Sequence and analysis of a swinepox virus homologue of the vaccinia virus major envelope protein P37 (F13L)

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P37 (F13L gene product), the most abundant protein in the envelope of the extracellular virus form of the prototype poxvirus, vaccinia virus (VV), is a crucial player in the process leading to acquisition of the envelope, virus egress and transmission. We have cloned and sequenced a swinepox virus (SPV) gene homologous to VV F13L. The SPV gene product, termed P42, was 54% identical to P37, the VV F13L gene product, and, among the poxviruses, was most similar (73% identity) to the myxoma virus homologue. The SPV P42 gene contained late transcription signals and was expressed only at late times during infection. The protein was palmitylated, and showed an intracellular distribution similar to that of VV P37, both by immunofluorescence and by subcellular fractionation. As with VV P37, SPV P42 was incorporated in extracellular enveloped SPV particles, but was absent from the intracellular mature virus form. To check the ability of SPV P42 to function in the context of VV infection, we inserted the SPV gene into a VV deficient in P37, which is severely blocked in virus envelopment and cell-to-cell transmission. Despite correct expression of SPV P42, the resulting recombinant VV showed no rescue of extracellular virus formation or cell-to-cell virus spread. The lack of function of SPV P42 in the VV genetic background suggests that specific interactions between SPV P42 or VV P37 and other viral proteins is required to drive the envelopment process.

Introduction

Swinepox virus (SPV) is the sole member of the genus Suipoxvirus, belonging to the family Poxviridae. Its biological characteristics, and particularly its narrow host range, make it a promising vector for recombinant vaccines for swine (Foley et al., 1991; Tuboly et al., 1993; van der Leek et al., 1994).

The best studied member of the poxvirus family, vaccinia virus (VV), belongs to the genus Orthopoxvirus. The classification of SPV in a separate genus, based originally on immunological studies, was later reinforced by DNA hybridization, restriction mapping and DNA sequencing (Massung & Moyer, 1991a, b). The limited sequence information available for SPV, including sequences close to the genome ends as well as internal regions (Feller et al., 1991; Massung et al., 1993; Schnitzlein & Tripathy, 1991) has revealed enough similarity to other poxviruses to allow alignment of gene maps, despite extensive sequence divergence.

The major protein component of the envelope of extracellular vaccinia virions (EEV), P37, is encoded by the F13L open reading frame (ORF) (Hirt et al., 1986). It is a 372 amino acid polypeptide expressed at late times during infection and is targeted to Golgi-derived membranes where it is incorporated into the virion by a wrapping process (Hiller & Weber, 1985; Schmelz et al., 1994). Thus P37 is present in the enveloped forms of the virus, but is absent from intracellular mature virus (IMV) (Payne, 1978). P37 is a peripheral membrane protein, and lines the inner surface of the EEV envelope (Roos et al., 1996; Schmutz et al., 1995). Palmitylation of the protein, a process that is facilitated by other viral proteins, is required for its membrane-association, proper intracellular targeting and correct functioning of the protein (Borrego et al., 1999; Grosenbach & Hruby, 1998; Grosenbach et al., 1997; Schmutz et al., 1995).

Deletion of the P37 gene results in a block of the virus envelopment process, and abolishes extracellular virus formation and cell-to-cell virus transmission (Blasco & Moss, 1991, 1992). In addition, other virus-induced phenomena that
require virus envelopment, such as engrossed actin tails and cell–cell fusion at acid pH, are also blocked (Blasco & Doms, 1993; Blasco & Moss, 1992; Cudmore et al., 1995; Sanderson et al., 1998).

Considering that P37 is the major protein in the EEV envelope, its topology relative to the EEV envelope and the phenotype of P37 knock-out mutants, it is likely that P37 acts by mediating the interaction between the surface of intracellular virions and wrapping membranes. Recent reports suggest that in addition to this structural role, P37 may have enzymatic activities related to lipid metabolism. Indeed, the P37 sequence contains motifs distinctive of the phospholipase D superfamily (Koonin, 1996; Ponting & Kerr, 1996), and shows phospholipase activities which may be important for function (Baek et al., 1997; Sung et al., 1997).

Because of the importance of P37 in VV envelope morphogenesis and dissemination, we mapped and sequenced the SPV homologous gene, and present here a characterization of the corresponding protein.

**Methods**

- **Cells and viruses.** SPV (Kasza strain, ATCC VR-363) was routinely grown in PK-15 cells, grown in EMEM containing 5% foetal calf serum. SPV titres were determined on ESK-4 (ATCC CL 184) cell monolayers as described previously (Barcena & Blasco, 1998). VV strain IHD-J was propagated on CV-1 or RK-13 cells and titrated on BSC-1 cell monolayers. VV mutant vRB10 (Blasco & Moss, 1991), derived from VV IHD-J by deleting 93% of gene F13L, was used to construct recombinant viruses.

- **Cloning and sequencing.** SPV DNA fragment HindIII B (Massung & Moyer, 1991a) was excised from an agarose gel and digested with EcoRI. A 5-5 kb HindIII–EcoRI fragment derived from the left-hand end of the HindIII B fragment was cloned into plasmid pUC19 generating plasmid pSPV-HE. A 1-9 kb Ncol–BamHI fragment derived from pSPV-HE was cloned in plasmid pUCPTS-3 (Barcena & Blasco, 1998) to generate pSPV-NB. Both pSPV-HE and pSPV-NB were used as templates for sequencing the P42 gene using the dyeoxy chain termination method. Both DNA strands were sequenced.

- **Sequence analysis.** Pairwise alignment of amino acid sequences was performed using the Align program (FASTA program package, version 2.0) (Pearson, 1990). Pairwise percentage identities (or similarities) were calculated by dividing the number of identical residues (and conservative changes) by the total number of positions in the aligned sequences, including gaps. Multiple alignment of amino acid sequences was generated using the program Clustal W version 1.7 (Thompson et al., 1994). Hydrophobicity analysis of the amino acid sequences was based on the algorithm of Kyte & Doolittle (1982).

- **Plasmid construction.** Plasmid pRB24 was constructed to insert foreign genes in place of F13L, under the control of the natural F13L promoter. The plasmid contains F13L recombination flanks, Xhol and BgIII restriction sites immediately downstream of the P37 promoter, and a β-glucuronidase (β-gus) cassette to provide visual identification of recombinant viruses.

The process of plasmid construction is outlined in Fig. 3(A) (see Results). First, the β-gus gene was amplified by PCR from plasmid pBI101 (Clontech) using primers 5’ CAGGTCGAGAATTCTATGGTACGTC 3’ (EcoRI site underlined) and 5’ GGAGAGGTGCCTAAGCCATTGTGTTGGCC 3’ (Nhel site underlined), and inserted into the EcoRI–Nhel sites of plasmid pRB21 (Blasco & Moss, 1995), to construct pRB21-βgus. Then, a Spnl–Xhol DNA fragment of pRB21-βgus, containing the P37 gene, was removed and replaced by a PCR product obtained using pRB21 as template and oligonucleotides 5’ GGGGCAATTCGATAGAGTTCTTGAACACGACAAAAA 3’ (Spnl site underlined) and 5’ CGATGGCTGAGAATCTATTTGTTACATAAAAAAC 3’ (Xhol and BgIII sites underlined) as primers, generating plasmid pRB24. Subsequently, the genes corresponding to SPV protein P42 or VV protein P37 were inserted into pRB24, generating plasmids pRB24-P42 and pRB24-P37 respectively. The gene encoding P42 protein was amplified by PCR from plasmid pSPV-HE using primers 5’ AAATAGGATCCTGATTGCGG-3’ (BamHI site underlined) and 5’ ACCTGCTGGATTCAAAATATTTTCC 3’ (BamHI site underlined). The PCR product containing the P42 gene was partially digested with BamHI, ligated to pRB24 plasmid digested with BgIII, and checked by DNA sequencing. Finally, a BamHI fragment from pSG-P37 plasmid (B. Borrego and others, unpublished results), containing the P37 gene, was isolated and ligated to BgIII-digested pRB24 to generate pRB24-P37.

- **Isolation of recombinant viruses.** VV recombinants 1-f, 1-fβ37 and 1-fP42 were obtained as follows. Monolayers of CV-1 cells were infected with the VV mutant vRB10 at an m.o.i. of 0.05 and transfected with pRB24, pRB24-P37 or pRB24-P42 by the calcium phosphate technique, following standard protocols. Recombinant viruses were selected by several rounds of plaque purification in the presence of X-Gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide) to reveal β-gus expression (Carroll & Moss, 1995). Subsequently, the recombinant viruses were amplified by infection of cell monolayers and their genomic structures were analysed by PCR.

- **Antiserum.** A rat monoclonal antibody, 15B6 (Schmeizl et al., 1994), was used to detect the P37 protein. Antiserum against an 18-mer synthetic peptide corresponding to the C terminus of P42 (NH2-CDIERFDWKNSNTPITNCOOH) was obtained. The peptide, conjugated with diphtheria toxoid protein, was injected subcutaneously into rabbits in three doses. The first dose contained 500 μg of protein in complete Freund’s adjuvant and the following two contained 500 μg of protein in incomplete Freund’s adjuvant.

- **Immunofluorescence.** PK-15 cells grown on coverslips were infected with SPV or VV (strain WR). After 23 h (for SPV) or 7 h (for VV) at 37°C, cells were washed twice with PBS at room temperature, fixed by addition of ice-cold 4% formaldehyde, and incubated for 12 min at room temperature. All subsequent incubations were carried out at room temperature. After washing once with PBS, cells were permeabilized by incubation with 0.1% Triton X-100 in PBS. After washing with PBS, cells were incubated for 5 min with PBS containing 0.1 M glycine, and then with primary antibodies diluted in PBS–20% foetal calf serum for 30 min. Anti-peptide antiserum was diluted 1:75, and anti-P37 hybridoma supernatant was diluted 1:50. After washing for 5 min in PBS, the cells were incubated for 30 min with secondary antibodies: rabbit anti-mouse IgG or swine anti-rabbit IgG conjugated with TRITC rhodamine (Dako) diluted 1:200 in PBS–20% foetal calf serum. After a final wash, coverslips were mounted using FluorSave mounting medium (Calbiochem).

- **Western blotting.** Proteins were electrophoresed in 12% SDS-polyacrylamide gels and transferred to nitrocellulose membranes by electroblotting. After transfer, the membranes were incubated overnight at 4°C in blocking buffer (PBS containing 0.1% Tween 20 and 5% non-fat dry milk). The membranes were then incubated with monoclonal antibody anti-P37 (diluted 1:2000) or antisera anti-P42 (diluted 1:100) in PBS–0.1% Tween 20 containing 1% BSA for 1 h at 37°C. After four
10 min washes with PBS–0·1% Tween 20, the membranes were incubated for 1 h at 37 °C with rat or rabbit anti-IgG antibody (diluted 1:2000) conjugated with horseradish peroxidase in PBS–0·1% Tween 20 containing 1% BSA. After four 10 min washes with PBS–0·1% Tween-20, bound antibodies were detected using the enhanced chemiluminescence (ECL) kit from Amersham.

Subcellular fractionation of infected cells. CV-1 cell monolayers were infected with recombinant VV at an m.o.i. of 10 p.f.u. per cell. At 12 h post-infection (p.i.) cells were scraped into the medium, collected by low-speed centrifugation, washed once with PBS and resuspended in 20 mM HEPES pH 7.0, 5 mM KCl, 1 mM MgCl₂, 150 mM NaCl. Subcellular fractionation was then performed as described by Grosenbach et al. (1997). Briefly, cells were lysed by Dounce homogenization to yield a total cell extract (TCE). The TCE was subjected to centrifugation at 700 g for 10 min at 4 °C to produce a nuclear pellet fraction (NP). The supernatant was centrifuged at 15000 g for 30 min at 4 °C to obtain the virus-containing fraction (P15). Finally, the supernatant of this centrifugation was further separated into soluble (S100) and insoluble (P100) protein fractions by centrifugation at 100000 g for 1 h at 4 °C. Portions of the resulting fractions were analysed by Western blot. Subcellular fractionation of SPV-infected cells was carried out as described above using PK-15 cell monolayers infected at an m.o.i. of 10 for 48 h.

Preparation and analysis of [³²P]palmitoylated VV and SPV proteins. Infected cells were radiolabelled with [³²P]palmitic acid essentially as described by Child & Hruby (1992). Briefly, confluent monolayers of CV-1 cells were infected with the different recombinant VVs at an m.o.i. of 10. At 16 h p.i., cell cultures were labelled for 4 h with 200 µCi/ml [³²P]palmitic acid (Amersham, 51 Ci/mmol). Radiolabelled SPV-infected cells were prepared as described above using PK-15 cells infected for 48 h. For SDS–PAGE analysis, infected-cell extracts were prepared by washing the cell monolayers with PBS followed by solubilization in 2% SDS. After electrophoresis in 11% SDS–polyacrylamide gels, the gels were fixed, fluorographed, dried, and exposed to an X-ray film at −70 °C.

Virion purification and CsCl gradient analysis. For equilibrium centrifugation, confluent monolayers of RK-13 cells in six-well plates were infected with virus at an m.o.i. of 10. At 7 h p.i., medium was replaced with 1 ml of solution comprising two-thirds of methionine-free MEM and one-third of complete MEM supplemented with 25 µCi [³⁵S]methionine. At 24 h p.i., 1 ml of complete MEM with 5% foetal calf serum was added, and the incubation was continued for another 24 h. Extracellular virus was then recovered from the medium after clarification by low-speed centrifugation and was pelleted through a cushion of 4 ml 40% sucrose cushion and subjected to buoyant density centrifugation as described above. Fractions were assayed for radioactivity and density. Finally, radioactive peaks corresponding to fractions with densities of 1.25 g/ml and 1.27 g/ml were pooled and used as purified EEV and IMV, respectively.

Results

Localization, cloning and sequence of the SPV P42 gene

The left-terminal genomic region of SPV DNA can be aligned with that of VV on the basis of ORF similarity (Massung et al., 1993). Based on the known SPV sequences and on the VV gene map, we predicted that an SPV homologue of the VV F13L gene could be located to the left side of the SPV HindIII B fragment (Massung & Moyer, 1991 a). Southern blot analysis revealed the presence of an EcoRI site located at 5·5 kb to the right of the SPV HindIII C/B boundary (not shown). Thus, the corresponding 5·5 kb HindIII–EcoRI fragment was isolated from SPV DNA and cloned. Subsequently, a 1·9 kb NcoI–BamHI fragment was isolated and cloned as described in Methods. From those clones, we obtained a 1381 bp sequence (Fig. 1) which contained an ORF (nucleotides 125–1239) with the potential to code for a protein of 371 amino acids.

We compared the SPV gene with that of other poxviruses: VV, fowlpox virus (FPV), myxoma virus (MV), orf virus (OV) and molluscum contagiosum virus (MCV). The SPV sequence had a high A + T content (69·4%), similar to previous data for SPV DNA. High A + T content was also apparent in the corresponding genes of FPV (67·8%) and VV (63·1%), whereas MV has a more equal A + T content (54%), and OV and MCV have a much lower A + T content (35 and 34% respectively). Analysis of the codon usage within the SPV ORF reflected its high A + T content, as there was a strong bias for A or T in codon position 3 (79·2%). The corresponding percentages for third codon position in other poxviruses are: 72% (FPV), 70·4% VV, 44·7% (MV), 4·5% (OV) and 18·0% (MCV). Coincident with these data, pairwise comparisons of the DNA sequence of the SPV gene with that of the other poxviruses only revealed significant homology with MV (66·8%), VV (62·5%) and FPV (57%) but not with OV or MCV genes.

Our sequencing revealed the typical poxvirus late promoter motif TATAAT, and the similar TAAATT motif, upstream of the putative ATG initiation codon (see Fig. 1). The early transcription termination sequence TTTTTNT occurs in three different places within the ORF, suggesting an exclusively late expression pattern.

SPV P42 amino acid sequence

The calculated molecular mass of the product of the SPV ORF is 41 779 Da, which is similar to the calculated size of VV P37, and somewhat larger than the size of 37 kDa estimated
Fig. 1. For legend see facing page.
previously size by its mobility in PAGE. On the basis of its predicted size, the putative SPV polypeptide was termed P42.

A comparison of the amino acid sequences of P37 homologues from different poxviruses is shown in Fig. 2. SPV P42 was most similar to the MV protein (73·1% identity) and VV P37 (54·3% identity), suggesting that SPV is more closely related to leporipoxviruses and orthopoxviruses than to the other poxvirus genera included in this study. Indeed, this result is in agreement with poxvirus phylogenies based on thymidine kinase sequences (Blasco, 1995).

A multiple alignment including VV P37 homologues from different poxviruses (Fig. 2A) showed several conserved features. In particular, a cysteine doublet in VV P37 (residues 185 and 186, located within a hydrophobic domain), which is the palmitylation site of the protein and is essential for P37 function (Groshenbach & Hruby, 1998; Groshenbach et al., 1997), is conserved in the SPV and MV genes. In addition, a partially conserved phospholipase D motif (HXXXXXD), which is also essential for P37 function (Cao et al., 1997; Ponting & Kerr, 1996; Sung et al., 1997), is also conserved.

**Construction of recombinant viruses**

In order to study SPV P42, we constructed several recombinant VVs in which either VV P37 or SPV P42 genes were placed downstream of the natural VV P37 promoter. To facilitate the isolation of recombinant viruses, plasmid pRB24 was constructed, as outlined in Fig. 3(A). This plasmid contains flanks for insertion into the P37 locus and a β-gus cassette to allow visual identification of recombinant virus plaques. The coding sequences of VV P37 or SPV P42 ORFs were inserted downstream of the P37 promoter, to generate recombinant viruses expressing either protein. As shown in Fig. 3(B), the DNA sequence between the promoter and the initiation ATG was slightly changed from wild-type virus due to the cloning. Recombinant viruses I-βP37 and I-βP42 were derived from VV vRB10, a VV (IHD-J strain) P37 deletion mutant, by recombination with plasmids pRB24, pRB24-P37 or pRB24-P42, respectively. Recombinant viruses were isolated by several consecutive rounds of plaque isolation in the presence of X-Gluc.

**Expression of SPV P42**

An antiserum was raised against a synthetic peptide corresponding to the C terminus of the protein. The antiserum specifically recognized SPV P42, when this was expressed from VV recombinant I-βP42 (Fig. 4A). In SPV-infected PK-15 cells, the antiserum recognized a polypeptide with an apparent molecular mass of 40 kDa, in good agreement with the predicted molecular mass for P42 (Fig. 4A).

The kinetics of accumulation of P42 in SPV-infected cells was followed by Western blot analysis with anti-P42 serum (Fig. 4B). The protein was first detected at 24 h p.i. and accumulated over several days. Taking into account the slower replication cycle of SPV compared to VV, these results showed, as predicted from the sequence analysis, that SPV P42 was expressed with a late expression pattern. Furthermore, P42 was not detected after 48 h of infection in the presence of araC, an inhibitor of DNA replication. Thus P42 showed an exclusively late expression pattern.

**Palmitylation of SPV P42**

Because of the overall sequence conservation between VV P37 and SPV P42, and the conservation of the two cysteine residues that are modified by palmitylation in VV P37, we wished to determine whether SPV P42 was modified by palmitylation. Labelling with [3H]palmitic acid during infection by SPV produced two major labelled proteins, one of which had an apparent molecular mass of 42 kDa (Fig. 5, lane SPV). This protein comigrated with immunoprecipitated, or palmitate-labelled, VV P37.

Several palmitylated proteins could be detected in I-βP37-infected cells, in agreement with results obtained for VV strain IHD-J (Child & Hruby, 1992). One of the labelled proteins had the same electrophoretic mobility as immunoprecipitated P37 protein, and was absent in I-β virus-infected cells, indicating that this protein is P37. Interestingly, expression of SPV P42 by VV recombinant I-βP42 resulted in a pattern of palmitylated proteins identical to that of I-βP37. These data indicate that SPV P42 is a palmityl protein, and that it is palmitylated efficiently when expressed via a VV recombinant.

**Localization of SPV P42**

In order to study the subcellular distribution of SPV P42, and compare it to that of VV P37, cells infected with I-βP37, I-βP42 or SPV were subjected to subcellular fractionation as detailed in Methods. After fractionation, samples were analysed by Western blot (Fig. 6A) with anti-P37 or anti-P42 antibody. P37 was detected in significant amounts in the nuclear fraction (NP) and the virus-containing fraction (P15), which potentially contains some of the cytoplasmic membrane-bound organelles. No P37 was detected in the particulate cytoplasmic fraction (P100) or in the soluble cytoplasmic fraction (S100). These results are consistent with those reported previously (Groshenbach & Hruby, 1998; Groshenbach et al., 1997).
Fig. 2. Comparison between poxvirus P37 homologues. (A) Multiple sequence alignment. Residues that are identical in at least four of the six sequences are highlighted. Cysteine residues that are palmitylated in VV P37 are indicated by asterisks. The conserved aspartate residues in phospholipase D motifs are indicated by arrowheads. (B) Percent identity between different proteins. Similarities are expressed as the percentage of identical residues derived from pairwise alignments between protein sequences. Sequences used are: SPV P42 (GenBank accession no. AI249689); MV, myxoma virus (U43549); VV, vaccinia virus (WR strain) P37 (M12882); OV, orf virus (U06671); FPV, fowlpox virus (M88587); MCV, molluscum contagiosum virus (subtype 1) (M63486).
Swinepox virus F13L homologue

Fig. 3. (A) Plasmid construction. Plasmid pRB21-βgus was obtained by inserting the β-gus gene in plasmid pRB21 (Blasco & Moss, 1992), downstream of the PE/L promoter. Plasmid pRB24 was derived from pRB21-βgus by substituting a fragment containing the left flank and P37 gene for an extended version of the left flank containing the natural F13L promoter. Subsequently, genes VV P37 and SPV P42 were cloned downstream of the F13L promoter to produce plasmids pRB24-P37 and pRB24-P42, respectively. LF, left recombination flank; RF, right recombination flank; PE/L, synthetic early/late VV promoter; PF13L, VV P37 gene promoter; β-gus, E. coli β-glucuronidase gene. (B) Viruses. All viruses used, except SPV, were derived from a VV strain IHD-J deficient in P37 (vRB10, Blasco & Moss, 1991). Plasmids used to introduce genes downstream of the natural P37 promoter are indicated. The sequence around the promoter/ATG region in each construct is shown. The late promoter motif TAAAT (TAAAAT in SPV) is shown in bold letters. The ATG initiation codon is underlined.

1997). After fractionation of SPV-infected cells, P42 showed roughly the same pattern, suggesting that P37 and P42 share a similar intracellular distribution. Fractionation of 1-βP42-infected cells also produced a similar pattern (Fig. 6A), indicating that the SPV P42 protein expressed in the context of a VV infection attains its correct subcellular distribution.

VV P37 is incorporated specifically to the outer envelope of the VV EEV form, but is absent from the IMV form (Hiller et al., 1981; Payne, 1978). To determine if P42 is incorporated into the EEV envelope, we sought to detect the protein in the two virus forms. SPV EEV and IMV particles were purified from infected PK-15 cells by CsCl gradients and equal amounts were analysed by Western blotting with anti-P42 serum (Fig. 6B). P42 was detected in EEV but was absent from purified IMV particles, indicating that SPV protein is a specific component of EEV, in a manner similar to P37 protein in VV.
Fig. 4. Expression of SPV P42. (A) Western blot analysis. An anti-peptide antiserum was used to detect the protein in lysates of uninfected PK-15 cells (Mock), SPV-infected PK-15 cells (SPV), uninfected CV-1 cells (Mock), VV-infected CV-1 cells (I-HDJ) or CV1 infected with recombinant VV expressing P42 (I-βP42). (B) Kinetics of P42 accumulation. Lysates of uninfected (M) or SPV-infected PK-15 cells harvested at the indicated times p.i. (in hours) were analysed by Western blotting with anti-P42 serum. Lane 48a, PK-15 cells infected with SPV for 48 h in the presence of araC. Molecular masses of the marker proteins are indicated in kDa at the left of each panel.

Fig. 5. Labelling with [3H]palmitate. CV-1 cells were infected with VV recombinants expressing VV P37 (lane I-βP37), SPV P42 (lane I-βP42) or none (lane I-β). At 16 h p.i., cell cultures were labelled for 4 h with [3H]palmitate. In parallel, PK-15 cells that had been infected with SPV for 48 h were similarly labelled for 4 h with [3H]palmitate. Lysates were prepared from the infected cells and the samples analysed by PAGE. As a control, VV P37 protein from VV-infected CV-1 cells radiolabelled with [35S]methionine/cysteine and immunoprecipitated with anti-P37 antibody was included (lane P37).

The intracellular localization of P42 was also explored by immunofluorescence staining of infected cells. Staining with anti-P42 antibody in SPV-infected cells revealed a strong juxtanuclear signal, and some dispersed punctate staining (Fig. 7B), similar to that of P37 in VV-infected cells (Fig. 7D). This localization pattern is characteristic of VV EEV envelope proteins, which at late times are enriched in the trans-Golgi network (juxtanuclear staining), and are also present in intracellular or cell-associated enveloped virus particles (punctated staining). The strong juxtanuclear labelling suggests efficient trans-Golgi network targeting of P42 in the context of SPV infection. Also, the punctate pattern obtained (which showed fewer and in general bigger structures than in the case of VV) is consistent with the incorporation of P42 in the enveloped forms of the virus, by a mechanism similar to that described for VV.

Functioning of SPV P42 in VV recombinants

The above results revealed that SPV P42 and VV P37 share, in addition to sequence homology, a number of properties.
Given these similarities between VV and SPV, we wondered if SPV P42 could substitute for P37 when expressed in VV. P37 is known to be required for VV envelopment and release. Consequently, enveloped virus formation and cell-to-cell virus transmission are blocked as a result of the P37 deletion. The EEV formation defect is better appreciated in the high EEV producer strain IHD-J. Therefore, we constructed a panel of recombinant viruses (see above, and Fig. 3) derived from virus vRB10, which is a P37 deletion mutant derived from VV IHD-J.

VV recombinants expressing VV P37 (I-βP37) or SPV P42 (I-βP42) as well as P37 deleted virus (I-β) were assayed for plaque formation, EEV release and EEV infectivity. Monolayers of BSC-1 cells were infected with wild-type VV (IHD-J) or the recombinant viruses and incubated under an agarose overlay. At 48 h p.i. the cell monolayers were stained with crystal violet to visualize virus plaques (Fig. 8A). I-βP37 virus formed clearly visible plaques, which were slightly smaller than normal IHD-J virus plaques, probably due to differences in the sequence around the P37 promoter (shown in Fig. 3B). As expected for a P37 deletion mutant (Blasco & Moss, 1991), virus I-β produced no visible plaques in 48 h and formed small plaques after 6–7 days (not shown). I-βP42 plaques were indistinguishable from I-β plaques, indicating that P42 expression did not rescue the defect of the VV P37 deletion mutant. When the monolayers were maintained under a liquid medium overlay, the characteristic comet-like plaques typical of IHD-J virus formed after 2 days for IHD-J and I-βP37, and after 7 days for I-β and I-βP42 (data not shown), indicating that EEV release was not enhanced by expression of P42. These observations were not dependent on the cell type, similar results being obtained with swine PK-15 cells (data not shown).

As P37 deficiency also results in a strong decrease in VV EEV formation, we determined the amount of infectious virus in the medium of infected RK-13 cells (Fig. 8B). All viruses used produced roughly equivalent titres of cell-associated virus. The P37-deficient virus I-β produced approximately 100-fold less extracellular virus than normal IHD-J virus. Expression of P37 in I-βP37 rescued EEV formation to a level similar to that of IHD-J. In contrast, I-βP42 virus exhibited a phenotype...
Fig. 8. Characterization of recombinant VVs. (A) Plaque formation. Monolayers of BSC-1 cells were infected with the indicated viruses and incubated under semi-solid medium. At 48 h p.i. the cell monolayers were stained with crystal violet.
(B) Extracellular virus production. RK-13 cell monolayers were infected at a multiplicity of 5 p.f.u. per cell and harvested at 24 h p.i. Virus from the cell lysates (white bars) and culture medium (black bars) was titrated by plaque assay on BSC-1 cell monolayers. For titration, monolayers were stained after 2 days for IHD-J and I-βP37, and after 7 days for I-β and I-βP42.
(C) Centrifugation of extracellular virus in CsCl density gradients. Infected RK13 cells were metabolically labelled with $^{[35}$$S$[methionine/cysteine. Virus from the medium of infected RK-13 cells was subjected to centrifugation in CsCl gradients as described in Methods. After centrifugation, fractions were collected from the bottom of the tube.

indistinguishable from that of I-β virus. Similar results were obtained using different cell lines such as CV-1 and BSC-1 (data not shown).

Both the development of virus plaques (Fig. 8 A) or the release of high virus titres into the culture medium (Fig. 8 B) are dependent on the production and release of infectious virus. The phenotype of I-βP42 virus could reflect either a block in EEV formation or a defect in EEV infectivity. To distinguish between these possibilities, infected RK13 cells were labelled with $^{[35}$$S$[methionine and the virus in the medium was analysed by CsCl gradient centrifugation (Fig. 8 C). I-βP37 produced a high peak of radioactivity in fractions corresponding to the density of the EEV band (1.23 g/ml). I-β and I-βP42 had drastically reduced levels of EEV particles, although detection of enveloped virions above background was possible. Thus, expression of P42 was not able to rescue EEV formation in a VV P37 deficient background.

Discussion
We describe here the sequence and characterization of an SPV EEV protein with similarity to VV P37, the major protein in the VV EEV envelope. P37 gene homologues have been previously reported for a number of poxviruses belonging to different genera: MCV (Blake et al., 1991), FPV (Calvert et al., 1992), OV (Sullivan et al., 1994) and MV (Jackson & Hall, 1998). Interestingly, as in other poxviruses, the SPV gene was located near the left-hand terminus of the genome, and its transcription orientation was towards the left genome end.

In addition to sequence homology, our characterization of SPV P42 revealed significant resemblance to its VV counterpart. Similar to VV P37, the SPV protein showed an exclusively late expression pattern, had a similar intracellular distribution, was incorporated into EEV and was absent from IMV. These data led us to propose that both proteins share
the same function in the morphogenesis of their respective enveloped virions. That SPV P42 is functional is supported by the observation that despite its slower replication kinetics with respect to VV (Massung et al., 1991b), SPV forms larger plaques, and releases more EEV than VV P37 deletion mutants (J. Bárcena, J. M. Sánchez-Puig & R. Blasco, unpublished). Deletion of the SPV gene, in a similar way to the deletion of its VV counterpart (Blasco & Moss, 1991) would be of interest to unequivocally determine the function of P42. However, SPV P42 deletion will presumably require the isolation of poorly plaquing or non-plaquing viruses, which is hampered by the low effectiveness of selection in SPV when using marker genes commonly used for VV selection (J. Bárcena & R. Blasco, unpublished).

Several studies point to a close connection between P37 function, palmitylation and intracellular targeting. By mutation of the palmityl acceptor cysteine residues, it has been shown that proper subcellular localization of P37, as well as functionality, are dependent on palmitylation of the protein (Grosenbach & Hruby, 1998; Grosenbach et al., 1997). Also, the infection context, and probably the interaction with other envelope proteins, is important for efficient palmitylation and targeting of P37 (Borrego et al., 1999). Since SPV P42 appears to have the same localization as VV P37, and the palmitylation site is conserved, we considered it likely that P42 was also modified by palmitylation. Indeed, our data suggest that P42 is the major palmitylated protein in a normal SPV infection (Fig. 5).

Broad similarities between SPV P42 and VV P37 led us to attempt to rescue the VV P37 deficiency by expressing SPV P42 in the VV genetic background. Notably, when expressed in VV, SPV P42 seemed to be normally palmitylated and targeted. However, no complementation of the P37 deficiency was apparent, either for virus transmission or EEV formation. There are several possible explanations for this lack of cross-species functionality. Despite all of the above observations, we cannot formally rule out the possibility that the two proteins have different functions. Also, it is possible that each protein is adapted to function exclusively in cells of the host of their respective viruses. However, we favour an explanation based on the likely requirements for protein–protein interactions between P37 (or P42) and other viral proteins involved in EEV formation. If those interactions are required for function, it is not surprising that a structural protein like SPV P42 may not function properly in the context of a different virus, like VV, provided that its protein partners have also undergone enough divergent evolution. From that point of view, the likely scenario is that concerted evolution of several virus proteins would result, eventually, in a lack of function of one single protein when isolated from its genetic context.

The absence of cross-species complementation is in sharp contrast with observations obtained with thymidine kinase genes, which are functional after being transferred between different poxviruses (Boyle & Coupar, 1986; Gruidl et al., 1992; Scheiflinger et al., 1997) or even between herpesviruses and poxviruses (Mackett et al., 1982; Panicali & Paoletti, 1982). Probably, complementation is easily achieved in the case of enzymes, where providing the enzymatic activity could be sufficient to provide successful complementation. Conversely, when protein interactions are required for function, the divergence between different viruses is expected to result in lack of complementation. This system, or similar situations when a particular function is affected by inter-genus divergence, has potential practical applications. For instance, one could study the protein(s) that interact with SPV P42 by incorporating additional SPV genes in the VV background. Also, recombinant viruses containing SPV P42–VV P37 protein chimaeras could allow study of the protein regions involved in the protein–protein interactions required for function.

SPV is a potential vector for the construction of recombinant vaccines for pigs. The possible widespread use of such a vector raises several concerns related to its safety. First, the possibility of horizontal transmission to an unintended host, and in particular to humans, should be addressed. In this respect, SPV shows an extremely narrow host range in vivo, and lack of transmission of SPV to humans and other species has been reported (Schwarte & Biester, 1941). A second concern regarding the use of SPV, or other poxviruses, is that the virus vector may change its biological properties, alter its host range or pathogenicity, through mutation or recombination with a naturally occurring poxvirus. Our results highlight the divergence of SPV with respect to VV, and reinforce the notion that suipoxviruses and orthopoxviruses are evolutionarily distant, and therefore unlikely to undergo genetic rearrangements.

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