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Phosphatidylserine is involved in gene expression from Sindbis virus subgenomic promoter

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Abstract

Sindbis virus replication is mediated by an RNA replicase translated from viral RNA genome. The replicase synthesizes progeny genomic RNA and shorter RNA (subgenomic RNA) carrying viral structural genes in association with cytoplasmic membranes. Here we examined the involvement of a membrane lipid, phosphatidylserine (PS), in Sindbis virus gene expression using Chinese hamster ovary cell mutants. When the mutant cells were transfected with viral replicon RNA, in which the structural genes downstream of the subgenomic promoter were replaced by a reporter gene, reporter expression was inhibited under PS-deficient conditions. In contrast, reporter expression from an SV40 promoter-driven construct was normal under similar conditions. Furthermore, expression of a viral replicase protein from the genomic RNA and accumulation of the subgenomic RNA were not inhibited by PS deficiency. These findings indicate that reduced cellular PS level impairs a posttranscriptional event of Sindbis virus subgenomic promoter-driven gene expression.

Keywords: Phosphatidylserines; Sindbis virus; Alphavirus; Gene expression; Membrane lipids; Phospholipids; Virus replication; RNA replicase; Replicon

Sindbis virus (SINV), the prototype of the genus *Alphavirus*, is a small enveloped RNA virus with a single capped positive-stranded RNA genome of approximately 12 kb [1]. The 5′ two-thirds of the genome encode four nonstructural or replicase proteins, whereas the 3′ one-third encodes three structural proteins. The replicase proteins nsP1–4 are indispensable for viral RNA synthesis [2,3] and initially translated from the genome as a large polyprotein, which is eventually processed into individual proteins [4]. Early in infection, a replicase composed of the processing intermediate polyprotein and the catalytic subunit nsP4 synthesizes a complementary negative-strand RNA, which serves as the template for subsequent positive-strand synthesis [5,6]. Later, mature nsP1–4 become constituents of the replicase, and subsequently synthesize a progeny genomic RNA and a subgenomic RNA encoding the structural proteins.

Alphavirus infection induces small invaginations on cytoplasmic membranes of endosomes and lysosomes in infected cells [7–10]. The invaginations, called spherules, appear to be the sites of RNA synthesis, because replicase proteins and newly synthesized viral RNA are located inside them [7,10]. The membrane association, and resultant membrane wrapping, of viral replicase are not unique to alphaviruses, being commonly seen upon infection by many other positive-strand RNA viruses, as reviewed [11–13]. Several studies suggested that membrane lipids are important for the replication of positive-strand RNA viruses. For example, RNA synthesis by Semliki forest virus (SFV) [14], another alphavirus, and other positive-strand RNA viruses [15–17] is sensitive to an inhibitor of de novo fatty acid synthesis. Moreover, RNA synthesis by brome mosaic virus, a member of the alphavirus-like superfamily, is inhibited by a host defect in fatty acid

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synthesis [18]. These studies have stimulated new interest in the possible function of specific lipids in the replication of viral RNA.

Membrane association of the alphavirus replicase is mediated by nsP1 [19,20], which is essential for negative-strand RNA synthesis [21] and viral RNA capping [22–24]. Analyses of the membrane association revealed a specific interaction between SFV nsP1 and membrane phospholipids, as reviewed [12]. In a cell-free system, phosphatidylserine (PS) and other anionic phospholipids associate with amino acid residues 245–264 of SFV nsP1, thereby activating its methyltransferase and guanylyltransferase activities [25]. The corresponding synthetic peptide forms an amphipathic α-helix and specifically binds to liposomes containing anionic phospholipids [26]. However, the physiological relevance of the interaction between nsP1 and anionic phospholipids remains unknown.

We have established Chinese hamster ovary (CHO) cell mutants defective in PS biosynthesis to investigate the biosynthetic pathway and function of the lipid in mammalian cells. PS, which accounts for approximately 5–10% of all phospholipids, is synthesized through the exchange of polar head groups of pre-existing phospholipids with free serine in mammalian cells. Our genetic studies revealed that PS synthesis is catalyzed by two enzymes: PS synthase (PSS) 1 converts phosphatidylcholine to PS, whereas PSS 2 converts phosphatidylethanolamine (PE) to PS [27]. A CHO cell mutant, PSA-3, is defective in PSS 1 and needs to be supplemented with PS to grow and to maintain a normal phospholipid composition [28]. Mutant cells not supplemented with PS exhibit decreases in amounts of PS and PE, the latter of which is synthesized via decarboxylation of PS, and eventually stop growing. By using PSA-3 cells, we have previously shown that host cell PS and PE deficiencies inhibit the production of progeny virus by SINV [29]. The binding and internalization of the virus seem to occur normally, but viral RNA synthesis is inhibited in the PS- and PE-deficient cells. Although any of the post-internalization steps could be potentially inhibited by a deficiency of PS, the fact that SINV nsP1 contains a sequence similar to that of the PS-binding peptide of SFV [26] prompted us to investigate the role(s) of PS in viral RNA synthesis and related events.

In the present study, we examined the effect of a deficiency of PS on SINV replicase-mediated gene expression using a SINV replicon expressing a reporter protein [30] and CHO mutant cells. The replicon system bypasses the virus entry and genome uncoating steps involved in infection, facilitating analysis of the RNA replication step. We found that the PS deficiency specifically impairs viral subgenomic promoter-driven gene expression. We discuss the possible role of PS in the gene expression process.

Materials and methods

Cell culture. CHO-K1 cells were obtained from the American Type Culture Collection, and maintained in Ham's F-12 medium (Invitrogen) supplemented with 10% (v/v) newborn bovine serum (ICN Biomedicals), 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate. PSA-3 [28] and PSB-2 [31] cells were maintained in the growth medium supplemented with 30 μM PS (from bovine brain; Sigma) as described [32]. Growth medium supplemented with 30 μM PE (from egg yolk; Sigma) was prepared as described [32]. All cultures were performed at 37 °C under a 5% CO_2 atmosphere and 100% humidity.

DNA constructs. A SINV replicon, SINrep/LacZ [30], was purchased from Invitrogen. For preparation of a plasmid, pSVOKneolacZ, a cDNA fragment carrying the *lacZ* gene was cleaved from SINrep/LacZ with *Xba*I and *Stu*I, and then ligated with the backbone of pSVOKneo [33] cleaved with *Xba*I and *Sna*BI.

Preparation of replicon RNA. Capped and polyadenylated replicon RNA was synthesized from *Xho*I-linearized SINrep/LacZ using an SP6 mMESSAGE mMACHINE™ kit (Ambion). After digestion with DNase I (Ambion), the replicon RNA was purified using an RNeasy® mini kit (Qiagen).

Transfection. CHO-K1 and PSA-3 cells ($1–2 \times 10^5$ cells) were seeded into each well of a 6-well plate 2 days before transfection and then grown in medium supplemented with PS. The medium was replaced with that supplemented with PS or unsupplemented 1 day before transfection. PSB-2 cells ($3–4 \times 10^5$ cells) were similarly seeded 1 day before transfection and grown in medium supplemented with PS. The cells were washed and then transfected with either 5–10 μg RNA or 4 μg DNA construct with the aid of 10 μl of DMRIE-C reagent (Invitrogen) in 1 ml of Opti-MEM® I reduced serum medium (Invitrogen). After 4 h, the cells were washed and further grown with or without lipid supplementation. At specific points in time, the cells were washed and harvested.

Preparation and fractionation of a post-nuclear supernatant. After swelling in hypotonic buffer [10 mM Tris–HCl (pH 7.5), 10 mM NaCl, and 0.1 mM (*p*-amidinophenyl)-methanesulfonyl hydrochloride] for 15 min on ice, cells were disrupted by sonication with a W-225R Ultrasonic disrupter (Heat System Ultrasonics) equipped with a No. 419 microtip, and then centrifuged at $1500g_{av}$ for 10 min at 4 °C to obtain a post-nuclear supernatant. For fractionation, cells were disrupted by Dounce homogenization and similarly centrifuged. After the addition of NaCl to a final concentration of 1 M, the post-nuclear supernatant was allowed to stand on ice for 30 min, and then centrifuged at $96{,}000g_{av}$ for 30 min at 4 °C to obtain membrane and soluble fractions. The membrane fraction was suspended in high-salt buffer [10 mM Tris–HCl (pH 7.5), 1 M NaCl, and 0.1 mM (*p*-amidinophenyl)-methanesulfonyl hydrochloride].

β-Galactosidase assay. The β-galactosidase assay was performed using the lactose analog orthonitrophenyl-β-D-galactoside (Sigma) as the substrate according to the manual for the Sindbis expression system (Invitrogen). One unit of β-galactosidase was defined as the amount of the enzyme that hydrolyzed 1 μmol of orthonitrophenyl-β-D-galactoside per minute at pH 7.5 and 37 °C.

Anti-nsP1 antibody. An anti-nsP1 antibody was raised by immunization of a rabbit with recombinant hexahistidine-tagged SINV nsP1 prepared as follows: a cDNA fragment encoding hexahistidine-tagged nsP1 was generated by polymerase chain reaction using a sense primer containing a *Bam*HI site (5′CGCGGATCCATGGAGAAGCCAGTAGTAAAC3′), an antisense primer containing an *Xho*I site (5′TATCTCGAGTTATGCTCCGATGTCCGCCTG3′), and SINrep/LacZ. The product was cleaved with *Bam*HI and *Xho*I, and then ligated with the similarly cut backbone of pQE-9 (Qiagen). After sequence confirmation, the *Escherichia coli* M15[pREP4] strain (Qiagen) was transformed with the construct, induced to express the tagged nsP1, and then lysed with extraction buffer [50 mM sodium phosphate (pH 7), 8 M urea, and 0.3 M NaCl]. The tagged protein was purified from the lysate by affinity chromatography on TALON® metal affinity resin (Clontech), followed by gel filtration chromatography on Superdex 200 HR 10/30 (GE Healthcare Biosciences). The identities of the purified protein and the tagged nsP1 were verified by mass spectrometry.

Immunoblotting. Protein samples dissolved in SDS sample buffer [62.5 mM Tris–HCl (pH 6.7), 2% SDS, 50 mM dithiothreitol, 10% (v/v) glycerol, and 0.025% bromophenol blue] were separated by 7.5% SDS–polyacrylamide gel electrophoresis as described [34], and then transferred