In a previous study, it was shown that the protein encoded by the gene B318L of African swine fever virus (ASFV) is a trans-prenyltransferase that catalyzes in vitro the condensation of farnesyl diphosphate and isopentenyl diphosphate to synthesize geranylgeranyl diphosphate and longer chain prenyl diphosphates (Alejo, A., Yáñez, R. J., Rodríguez, J. M., Viñuela, E., and Salas, M. L. (1997) J. Biol. Chem. 272, 9417–9423). To investigate the in vivo function of the viral enzyme, we have determined, in this work, its subcellular localization and activity in cell extracts. Two systems were used in these studies: cells infected with ASFV and cells infected with a recombinant pseudo-Sindbis virus carrying the complete B318L gene. In this latter system, the trans-prenyltransferase was found to colocalize with the endoplasmic reticulum marker protein-disulfide isomerase, whereas in cells infected with ASFV, the viral enzyme was present in cytoplasmic viral assembly sites, associated with precursor viral membranes derived from the endoplasmic reticulum. In addition, after subcellular fractionation, the viral enzyme partitioned into the membrane fraction. Extraction of membrane proteins with alkaline carbonate and Triton X-114 indicated that the ASFV enzyme behaved as an integral membrane protein. The membrane enzyme synthesized predominantly all-trans-geranylgeranyl diphosphate from farnesyldiphosphate and isopentenyl diphosphate. These results indicate that the viral B318L protein is a trans-geranylgeranyl-diphosphate synthase, being the only enzyme of this type that is known to have a membrane localization.

The African Swine Fever Virus Prenyltransferase Is an Integral Membrane trans-Geranylgeranyl-diphosphate Synthase*

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The abbreviations used are: IPP, isopentenyl diphosphate; FPP, farnesyl diphosphate; GGPP, geranylgeranyl diphosphate; ASFV, African swine fever virus; ER, endoplasmic reticulum; BHK, baby hamster kidney; Ara-C, cytosine arabinoside; Q, ubiquinone.

The biosynthetic pathway of isoprenoid compounds, different enzymes belonging to the family of prenyltransferases catalyze the condensation of isopentenyl diphosphate (IPP) with allylic diphosphates to give rise to prenyl diphosphates of different chain lengths and double-bond stereochemistry (1–3). Thus, farnesyl-diphosphate synthase and geranylgeranyl-diphosphate synthase produce the 15-carbon all-trans-FPP and the 20-carbon all-trans-GGPP isoprenoids, respectively, which can serve as substrates for protein farnesylation or geranylgeranylation (4, 5). FPP is also the direct precursor for the synthesis of cholesterol and serves as allylic substrate for cis- and trans-polyprenyl-diphosphate synthases involved in the synthesis of the long chain isoprenoids with cis- or trans-stereocchemistry, which are the precursors of dolichols and the lateral chain of ubiquinones, respectively (6, 7).

Prenyltransferases have been described in a wide variety of organisms, both prokaryotic and eukaryotic, where their isoprenoid products play essential roles in cellular processes. More recently, it has been shown that prenylation of cellular or viral proteins is required for the multiplication of certain viruses (8–11). In this connection, we have previously reported (12) the characterization of the first viral trans-prenyltransferase encoded by African swine fever virus (ASFV), a large enveloped DNA virus with an icosahedral morphology that assembles in the cytoplasm of the infected cell and causes a severe disease in domestic pigs (13, 14). The ASFV gene, designated B318L, contains an amino-terminal hydrophobic sequence and conserves all the regions characteristic of prenyltransferases. After cloning of the gene without the hydrophobic sequence, expression in Escherichia coli as a fusion to the E. coli protein thioredoxin, and purification, it was shown that the recombinant enzyme catalyzed the trans-addition of IPP to allylic diphosphates, with FPP being the best substrate of the reaction (12). However, under a variety of reaction conditions, the enzyme did not synthesize a unique product with a specific chain length. Although the major product of the reaction was GGPP, using IPP and FPP as substrates, additional longer chain prenyl diphosphates, containing up to 13 isoprene units, were synthesized in significant amounts. In addition, the viral enzyme could use GGPP efficiently as substrate. These properties are clearly distinct from those described for the cytosolic trans-GGPP synthase, the enzyme involved in the synthesis of the prenyl donor for protein geranylgeranylation, since this enzyme synthesizes GGPP as the ultimate product and is unable to use GGPP as substrate (15–20). On the other hand, specific all-trans-polyprenyl-diphosphate synthases, such as octaprenyl-diphosphate synthase (21) and solanesyl-diphosphate synthase (22), have also been shown to catalyze the synthesis of a defined length product.

Since the ASFV trans-prenyltransferase contains a putative transmembrane domain at the amino-terminal end, it was possible that its interaction with membranes in the cell environment could determine the chain length of the product synthesized, as in the case of some long chain polyprenyl-diphosphate synthases (23). Therefore, as a step toward a better understanding of the in vivo activity and function of the ASFV trans-prenyltransferase, we have examined the subcellular localization and activity of the viral enzyme in cells infected with ASFV or with a recombinant pseudo-Sindbis virus carrying the
ASVF gene. We show that the viral protein is associated with endoplasmic reticulum (ER) membranes in cells infected with the recombinant Sindbis virus, whereas in ASVF-infected cells, the enzyme is localized in the cytoplasmic viral assembly sites, where it is found associated with membrane structures that have been shown recently by Rouiller et al. (24) and André et al. (25) to derive from the ER. We also show that the ASVF enzyme is an integral membrane protein and that the membrane-bound enzyme synthesizes preferentially all-trans-GPP.

**Experimental Procedures**

**Cells, Viruses, and Reagents—**Vero (African green monkey kidney) cells were obtained from American Type Culture Collection and grown in Dulbecco’s modified Eagle’s medium containing 5% newborn calf serum. Baby hamster kidney (BHK) cells were from Invitrogen and grown in modified Eagle’s medium supplemented with 2 mM l-glutamine and 5% fetal bovine serum. The Vero adapted ASVF strain B714 was propagated and titrated as described previously (26). [1-4C]IPP (specific activity of 56 Ci/mol) was obtained from Amersham Pharmacia Biotech; trans-[1-3H]geranylgeraniol was from American Radiolabeled Chemicals, Inc.; and unlabeled isoprenoids compounds and cytosine arabinoside (Ara-C) were from Sigma. Silica Gel 60 and RP-18F254 thin-layer plates were from Merck, and acid phosphatase was from Roche Molecular Biochemicals.

**Antibodies—**The ASVF B318L gene was cloned into a pRSET vector (Invitrogen), and the recombinant protein was expressed in E. coli and purified under denaturing conditions using single-step Ni2+-nitrilotriacetic acid affinity chromatography. Antibodies against the purified recombinant trans-prenyltransferase (B318L protein) were raised in rabbits. The immune serum obtained was recognized the recombinant protein on Western blots. The anti-protein-disulfide isomerase mouse monoclonal antibody (1D3) was from Stressgen Biotech Corp.

**Metabolic Labeling, Immunoprecipitation, and Electrophoretic Analysis—**Preconfluent monolayers of Vero cells were infected with ASVF at a multiplicity of infection of 10 plaque-forming units/cell, and at different times post-infection, the cells were lysed in radioimmune precipitation assay buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% sodium deoxycholate, 1% Nonidet P-40, and 0.1% SDS), diluted 10-fold in the same buffer, preimmunoprecipitated with preimmune serum and protein A-Sepharose, and then immunoprecipitated with a 1:100 dilution of the anti-B318L serum in Tris-buffered saline/Tween with 5% dry milk powder for 1 h and then incubated overnight at 4 °C with a 1:500 dilution of the anti-pB318L serum in Tris-buffered saline/Tween with 5% dry milk powder. The membranes were blocked in Tris-buffered saline/Tween buffer (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, and 0.1% Tween 20) with 5% dry milk powder for 1 h and then incubated overnight at 4 °C with a 1:500 dilution of the anti-pB318L serum in Tris-buffered saline/Tween with 5% dry milk powder. The membranes were washed with Tris-buffered saline/Tween and incubated with a peroxidase-labeled anti-rabbit serum (Amersham Pharmacia Biotech), and the proteins were detected using an electrochemiluminescence system (ECL system, Amersham Pharmacia Biotech), and the proteins were detected using the ECL substrate system (ECL-Plus detection system, Amersham Pharmacia Biotech) according to the manufacturer’s recommendations.

**Construction of Recombinant Pseudo-Sindbis Virus—**The ASVF gene B318L, cloned in the pBluescript plasmid, was subcloned in the BbrFI restriction site of the pSinRep5 plasmid (Invitrogen Sindbis Expression System, Version C) to obtain the recombinant plasmid pSinRep/B318L, which was linearized with NotI. The linearized plasmid pSinRep/B318L and the DH-BB helper template were transcribed in vitro using the InvitroScript CAP SP6 in vitro transcription kit (Invitrogen). To obtain recombinant pseudovirus, the in vitro transcripts were transfected by electroporation into BHK cells following the instructions provided by Invitrogen. At 24 h after transfection, the supernatants were collected, and pseudovirions were concentrated by ultracentrifugation onto sucrose cushions.

**Immunofluorescence—**Vero cells, grown on chamber slides, were mock-infected or infected with ASVF at a multiplicity of infection of 1 plaque-forming unit/cell and fixed at 14 h post-infection with methanol at −20 °C for 5 min. The cells were then incubated with anti-B318L and anti-protein-disulfide isomerase antibodies, as indicated in the legend to Fig. 2, in phosphate-buffered saline containing 0.1% bovine serum albumin at 37 °C for 1 h; rinsed three times for 10 min with the same buffer; and incubated with fluororescinated goat anti-rabbit (Tago, Inc.) and Texas Red-linked sheep anti-mouse (Amersham Pharmacia Biotech) antibodies. Nuclear staining was performed by cotransfection with 5 μg of biotinylated (Hoechst 33258, Sigma) per ml of phosphate-buffered saline for 5 min. Cells were examined under a Zeiss Axiosview microscope. BHK cells were grown as described above and infected at a multiplicity of infection of 0.2 infective units/cell with recombinant pseudo-Sindbis virus carrying the ASVF B318L gene (Sind/B318L), and at 24 h post-infection, the cells were fixed and processed for immunofluorescence as described above.

**Immunoelectron Microscopy—**Vero cells were mock-infected or infected with ASVF at a multiplicity of infection of 10 plaque-forming units/cell and fixed, at 20 h post-infection, with 4% formaldehyde and 0.1% glutaraldehyde in 200 mM HEPES, pH 7.2, for 1 h at room temperature. After fixation, the cells were processed for cryosectioning as detailed by André et al. (25). Ultrathin thawed cryosections were incubated with a 1:40 dilution of the anti-B318L antibody followed by protein A-gold (10 nm).

**Subcellular Fractionation—**Subcellular fractionation of Vero cells was performed as described (28) with slight modifications. Vero cells were mock-infected or infected with ASVF at a multiplicity of infection of 5 plaque-forming units/cell, and at 19 h post-infection, the cells were resuspended in homogenization buffer containing 20 mM HEPES, pH 7.4, 0.28 M sucrose, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride and then disrupted by N2 cavitation at 400 p.s.i for 10 min at 4 ºC or by passing through a 25-gauge syringe. In the case of mock- or Sindbis virus-infected BHK cells, the cells were resuspended in the same homogenization buffer as described above and disrupted manually using a Kontes homogenizer. In all cases, the homogenate was centrifuged at 700 × g for 5 min to sediment nuclei, and the supernatant fraction was centrifuged at 150,000 × g for 30 min at 4 ºC to obtain the membrane and cytosolic fractions. For prenyltransferase assays, the membrane fraction was resuspended in homogenization buffer at a protein concentration of 3–7 mg/ml and stored, as the cytosolic fraction, at −70 ºC until use.

For the analysis of membrane association of the B318L protein, membrane fractions from mock-or ASVF-infected Vero cells were treated with sodium carbonate or Triton X-114 according to the methods of Fujiaki et al. (29) and Pryde and Phillips (30). For carbonate treatment, membrane fractions from 4 × 107 cells were resuspended in 100 μl of 0.1 M Na2CO3, pH 11.5, incubated for 30 min at 4 °C, and subsequently centrifuged at 100,000 × g for 30 min. The supernatant and the sediment were dissociated with electrophoresis sample buffer. For treatment with Triton X-114, an equivalent amount of the membrane fraction was resuspended in 100 μl of 2% Triton X-114, 150 mM NaCl, and 10 mM Tris-HCl, pH 7.5, incubated for 10 min at 4 °C, and then transferred to 30 °C for 20 min. The samples were briefly spun in a microcentrifuge to separate the lower detergent-rich phase from the upper phase, and both phases were dissociated as described above.

Equivalent amounts of each sample were subjected to Western blot analysis.

**Assay of Prenyltransferase Activity and Product Analysis—**The regular assay mixture contained, in a final volume of 0.1 ml, 50 μM [1-14C]IPP (specific activity of 24 cpnmol), 100 mM all-trans-FPP, 2 mM MgCl2, 1 mM dithiothreitol, 20 mM Tris-HCl, pH 7.5, 50 μg/ml bovine serum albumin, 20 mM KF, and enzyme protein as indicated. For 4 h at 37 °C, the products were extracted with butanol as described before (12), and the butanol extracts were treated with acid phosphatase by the method of Fujiaki et al. (31). The hydrolysates were extracted with hexane, and the hexane-soluble products were analyzed by reversed-phase RP-18 thin-layer chromatography in aceton/H2O (95:5). The positions of authentic standards were visualized with iodine vapor. The thin-layer plates were exposed for autoradiography on a Fuji BAS-MP 20405 imaging plate, and the exposed imaging plate was analyzed with a Fuji BAS 1500 analyzer. Densitometric analysis of the bands was performed using TINA Version 2.0 software.

**Results**

**Synthesis and Localization of ASVF trans-Prenyltransferase in Infected Cells—**We have previously shown that the ASVF trans-prenyltransferase gene B318L is transcribed at late times of infection (12). In agreement with this, the results of immunoprecipitation experiments presented in Fig. 1A indicate that the synthesis of the enzyme in ASVF-infected Vero
cells was initiated at 8–10 h post-infection, a time at which viral DNA replication is already underway (32); reached a maximum at 13–15 h post-infection; and decreased at later times. Furthermore, the protein was not synthesized in the presence of Ara-C, an inhibitor of viral DNA replication and late transcription (32), indicating that the ASFV trans-prenyltransferase is a late protein. Western blot analysis revealed the presence of a specific band of \(~37 \text{kDa}\) in ASFV-infected cells. This band was first detected at 12 h post-infection and accumulated up to 24 h post-infection (Fig. 1B). In keeping with the immunoprecipitation results, the protein band was not seen in the presence of Ara-C (Fig. 1B).

As mentioned in the Introduction, the trans-prenyltransferase of ASFV contains a putative transmembrane domain at its amino-terminal end, suggesting an association of the protein with cellular or viral membranes. To test this possibility, we first examined the intracellular localization of the trans-prenyltransferase in BHK cells infected with a recombinant pseudo-Sindbis virus carrying the ASFV gene to determine whether the enzyme was targeted to cellular membranes independently of other ASFV proteins or processes related to the infection with ASFV. The results of double immunofluorescence experiments using antibodies against the ASFV trans-prenyltransferase (B318L protein) and the ER marker protein protein-disulfide isomerase indicated that the viral enzyme localized to the ER (Fig. 2, B and C). A very low background immunofluorescence was observed with the anti-B318L antibody in the case of mock-infected BHK cells, which served as a control (Fig. 2A). A similar low background signal was detected with this antibody in mock-infected Vero cells (Fig. 2D). On the other hand, in Vero cells infected for 14 h with ASFV, the trans-prenyltransferase was located in discrete cytoplasmic areas close to the nucleus, which were identified as viral assembly sites by staining of the viral DNA with bisbenzimide (Fig. 2, E and G). It should also be noted that at an earlier time of infection (10 h), when the synthesis of the protein has just been initiated (see Fig. 1), the same immunofluorescent pattern was obtained (data not shown). In contrast, the protein-disulfide isomerase signal was excluded from these areas and was found surrounding the viral factories, whereas in cells lacking factories, the signal showed a pattern characteristic of the ER (Fig. 2F).

To examine in detail the localization of the trans-prenyltransferase within the viral factories, immunocytochemical studies with electron microscopy were performed. For this, ultrathin thawed cryosections of Vero cells mock-infected or infected with ASFV for 20 h were incubated with the anti-B318L protein antibody followed by protein A-gold. A low immunogold labeling was observed in the case of mock-infected cells (Fig. 3A). In cells infected with ASFV, the antibody strongly labeled the region of the viral factories, in keeping with the immunofluorescence results (Fig. 3B). Within the factories, the gold grains were seen decorating mainly membrane-like structures (Fig. 3C), which are precursors of the inner envelope of ASFV (33) and are derived from the ER (24, 25). Gold grains were essentially excluded from mature virus...
particles (Fig. 3B). In agreement with this, Western blot analysis of highly purified ASFV using the antibody against the B318L protein has indicated that the trans-prenyltransferase is not a structural component of the virus (data not shown).

ASFV trans-Prenyltransferase Is an Integral Membrane Protein—The above studies indicated that the ASFV trans-prenyltransferase was associated in the viral factories with membranes that are derived from the ER. To further investigate the association of the viral enzyme with membranes, cell fractionation experiments were performed, and the distribution of the viral enzyme into membrane and cytosolic fractions from ASFV-infected cells was examined using the anti-B318L protein antibody. As a control, a similar fractionation of mock-infected cells was carried out. In the membrane fraction from ASFV-infected cells, a specific band corresponding to the ASFV prenyltransferase was detected (Fig. 4, lane 4). This band was not found in the cytosolic or membrane fractions from mock-infected cells (Fig. 4, lanes 1 and 2). On the other hand, a cellular protein that partitioned exclusively into the cytosolic fraction from mock-infected and infected cells was detected (Fig. 4, lanes 1 and 3).

To analyze the nature of the association of the viral enzyme with membranes, washes with alkaline carbonate and centrifugation with Triton X-114 were performed as indicated under “Experimental Procedures.” After washing the membranes from infected cells with alkaline carbonate and centrifugation, the ASFV B318L protein remained associated with the sediment (Fig. 4, lanes 5 and 6), indicating that it is not peripherally bound to the membranes. On the other hand, phase fraction of membrane proteins with Triton-X114 showed that the viral protein behaved as an integral membrane protein since it partitioned into the detergent phase (Fig. 4, lanes 7 and 8).

Prenyltransferase Activity in Membrane Fractions of ASFV-infected Cells—To determine the activity and products of the ASFV prenyltransferase in extracts from infected cells, the cytosolic and membrane fractions were incubated with [1-14C]IPP and FPP in a standard reaction mixture (see “Experimental Procedures”), and the reaction products were analyzed by reversed-phase thin-layer chromatography after butanol extraction and hydrolysis with acid phosphatase to the corresponding alcohols as described under “Experimental Procedures.” A low GGPP synthase activity was detected in the cytosolic fraction of mock-infected cells, but this activity did not increase in the virus-infected cells (data not shown). The results obtained with the membrane fractions are shown in Fig. 5. In the case of mock-infected cells, the major products formed were prenyl diphosphates of 30 and 50 carbons, whereas the levels of GGPP synthesized were very low (Fig. 5A, lane 2). In contrast, the membrane fraction of ASFV-infected cells produced predominantly GGPP, together with geranylgeranyl diphosphate, although in considerably lower amounts (Fig. 5A, lane 4). On the other hand, the synthesis of the C30 and C50 products was comparable to that found in mock-infected cells. In the presence of 1% Triton X-100, the products synthesized remained essentially unchanged (Fig. 5A, lanes 3 and 5). In all cases, similar levels of 14C-labeled FPP were produced, probably due to the presence of IPP isomerase in the membrane preparations. The product distribution obtained with the enzyme from the membrane fraction of infected cells was very similar using different amounts of protein (between 50 and 150 μg) in the assay and various reaction times (1–16 h) (data not shown).

A densitometric quantification of the products synthesized by the membrane fractions of mock- and ASFV-infected cells showed an increase of ~25-fold in the amount of GGPP generated by the prenyltransferase of infected cells with respect to that of mock-infected cells (Table I). On the contrary, no significant differences were found in the levels of the C30 and C50 polyprenyl diphosphates. The C50 isoprenoid detected in the mock-infected and infected cells could be a precursor of the ubiquinone (Q) side chain. In keeping with this, an analysis of the Q species present in Vero cells mock-infected or infected with ASFV revealed that Q-10, which contains a lateral chain of 50 carbons, was the predominant form in both cases.  

As described previously (12) and as shown in Fig. 5A (lane 1),

\[ \text{Geranylgeranyl-diphosphate Synthase of ASFV} \]

\[ \text{FIG. 4. Subcellular distribution and membrane association of trans-prenyltransferase in ASFV-infected Vero cells.} \]

Mock-infected (lanes 1 and 2) or ASFV-infected (lanes 3 and 4) cells were disrupted at 20 h post-infection and fractionated into cytosolic (lanes 1 and 3) and membrane (lanes 2 and 4) fractions as described under “Experimental Procedures.” Membrane fractions from infected cells were treated with sodium carbonate or Triton X-114 as indicated under “Experimental Procedures”: the supematant (lane 5) and sediment (lane 6) from carbonate-treated membrane fractions from ASFV-infected cells and the upper (lane 7) and lower (lane 8) phases from Triton X-114-extracted membrane fractions from infected cells. Samples were analyzed by Western blotting with the anti-B318L antibody. The migration positions of molecular mass markers are shown on the left. The arrow indicates the band corresponding to the B318L protein.

Fig. 3. Immunoelectron microscopy with anti-B318L antibody in ASFV-infected Vero cells. Vero cells were mock-infected or infected with ASFV, fixed at 20 h post-infection, and processed for cryosectionning. Ultrathin sections were incubated with the anti-B318L antibody followed by protein A-gold (10 nm) as described under “Experimental Procedures.” A, section of a mock-infected cell showing a region of the cytoplasm. M, mitochondria; G, Golgi. B, section of an infected cell showing a region of the cytoplasm containing a viral factory (VF). The upper left area with very few gold particles corresponds to the cytoplasmic region surrounding the viral factory. The arrow indicates a mature virion at the limit of the factory. C, region of a viral factory at a higher magnification. Arrowheads indicate gold particles decorating membrane-like structures. Bars = 200 nm in A–C.

\[ \text{A. Alejo and M. L. Salas, unpublished results.} \]
iodine vapors. The positions of the markers are indicated on the right.

The chromatogram was cut into rectangles, and the radioactivity was determined by scintillation counting. The unlabeled marker was revealed with 

ASFV cells were used. 

performed in the absence of detergent. After 37 °C, the products were treated with acid phosphatase and analyzed by reversed-phase RP-18F254-S thin-layer chromatography as described under “Experimental Procedures.” The sizes of some of the most relevant products synthesized are indicated on the right. B, Silica Gel 60 thin-layer chromatogram. To determine the stereochemistry of the double bond, the radioactivity corresponding to the geranylgeraniol bands was eluted with ether and analyzed on silica gels. Shown is geranylgeraniol from reactions catalyzed by membrane extracts from ASFV-infected cells (lane 1) or by the recombinant enzyme (lane 2). Unlabeled all-trans-geranylgeraniol (t,t,t-GGOH) and 3H-labeled trans,trans-cis-geranylgeraniol (t,c-GGOH) were used as markers. The 3H-labeled geranylgeraniol product was detected as described under “Experimental Procedures.” To determine the migration position of the 3H-labeled trans,trans,cis-geranylgeraniol marker, the chromatogram was cut into rectangles, and the radioactivity was determined by scintillation counting. The unlabeled marker was revealed with iodine vapors. The positions of the markers are indicated on the right. ori, origin. 

TABLE I 

Quantification of isoprenoid compounds produced by membrane-associated enzyme

The products were quantified by densitometric scanning, and the data were then normalized with respect to the farnesol band in each experiment. The data are means ± S.E. of at least eight independent experiments.

| Product          | Membrane fractiona | Increase 
|------------------|---------------------|---------|
|                  | Mock                | ASFV    |
| Geranylgeraniol  |                     | 1.78 ± 0.67 | 25.4 |
| C50              | 0.11 ± 0.11         | 0.17 ± 0.12 | 1.5 |
| C50              | 0.12 ± 0.12         | 0.66 ± 0.29 | 1.5 |

a Membrane fractions from mock-infected (Mock) or ASFV-infected (ASFV) cells were used.

The purified recombinant ASFV trans-prenyltransferase produced prenyl diphosphates containing 4–12 isoprene units. In contrast, chain elongation by the enzyme present in the membrane fraction from infected cells was more limited (Fig. 5A, lanes 4 and 5). Thus, the ratio of GGPP to geranylgeraniol diphosphate (C25) was 3.6 ± 0.7 (n = 6) in the case of the recombinant enzyme and 8.5 ± 1.5 (n = 9) for the membrane prenyltransferase. Additional longer chain isoprenoids were synthesized in very low amounts by the enzyme from infected cells (Fig. 5A).

To determine the trans,cis-configuration of GGPP synthesized by the viral enzyme, the radioactivity of the geranylgeraniol band was eluted from the reversed-phase chromatogram and analyzed by silica gel thin-layer chromatography. The resulting autoradiogram revealed a main spot comigrating with all-trans-geranylgeraniol (Fig. 5B, lane 1), as in the case of the recombinant ASFV trans-prenyltransferase (lane 2), whereas no product comigrating with trans,trans,cis-geranylgeraniol was detected.

Taken together, the above results suggested that the activity detected in the membrane fraction of infected cells corresponded to the ASFV trans-prenyltransferase. To confirm this, we first tested the effect of the anti-B318L antibody on the reaction catalyzed by this subcellular fraction. Fig. 6A shows that the specific antibody strongly inhibited the formation of GGPP and geranylgeraniol diphosphate by the enzyme from infected cells (lanes 4 and 5), whereas a preimmune serum had no effect (lane 6). In contrast, the reaction catalyzed by the membrane fraction of mock-infected cells was not affected by the anti-B318L antibody (Fig. 6A, lanes 2 and 3), and this antibody did not decrease the formation of the C30 and C50 products obtained with the membrane preparation of infected cells (lanes 4 and 5), suggesting that these compounds are synthesized by the cellular polyprenyl-diphosphate synthase.

In a different approach, we assayed prenyltransferase activity in a membrane fraction prepared from BHK cells infected with the recombinant Sind/B318L virus expressing the ASFV B318L gene. When compared with mock-infected BHK cells (Fig. 6B, lane 1), a considerable increase (16-fold as determined by densitometric scanning) was observed in the amount of GGPP synthesized by the membrane fraction from cells infected with the recombinant Sind/B318L virus (lane 2). With this fraction, lower amounts of geranylgeraniol diphosphate and C50–40 isoprenoid compounds were also produced. On the other hand, the synthesis of C45 and C50 polyprenyl diphosphates increased only 2.5-fold in the infected cells relative to mock-infected cells. Thus, the products generated by the enzyme present in the membrane fraction from cells infected with the recombinant Sindbis virus were very similar to those synthesized by the corresponding fraction from ASFV-infected cells.

DISCUSSION

A previous characterization of the trans-prenyltransferase encoded by ASFV showed that the purified recombinant enzyme, lacking an amino-terminal hydrophobic region and expressed as a fusion with the E. coli protein thioredoxin, catalyzed the synthesis of GGPP as the major product, as well as longer prenyl diphosphates containing up to 13 isoprene units, using FPP and IPP as substrates (12). On the basis of these results, two possible roles for the viral enzyme during infection were proposed; one of them was the formation of GGPP, a product that could then be used for prenylation of proteins during the virus life cycle. If this were the case, the synthesis of longer isoprenoid compounds could be due to an inability of the recombinant enzyme to terminate chain elongation at the GGPP step. As an alternative, the possibility was also considered that the long chain polyprenyl diphosphates synthesized could serve as precursors of the Q side chains. The synthesis of Q, in turn, could be related to an increase in mitochondrial...
function or to a role of Q as antioxidant during the infection of macrophages, the target cells in natural ASFV infection, to prevent oxidative damage to viral components since these cells generate large amounts of reactive oxygen species for their microbicidal functions.

To obtain further information on the natural products of the ASFV trans-prenyltransferase and on its function during the infectious cycle, we have examined, in this study, the subcellular localization of the viral enzyme and its activity in extracts from infected cells. Expression of the trans-prenyltransferase in a recombinant Sindbis virus has allowed us to demonstrate its targeting to the ER, a result that is consistent with the presence of a transmembrane domain in the protein. On the other hand, in cells infected with ASFV, the enzyme is found in the cytoplasmic virus assembly sites, associated with membrane structures that are precursors of the viral inner envelope and are derived from the ER (24, 25, 33). Therefore, it is possible that in ASFV-infected cells, the enzyme is synthesized within the viral factories, being thus directed to the precursor viral membranes of the assembly areas or to nearby ER membranes, which would then be rapidly incorporated into the factories to form the viral membranes. During the maturation process of the virus particles from these membranes, the trans-prenyltransferase must be excluded from them since it is not found in the mature virions.

The enzyme present in membrane preparations from cells infected with ASFV or with the recombinant Sindbis virus expressing the ASFV gene catalyzes predominantly the synthesis of all-trans-GGPP, with little further elongation to longer chain polypropenyl diphosphates, in contrast to the results obtained in the case of the recombinant enzyme expressed in E. coli. Although this difference could reflect a better control of polymerization by the membrane-associated enzyme, the fact that the pattern of isoprenoid distribution is not altered by the presence of detergent suggests that an integral membrane structure is not required to determine the specificity of products. It is possible that other factors might influence chain polymerization.

On the other hand, the finding that the formation of GGPP by the membrane fraction of ASFV-infected cells is greatly increased with respect to that of mock-infected cells, whereas the synthesis of the C50 precursor of Q remains essentially unchanged, strongly supports that the natural product of the ASFV enzyme is all-trans-GGPP, allowing its classification as a trans-GGPP synthase. However, two features of the viral enzyme distinguish it from the cellular trans-GGPP synthases. Thus, the cellular enzymes are cytosolic proteins (17, 20) and strictly control chain termination at the GGPP step (15, 16). In contrast, the ASFV trans-GGPP synthase is an integral membrane protein and elongates to some extent the GGPP product, even when assayed in a membrane-associated form.

As mentioned above, GGPP synthesized by the ASFV trans-prenyltransferase could serve as substrate for prenylation of cellular or viral proteins. The synthesis of the enzyme at the late stage of infection and its localization in precursor viral membranes within the viral factories are consistent with the view that this modification might be required in the morphogenetic processes of the virus, as has been described for hepatitis δ virus and murine leukemia virus (8–11). In the first case, it has been shown that the large antigen of the virus is farnesylated (34) and that this modification facilitates virion assembly (8, 9, 11). On the other hand, the studies with murine leukemia virus indicate that prenylation of cellular Rab proteins is required for the processing and incorporation of viral envelope precursors into the virions (10). We are presently examining whether viral or cellular proteins are prenylated during the ASFV infectious cycle. A possible candidate to be modified by prenylation is the viral protein encoded by the open reading frame L83L, which contains a carboxyl-terminal CTIL type I geranylgeranylation motif (35) and is transcribed at a late time of infection.2 The use of drugs known to interfere with the prenylation of proteins may also help to understand the role, if any, of protein prenylation during the infectious cycle.

Attempts to construct ASFV recombinants depleted of the trans-prenyltransferase gene have failed,2 suggesting that the gene is essential for virus multiplication. A system for inducible gene expression from ASFV recombinants, recently developed in our laboratory (36), might be useful to investigate the role of the trans-prenyltransferase during virus replication.

The association with membranes of the ASFV enzyme is intriguing in view of the fact that the cellular trans-GGPP synthases have been described as cytosolic proteins. Recent studies on the mammalian protein farnesyltransferase provide clues to the possible physiological significance of that association. Thus, it has been shown that product release is the rate-limiting step in catalysis (37) and that dissociation of the product from the enzyme requires the provision of additional FPP substrate (38). It has been proposed that the farnesyltransferase-product complex is directed to a membrane compartment where FPP would be located, thus triggering the release
of the farnesylated protein (38). In a similar fashion, GGPP generated by the membrane-bound trans-prenyltransferase of ASFV might efficiently promote the dissociation of geranylgeranyltransferase-product complexes targeted to the viral membranes in the factories. This could facilitate the prenylation of proteins playing a role in the virus replication cycle.

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REFERENCES

1. Poulter, C. D., and Rilling, H. C. (1981) in Biosynthesis of Isoprenoid Compounds (Porter, J. W., and Spurgeon, S. L., eds) Vol. 1, pp. 161–282, John Wiley & Sons, Inc., New York
2. Rilling, H. C., and Chayet, L. T. (1985) in Sterols and Bile Acids (Danielson, H., and Stovall, J., eds) pp. 17–23, Elsevier Science Publishing Co., Inc., New York
3. Goldstein, J. L., and Brown, M. S. (1990) Nature 343, 425–430
4. Glomset, J. A., Gelb, M. H., and Farnsworth, C. C. (1990) Trends Biochem. Sci. 15, 139–142
5. Zhang, F. L., and Casey, P. J. (1996) Annu. Rev. Biochem. 65, 241–269
6. Chojnacki, T., and Dallner, G. (1988) Biochem. J. 251, 1–9
7. Olson, R. E., and Rudney, H. (1983) Vitam. Horm. 40, 1–43
8. Glenn, J. S., Watson, J. A., Havel, C. M., and White, J. M. (1992) Science 256, 1313–1333
9. Hwang, S. B., and Lai, M. C. (1993) J. Virol. 67, 7659–7662
10. Overmeyer, J. H., and Maltese, W. A. (1992) J. Biol. Chem. 267, 22686–22692
11. Lee, C.-Z., Chen, P.-J., Lai, M. M. C., and Chen, D.-S. (1994) J. Virol. 68, 169–175
12. Alejo, A., Yáñez, R. J., Rodriguez, J. M., Viñuela, E., and Salas, M. L. (1997) J. Biol. Chem. 272, 9417–9423
13. Viñuela, E. (1985) Curr. Top. Microbiol. Immunol. 116, 151–170
14. Costa, J. V. (1990) in Molecular Biology of Iridoviruses (Darai, G., ed) pp. 247–270, Kluwer Academic Publishers, Dordrecht, Holland
15. Hugueney, P., and Camara, B. (1996) FEBS Lett. 374, 238–238
16. Sagami, H., Morita, Y., and Ogura, K. (1994) J. Biol. Chem. 269, 20561–20566
17. Reed, B. C., and Rilling, H. C. (1975) Biochemistry 14, 50–54
18. Yeh, L.-S., and Rilling, H. C. (1977) Arch. Biochem. Biophys. 183, 718–725
19. Barnard, G. F., and Popjak, G. (1981) Biochim. Biophys. Acta 661, 87–99
20. Ericsson, J., Runquist, M., Thelin, A., Andersson, M., Chojnacki, T., and Dallner, G. (1993) J. Biol. Chem. 268, 832–838
21. Fujisaki, S., Nishino, T., and Katsuki, H. (1986) J. Biochem. (Tokyo) 99, 1327–1337
22. Ohnuma, S., Koyama, T., and Ogura, K. (1991) J. Biol. Chem. 266, 23706–23713
23. Matsuoka, S., Sagami, H., Kurisaki, A., and Ogura, K. (1991) J. Biol. Chem. 266, 3464–3468
24. Rouiller, I., Brookes, S. M., Hyatt, A. D., Windsor, M., and Wileman, T. (1998) J. Virol. 72, 2373–2387
25. Andrés, G., García-Escudero, R., Simón-Mateo, C., and Viñuela, E. (1998) J. Virol. 72, 8988–9001
26. Enjuanes, L., Carrascosa, A. L., Moreno, M. A., and Viñuela, E. (1976) J. Gen. Virol. 32, 471–477
27. Ausubel, F. M., Brub, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1994) Current Protocols in Molecular Biology Unit 16.20, Greene Publishing Associates/Wiley-Interscience, New York
28. Rome, L., Garvin, A. J., Allietta, M. M., and Neufeld, E. F. (1979) Cell 17, 143–153
29. Fujiki, Y., Hubbard, A. L., Fowler, S., and Lazarov, P. B. (1982) J. Cell Biol. 93, 97–102
30. Pryde, J. G., and Phillips, J. H. (1986) Biochem. J. 233, 525–533
31. Fujii, H., Koyama, T., and Ogura, K. (1982) Biochim. Biophys. Acta 712, 716–718
32. Salas, M. L., Rey-Campos, J., Ahmendral, J. M., Talavera, A., and Viñuela, E. (1986) Virology 152, 228–240
33. Andrés, G., Simón-Mateo, C., and Viñuela, E. (1997) J. Virol. 71, 2331–2341
34. Otto, J. C., and Case, P. J. (1996) J. Biol. Chem. 271, 4569–4572
35. Yáñez, R. J., Rodríguez, J. M., Nogal, M. L., Yuste, L., Enríquez, C., Rodríguez, J. F., and Viñuela, E. (1995) Virology 208, 249–278
36. García-Escudero, R., Andrés, G., Almazán, F., and Viñuela, E. (1998) J. Virol. 72, 3185–3195
37. Furine, E. S., Leban, J. J., Landavazo, A., Moonaw, J. F., and Casey, P. J. (1995) Biochemistry 34, 6877–6882
38. Tschante, W. R., Furine, E. S., and Casey, P. J. (1997) J. Biol. Chem. 272, 9989–9993