUSSION**

Protein palmitoylation is known to have multiple consequences in a number of processes, including protein trafficking, sorting to specific membrane domains, protein activity and degradation. Palmitoylation is a common feature in the proteins of the vaccinia virus envelope, as at least four of these proteins (F13, B5, A33 and A36) are modified by palmitoylation. This fact led us to hypothesize that the presence of the fatty acid might be related to the incorporation of those proteins into the viral membrane. In this work, we have isolated and characterized a non-palmitoylated version of protein B5, as a step in determining the functional significance of protein palmitoylation in the biogenesis of the virus envelope. Precise mutation of two serine residues located adjacent to the transmembrane domain produced a version of B5 that did not incorporate palmitate. Previous reports showed that, in transfection experiments, single mutation of these residues did not block palmitoylation of B5 (Grosenbach et al., 2000). Our results with the double mutation are compatible with the explanation given by Grosenbach et al. (2000) in the sense that both cysteine residues can be palmitoylated. Further, the complete absence of palmitate incorporation in our double mutants demonstrates that these two residues are the only functional palmitoylation sites in the protein.
One unanticipated result was the lack of a major functional consequence derived from the absence of B5 palmitoylation. After careful examination of the mutant viruses, we have detected only minor effects in EEV formation and cell-to-cell transmission as a consequence of the mutations. This small phenotypic effect is in sharp contrast with the profound effect that removal of the F13 palmitoylation site has on the virus phenotype (Grosenbach & Hruby, 1998; Grosenbach et al., 1997). However, the situation of F13 varies from that of B5, because F13 is a peripheral membrane protein (Schmutz et al., 1995), whereas B5 is an integral, type I transmembrane protein. Several reports indicate that association of F13 with the membrane critically depends on palmitate addition, as non-palmitoylated F13 is not associated with membranes (Borrego et al., 1999; Grosenbach & Hruby, 1998; Husain & Moss, 2001; Schmutz et al., 1995). Therefore, one essential function of F13 palmitoylation is to anchor the protein to the membrane, which in turn is required for F13 function. In contrast to F13, B5 is an integral membrane protein anchored by a transmembrane domain and, consequently, does not depend on palmitate for membrane association. Therefore, mutation of the B5 palmitoylation site offered us an opportunity to evaluate the role of this post-translational modification separately from its potential role in membrane association.

Our results revealed a small effect of the mutations on the formation and transmissibility of enveloped virus. The relative lack of effect on virus transmission and extracellular virus formation was unexpected, and can be explained by the existence of multiple interactions between B5 and other envelope proteins, where disruption of a single interaction site might not be able to abrogate the formation of the protein complexes required for formation of the virus envelope. Considering this possibility, it is likely that a more evident effect of the mutations in the cytoplasmic tail of B5 will be seen in conjunction with second mutations. Of note, the palmitoylated cysteine residues are conserved in different orthopoxviruses as well as more divergent poxviruses, such as myxoma virus and rabbit fibroma virus. Other poxviruses, such as Yaba monkey tumor virus, lumpy skin disease virus, Yaba-like disease virus, tanapox virus, swinepox virus and sheeppox virus, maintain only one cysteine in a similar position. The degree of conservation suggests that the palmitoylation site, including one or two cysteine residues, fulfills an important function in virus biology.

Despite the small phenotypic effect on virus transmission, we have shown here a role of B5 palmitoylation in the intracellular transport of B5. The results presented here extend previous studies on the role of the B5 cytoplasmic tail (Lorenzo et al., 1998; Mathew et al., 2001). Notably, a similar deletion of the cytoplasmic tail was shown to cause a retention of the protein in the ER/nuclear membrane staining and a reduction in cell-surface staining (Mathew et al., 2001). The deletion of the cytoplasmic tail made by Mathew et al. (2001) retained cysteines 301 and 303, and thus is probably palmitoylated. We have shown here that further mutation of the palmitoylation site results in increased targeting to the Golgi complex. Therefore, the palmitate and the cytoplasmic tail (residues 306–317) seem to have opposite effects on the targeting of B5.

In a previous report (Ward & Moss, 2000), the presence of localization signals in B5 was studied using B5–GFP fusions expressed in uninfected cells. B5–GFP labelled perinuclear and juxtanuclear structures characteristic of the ER and Golgi complex, respectively. That distribution differs from the distribution of unmodified B5 in both infected and transfected cells (Lorenzo et al., 1998; Mathew et al., 2001; Fig. 7 of the current study). Also, B5–GFP has been shown to have an altered behaviour with respect to unmodified B5 in the absence of A33 (Chan & Ward, 2010). The easiest interpretation of the different results is that GFP might interfere with some of the functions of the cytoplasmic tail of B5, leading to divergent results.

The non-palmitoylated versions of B5 showed decreased interaction with A33 and F13. This observation complements previous studies, where the interaction between B5 and A33 has been shown to be independent of deletion of the cytoplasmic tail and most of the extracellular domain (Perdiguero & Blasco, 2006). Of note, B5 versions lacking the cytoplasmic domain in previous reports (Lorenzo et al.,
1998; Perdiguero & Blasco, 2006) are identical to our RST versions, and therefore did not remove Cys 301 and 303 (see Fig. 1). Thus, it can be inferred that those versions were palmitoylated, and were shown to interact with A33. We have further defined the requirements for the interaction of B5 with A33, by mutation of the B5 palmitoylation site. Our results suggest that an important part of the A33-interacting domain of B5, which was previously assigned to the transmembrane domain, includes the palmitate. Similarly, interaction of B5 with F13 was also dependent on palmitoylation of B5.

Despite the role of the palmitate in the interaction of B5 with A33 and F13, both proteins were incorporated efficiently into the virus envelope when the palmitoylation site of B5 was mutated. Furthermore, incorporation of both F13 and A33 into extracellular particles was independent of B5, as both proteins were detected in medium from the B5-deletion mutant.

In conclusion, mutation of the palmitoylation site in B5 results in significant effects on several aspects of B5, including its transport along the exocytic pathway and the establishment of protein–protein interactions. However, this is translated into modest consequences in the functions carried out by the virus envelope. The simplest explanation for the observations herein is that multiple interactions between envelope proteins determine the formation of protein complexes that are incorporated into the virus envelope. It is likely that multiple mutations will be required to achieve strong phenotypic effects.

**METHODS**

**Cells, viruses and antibodies.** BSC-1 cells were propagated in Eagle’s minimal essential medium (EMEM) supplemented with 5% FCS, 2 mM glutamine and 100 U penicillin/streptomycin ml⁻¹. BHK-21 cells were grown in BHK-21 Glasgow minimal essential medium (Gibco) containing 5% FCS, 3 mg tryptase phosphate broth ml⁻¹, 0.01 M HEPES, 100 U penicillin ml⁻¹, 100 U streptomycin ml⁻¹ and 2 mM glutamine. Vaccinia virus vAB5R (Wolfe et al., 1993) was made available by B. Moss, National Institutes of Health, Bethesda, MD, USA. Mouse mAb VMC-34 (anti-A33) was provided by G. H. Cohen, University of Pennsylvania, PA, USA. Rat mAbs 19C2 (anti-B5) and 15B6 (anti-F13) were kindly made available by G. Hiller. Anti-A34 rabbit antiserum was obtained by immunization.
Bam PCR product was cut with BamHI and B5RAcc65I (B5Ra301-303, pSG-B5Rb and pSG-B5Rb301-303. Primers B5RE (9301s/c303s, pG-V5-B5Rb and pG-V5-B5Rb301-303 using oligonucleotide were amplified by PCR from plasmids pG-V5-B5Ra, pG-V5-B5Ra301-303, pSG-B5Rb and pSG-B5Rb1-3, termed here RSTC.

Mutated versions of plasmid pSG5-B5R, namely pSG-B5Ra, pSG-B5Ra301-303, pSG-B5Rb and pSG-B5Rb1-3, termed here RSTC, Mutated plasmids pG-V5-B5Ra301-303 and pG-V5-B5Rb301-303 were generated using site-directed mutagenesis (QuickChange kit; Stratagene) generated using site-directed mutagenesis (QuikChange kit; Stratagene) with mutagenic primers B5R c301s/c303s 5'-GTTTCCTCCTCTGACAAAAATAATG-3' and B5R c301s/c303s 3' 5'-GTTATAGTATTA-CTCATGAATTCAAATGAAAACGATTTCC-3'. The mutated genes, termed RSTCnp and RSTnp respectively, allowed the isolation of virus mutants.

For transient expression in uninfected cells, the different versions of B5R were placed downstream of a simian virus 40 early promoter by cloning into expression plasmid pSG5. The wild-type B5R gene was obtained by digestion of plasmid pSFV-B5R (Lorenzo et al., 2000) with Smal and ligated into plasmid pSG5 (Stratagene) previously digested with BamHI and treated with the Klenow fragment of Escherichia coli DNA polymerase I. The resulting plasmid was termed pSG5-B5R. Mutated versions of plasmid pSG5-B5R, namely pSG-B5Ra, pSG-B5Ra301-3, pSG-B5Rb and pSG-B5Rb301-3, termed here RSTC, RSTCnp, RST and RSTnp, respectively, were generated by swapping a BamHI/Ace65I fragment into plasmid pSG5-B5R, corresponding to the 3’ portion of the B5R gene. For this, the mutant versions of B5R were amplified by PCR from plasmids pG-V5-B5Ra, pG-V5-B5Ra301-303, pG-V5-B5Rb and pG-V5-B5Rb301-303 using oligonucleotide primers B5RE (5'-CTCATGAATTCAATGGAAAACGATTCC-3') and B5Racc65I (5'-TTCATTAGTGTACCGGATTTATG-3'). The PCR product was cut with BamHI and Ace65I and inserted into BamHI/Ace65I-digested pSG-B5R to generate pSG-B5Ra, pSG-B5Ra301-303, pSG-B5Rb and pSG-B5Rb301-303.

**Plasmid construction.** Non-palmitoylated (np) versions of the B5R gene, depicted schematically in Fig. 1, were generated by site-directed mutagenesis of the previously described plasmids pG-V5-B5Ra (version RSTC) and pG-V5-B5Rb (version RST) (Perdiguero & Blasco, 2006). Mutated plasmids pG-V5-B5Ra301-303 and pG-V5-B5Rb301-303 were generated using site-directed mutagenesis (QuickChange kit; Stratagene) using mutagenic primers B5R c301s/c303s 5'-GTTATAGTATTA-CTCATGAATTCAAATGAAAACGATTTCC-3'. The correct sequence around the B5 locus was verified by PCR and sequencing of the regions.

**Construction of recombinant viruses.** Virus recombinants expressing different mutated versions of the B5 protein tagged with the V5 epitope were isolated following plasmid transfection in vAb5R-infected cells. Transfections were carried out using Fugene transfection reagent (Roche) following the manufacturer’s instructions. BSC-1 cells were infected with WRAb5R at 0.05 p.f.u. per cell and transfected 1 h later with pG-V5-B5Ra, pG-V5-B5Ra301-303, pG-V5-B5Rb or pG-V5-B5Rb301-303. Recombinants were isolated following a transient marker expression strategy using a GFP cassette. Single recombinants were isolated by rounds of plaque purification on BSC-1 cells, during which the plaques were screened for GFP fluorescence. Finally, a stable double cross-over virus was isolated by isolating a GFP(−) virus. The correct sequence around the B5 locus was verified by PCR and sequencing of the regions.

**Quantification of virus plaque area.** BSC-1 cell monolayers in six-well plates were inoculated with <30 p.f.u. per well. After 48 h, monolayers were stained with crystal violet and photographed with a Nikon D-70s digital camera. The area of virus plaques in digital images was measured in pixels, using the program ImageJ (http://rsweb.nih.gov/ij/). One-way analysis of variance was performed using Instat 3 for Windows. For multiple comparisons, Student–Newman–Keuls correction was applied. Data are presented as means ±SD. Differences were considered statistically significant at a P-value of <0.05.

**[3H]Palmitate labelling.** BSC-1 cells grown in a T25 flask were infected with different recombinant viruses at an m.o.i. of 3. At 2 h p.i., medium was replaced by 4 ml EMEM–pyruvate medium with no FCS (EMEM supplemented with 20 mM glutamine, 10 U penicillin/streptomycin ml⁻¹ and 2 mM sodium pyruvate). Five hours p.i., medium was replaced by EMEM–pyruvate containing 430 μCi (15.9 MBq) [3H]palmitate ([9,10-(2H) hyaluronic acid; Amersham] and incubated for an additional 4.5 h. Cell lysates were prepared by covering the cell monolayer with 1 ml digitonin-containing lysis buffer (1% digitonin, 10 mM triethanolamine pH 7.8, 150 mM

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Fig. 8. Coexpression of B5 and A33 in transfected cells. BHK cells were transfected with plasmids harbouring the B5 versions indicated (left column) or cotransfected with a plasmid harbouring the A33R gene. Cells were incubated for 24 h and subjected to immunofluorescence. Cotransfection/double-labelling experiments show B5 staining with rat anti-B5 antibody followed by secondary anti-rat Alexa Fluor 488 antibodies [column B5 (green)]. A33 staining of the same cells was done with mouse anti-A33 antibody followed by anti-mouse IgG–Alexa Fluor 594 [column A33 (red)].
NaCl, 1 mM EDTA, 10 mM PMSF) and incubation for 30 min at 4 °C. After scraping, cell lysates were collected and used for SDS-PAGE analysis or immunoprecipitation.

**Immunoprecipitation and Western blotting.** Immunoprecipitation and Western blotting were carried out as described previously (Perdiguero & Blasco, 2006). For Western blotting, antibody dilutions were 1:25 for anti-B5, 1:50 for anti-F13, 1:300 for anti-A33, 1:700 for anti-rVB12, 1:700 for anti-A34 and 1:5000 for anti-V5–HRP (Invitrogen). Secondary antibodies conjugated with HRP were obtained from Amersham and diluted 1:3000. Blots were finally imaged using a Molecular Imager Chemi Doc-XRS (Bio-Rad). Western blot analysis of the proteins present in the medium of infected cells was done as described previously (Perdiguero et al., 2008).

**Immunofluorescence microscopy.** BHK-21 cells grown to 70% confluence on round 13 mm coverslips were infected with vaccinia virus at 5 p.f.u. per cell or transfected with plasmids using FuGene transfection reagent (Roche). At 18 h p.i., cells were incubated with virus at 5 p.f.u. per cell or transfected with plasmids using Fugene 742. At 18 h p.i., cells were incubated with virus at 5 p.f.u. per cell or transfected with plasmids using FuGene transfection reagent (Roche). At 18 h p.i., cells were incubated with virus at 5 p.f.u. per cell or transfected with plasmids using FuGene transfection reagent (Roche). At 18 h p.i., cells were incubated with virus at 5 p.f.u. per cell or transfected with plasmids using FuGene transfection reagent (Roche).

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