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Abortive infection with Sindbis virus of a Chinese hamster ovary cell mutant defective in phosphatidylserine and phosphatidylethanolamine biosynthesis

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The effects of phosphatidylserine starvation on the infection with Sindbis virus (an enveloped RNA virus) have been investigated in a Chinese hamster ovary (CHO) cell mutant (strain PSA-3) which requires exogenously added phosphatidylserine for cell growth because it lacks the ability to synthesize this phospholipid. When PSA-3 cells were grown in the absence of phosphatidylserine, the cellular contents of phosphatidylserine and also phosphatidylethanolamine produced through decarboxylation of phosphatidylserine decreased. Sindbis virus production in the mutant cells decreased immediately upon phosphatidylserine deprivation as did the contents of phosphatidylserine and phosphatidylethanolamine, whereas the cell growth, viability, and syntheses of protein, DNA and RNA remained normal for approx. 40 h phosphatidylserine starvation. Although PSA-3 cells grown without phosphatidylserine for 24 h were able to bind and internalize Sindbis virus almost normally, viral RNA synthesis was greatly reduced in the cells, suggesting that nucleocapsids of internalized Sindbis virus are not normally released into the cytoplasm. Unlike mammalian cell mutants defective in endosomal acidification, PSA-3 cells grown without phosphatidylserine were not resistant to diphtheria toxin. Furthermore, the yield of virions and viral RNA synthesis in PSA-3 cells were not completely restored on brief exposure of the cells to low pH medium following virus adsorption, which is known to induce artificial fusion of the viral envelope with the plasma membrane of normal host cells and then injection of viral nucleocapsids into the cytoplasm. Our data demonstrate the requirement of membrane phospholipids, such as phosphatidylserine and/or phosphatidylethanolamine, in CHO cells for Sindbis virus infection, and we discuss their possible roles.

Introduction

The isolation and biochemical characterization of cell mutants with specific defects in phospholipid metabolism constitutes a powerful means for understanding the biological significance of and metabolic pathways for membrane phospholipids [1]. Recently, we isolated Chinese hamster ovary (CHO) cell mutants that require exogenously added phosphatidylserine for cell growth, using the replica technique with polyester cloths [2]. Analysis of one of the mutants (PSA-3) provided genetic evidence that phosphatidylserine in CHO cells is biosynthesized by two different serine-exchange enzymes (I and II) through the following sequential reactions: phosphatidylcholine → phosphatidylserine → phosphatidylethanolamine → phosphatidylserine [2,3]. The three reactions are catalyzed by serine-exchange enzyme I, phosphatidylserine decarboxylase and serine-exchange enzyme II, respectively.

In addition to studies on the mechanism of phosphatidylserine biogenesis, PSA-3 cells should also be useful for determining the biological functions of cellular phosphatidylserine and metabolically related derivatives, because the contents of phosphatidylserine and phosphatidylethanolamine in the mutant cells can be changed by growing the cells in the presence or absence of exogenous phosphatidylserine. Such genetic alteration of the contents of membrane phospholipids may facilitate the elucidation of the roles of phospholipids in a variety of membrane-associated reactions.

Abbreviations: CHO, Chinese hamster ovary; SFV, Semliki Forest virus; VSV, vesicular stomatitis virus; pfu, plaque forming unit; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

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Enveloped animal viruses, such as the Sindbis virus, Semliki Forest virus (SFV) and vesicular stomatitis virus (VSV), constitute suitable experimental systems for studying the biological functions of cellular membrane components. These viruses consist of a RNA-containing nucleocapsid and a surrounding envelope composed of a lipid bilayer and proteins [4]. In recent years, a great deal of evidence has accumulated, indicating that host cell membranes play important roles in many viral infection processes, including internalization via adsorptive endocytosis in coated vesicles, fusion of the viral membrane with endosomes, whereby the nucleocapsids enter the cytosol, and biosynthesis and assembly of the viral envelope (for a review, see Ref. 5). Although our knowledge of the mechanisms of these processes has been accumulating, only a few reports have appeared on the functions of cellular phospholipids in infections by enveloped viruses [6,7].

In the present study, we examined the effects of the modification of membrane phospholipids on the proliferation of the Sindbis virus in PSA-3 cells and found that when PSA-3 cells are grown without phosphatidylserine, the binding and internalization of the virus occur normally but the yield of virions and viral RNA synthesis greatly decreased. These results indicate that cellular phosphatidylserine and/or phosphatidylethanolamine participate in a certain step of Sindbis virus infection, after the internalization of virus particles, but before penetration of the viral nucleocapsids into the cytoplasm. The possible roles of these phospholipids in the fusion of the Sindbis virus with the endosomal membrane are discussed.

Materials and Methods

Materials. The sources of the materials used in this work were as follows; L-[U-^{14}C]leucine, [5-^{3}H]uridine, [*methyl*-^{3}H]thymidine and L-[^{35}S]methionine were from the Radiochemical Centre, Amersham, U.K.; Ham's F-12 medium and newborn calf serum from Flow Laboratories; trypsin (1 : 250) and agar (Special Agar-Noble) from Difco Laboratories; phosphatidylserine from Sigma; and proteinase K and neutral red from E. Merck. Diphtheria toxin (0.35 Lf/μg) was a gift from Drs. Akio Yamamoto and Tyoku Matsuhashi of our institute. All other chemicals used were of analytical grade.

Cell lines and cell cultures. Strains CHO-K1 (ATCC CCL 61) and BHK-21 (ATCC CCL 10) were from the American Type Culture Collection and maintained in plastic tissue culture dishes in Ham's F-12 medium supplemented with 10% (v/v) newborn calf serum, penicillin G (100 units/ml), streptomycin sulfate (100 μg/ml) and $NaHCO_3$ (1.176 g/ml) (standard growth medium) under a 5% CO_2 atmosphere at 100% humidity and 37°C. Mutant PSA-3 was isolated as described previously [2] and maintained under the same conditions, except that the growth medium was supplemented with 30 μM phosphatidylserine liposomes, prepared as described [8].

Virus. The Sindbis virus (strain HR) was provided by Drs. A. Oya and K. Hashimoto of our institute. Stocks of the virus were prepared by infecting BHK-21 cells with 0.1 plaque forming units (pfu) per cell. After 24-h incubation at 37°C, the medium, with a titer of about 10^9 pfu/ml, was harvested, clarified by centrifugation at $1000 \times g$ for 10 min and then stored at −70°C.

Viral titers were determined with confluent monolayers of BHK-21 cells [7]. For this, cells were plated at approx. $5 \cdot 10^6$ cells per 60-mm plastic tissue culture dish containing 5 ml of the standard growth medium, followed by incubation for 1 or 2 days. The diluted virus (0.2 ml/dish) was allowed to adsorb to the resulting monolayers for 1 h at 37°C, and then 5 ml of the standard growth medium supplemented with 1% agar was added to the dishes. After 24 h at 37°C, 5 ml of the standard growth medium supplemented with 1% agar and 25 μg/ml of neutral red was added, and then the plaques were counted 16 h later.

Sindbis virus labeled with [^{35}S]methionine was prepared by the method of Robbins et al. [9] with slight modifications. BHK-21 cells were infected as above with about 1 pfu/cell. After 1 h adsorption and then 3 h incubation in the standard growth medium at 37°C, the cells were rinsed three times with 3 ml of Ham's F-12 medium without methionine and then incubated for 16 h in the methionine-free Ham's F-12 medium supplemented with 0.2 mCi/ml [^{35}S]methionine, 1.5 μg/ml nonradioactive methionine and 10% (v/v) newborn calf serum. The medium was harvested as above, and then the viruses were pelleted by centrifugation at $100\,000 \times g$ for 90 min. The viruses were resuspended in 10 mM Tris, 0.1 M NaCl and 1 mM EDTA (pH 7.4), and again pelleted at $100\,000 \times g$ for 90 min. Analysis of the labeled virus on SDS-polyacrylamide gels [10] revealed that almost all of the radioactivity was in the viral proteins.

Rates of macromolecular synthesis. The rates of synthesis were determined by briefly labeling cells with specific precursors. Cells grown in 60-mm diameter dishes were washed with 5 ml of PBS and then incubated with 2 ml of Ham's F-12 without leucine or thymidine, supplemented with 10% (v/v) dialyzed newborn calf serum and 0.1 μCi/ml of L-[U-^{14}C]leucine (340 mCi/mmol) or 1 μCi/ml of [*methyl*-^{3}H]thymidine (25 Ci/mmol) for 1 h at 37°C to estimate the rate of protein or DNA synthesis, respectively. The synthetic rates for RNA were estimated by incubating cells with 2 ml of Ham's F-12, supplemented with 10% (v/v) dialyzed newborn calf serum and 2 μCi/ml of [5-^{3}H]uridine (29 Ci/mmol). After the incubation, the radioactive medium was removed, and then 5% (wt./vol.)

trichloroacetic acid was added. The trichloroacetic acid-treated cells were scraped off with a rubber policeman onto glass microfibre filters (Whatman, GF.C) and then assayed for radioactivity by liquid scintillation spectrometry using a toluene scintillator.

Association and degradation of [^{35}S]methionine-labeled Sindbis virus in cells. After confluent cells on 60-mm diameter dishes had been washed three times with F-medium (Ham's F-12 containing 10 mM Hepes (pH 7.4) and 0.1% BSA), 1 ml of [^{35}S]methionine-labeled Sindbis virus in F-medium was added to each dish either at room temperature or on ice. The cells were then incubated at 37°C or on ice for different periods of time. The medium was removed and the cells were washed three times with 1 ml of cold F-medium. 1 ml of the combined medium was mixed with 0.4 ml of 50% TCA. After 1 h at 0°C, the medium was centrifuged (10 min, 2000 rpm, 4°C), and 1 ml of the supernatant was assayed for radioactivity by liquid scintillation spectrometry using ACS II (Amersham) scintillation fluid. The cells were scraped off with a rubber policeman into 1 ml of PBS containing 1 mM phenylmethylsulfonyl fluoride (PMSF), and then the dishes were washed twice with 1 ml of cold 10% TCA. After 1 h on ice, the combined cells and washes were centrifuged, and the precipitates were collected and assayed for radioactivity as above.

Other methods. Internalization of the Sinbis virus, production of virus RNA and the effect of low-pH treatment on the virus RNA synthesis were investigated according to Helenius et al. [11], Robbins et al. [12] and Robbins et al. [9], respectively, with the modifications indicated in the legends. Analysis of phospholipids was performed as described in our previous report [8].

Results

Phospholipid composition, cell growth and rates of macromolecular synthesis during phosphatidylserine deprivation

The effect of phosphatidylserine starvation on the contents of individual phospholipids in PSA-3 cells was examined by analyzing the phospholipids in cells grown without phosphatidylserine for various times. As shown in Fig. 1, the contents of phosphatidylserine and phosphatidylethanolamine in PSA-3 cells immediately decreased upon deprivation of supplemental phosphatidylserine. After 48 h cultivation in the absence of phosphatidylserine, the mutant cells contained about 67 and 57% less phosphatidylserine and phosphatidylethanolamine (4.0 and 12.6 nmol per mg protein, respectively) than those grown in its presence (12.2 and 29.3 nmol per mg protein, respectively), in agreement with our previous data [2]. The levels of other phospholipids such as phosphatidylcholine, phosphatidylinositol and sphingomyelin remained almost constant on phosphatidylserine starvation for at least 48 h (data not shown).

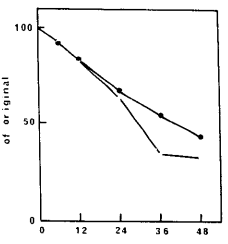

Fig. 1. Effect of phosphatidylserine starvation on the phosphatidylserine and phosphatidylethanolamine contents of mutant PSA-3 cells. Cells were seeded at $5 \cdot 10^5$ cells per 100-mm diameter dish in growth medium containing 30 μM phosphatidylserine at 37°C. After 2 days (zero time), the medium was replaced with fresh growth medium without phosphatidylserine. At the times indicated, the cells were washed with phosphate-buffered saline and then harvested with a rubber policeman. The cellular phospholipids were extracted and analyzed by two-dimensional thin-layer chromatography, as described [8]. To quantitate the individual phospholipids, the phosphate in each spot on a chromatogram was determined chemically. At zero time, the amounts of phosphatidylserine and phosphatidylethanolamine of strain PSA-3 (12.2 and 29.3 nmol per mg protein, respectively) were almost the same as those in the case of parental strain CHO-K1. ○, phosphatidylserine; ●, phosphatidylethanolamine.

We had previously shown that the growth rate of PSA-3 cells was quite similar to that of CHO-K1 cells in a growth medium supplemented with phosphatidylserine [2]. Fig 2A shows the growth curves for PAS-3 and CHO-K1 cells in the medium without exogenous phosphatidylserine. PSA-3 cells grew normally for at least 40 h in the absence of phosphatidylserine. More than 80% viability was retained during that period of phosphatidylserine starvation (Fig. 3). After 40 h deprivation, however, PSA-3 cells stopped growing and their viability gradually decreased (Figs. 2A and 3).

To examine the effect of phosphatidylserine starvation on macromolecular synthesis, the rates of protein, DNA and RNA synthesis were estimated by briefly labeling cells with [^{14}C]leucine, [^{3}H]thymidine or [^{3}H]uridine, respectively, at various times after deprivation of phosphatidylserine. As in the case of cell growth, the biosyntheses of protein, DNA and RNA in PSA-3 cells occurred normally for 40 h, after which the protein synthetic rate slowed down, and the rates of DNA and RNA synthesis significantly decreased.

Effect of phosphatidylserine starvation on Sindbis virus production

The yields of Sindbis virus in PSA-3 and CHO-K1 cells grown for various times in the medium without

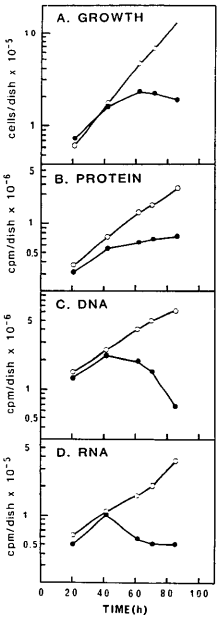

Fig. 2. Cell growth and macromolecular synthesis of phosphatidylserine-deprived cultures of PSA-3 and CHO-K1 cells. Cells were seeded at approximately $5 \cdot 10^4$ cells per 60-mm diameter dish in growth medium without phosphatidylserine followed by incubation at 37 °C. At the times indicated, cell growth (A) was determined by dispersing the cells with trypsin and then counting with a Coulter model ZB1 Counter, and the synthetic rates for protein (B), DNA (C) and RNA (B) were estimated by labeling cells for 1 h at 37 °C with [^{14}C]leucine, [^{3}H]thymidine or [^{3}H]uridine, respectively, as described under Materials and Methods. ○, CHO-K1; ●, PSA-3.

phosphatidylserine were determined by means of a plaque assay, as described under 'Materials and Methods'. Although there was no significant difference in Sindbis virus production between the two strains when grown in the presence of phosphatidylserine, the virus production in PSA-3 cells after 24 and 34 h phosphatidylserine deprivation strikingly decreased, by approx. 100- and 1000-fold, respectively, in comparison with in CHO-K1 cells (Fig. 4). These results, together with the results in Fig. 1, suggest that a supply of phosphatidyl-

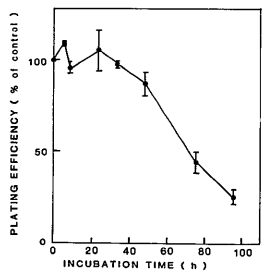

Fig. 3. Colony-forming ability of mutant PSA-3 cells incubated without phosphatidylserine for various times. Cells were seeded at approx. 300 cells per 100-mm diameter dish in growth medium containing 30 μM phosphatidylserine. After incubation for 1 day at 37 °C, the medium in all of the cultures except two was replaced with fresh growth medium without phosphatidylserine. At the indicated times, the medium in a pair of cultures was again replaced with fresh medium supplemented with 30 μM phosphatidylserine. After 4 days, from zero time, the cells were overlayed with Whatman filter paper [13] and then incubated for an additional 6 days at 37 °C. At the end of the experiment the plates were stained as described [13]. All of the data are expressed relative to cultures which were continuously incubated in the presence of phosphatidylserine.

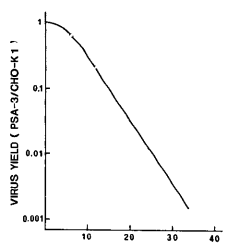

Fig. 4. Effect of phosphatidylserine starvation on Sindbis virus production in CHO-K1 and PSA-3 cells. Cells were seeded at $2.5 \cdot 10^5$ cells per 60-mm diameter dish in growth medium containing 30 μM phosphatidylserine. After 2 days (zero time), the medium was replaced with fresh medium without phosphatidylserine. At the indicated times, the cells were washed with Ham's F-12 medium and then infected with Sindbis virus at a moi of approx. 10 in 0.5 ml of the medium. After incubation for 1 h at 37 °C, the cells were washed and then incubated for an additional 12 h in growth medium without phosphatidylserine. The viral titers in the medium were determined as described under Materials and Methods. Data are expressed as the ratio of the virus yield in PSA-3 to that in CHO-K1 cells.

serine and/or phosphatidylethanolamine is required for Sindbis virus proliferation. In addition, the fact that cell growth, cell viability and macromolecular synthesis remained almost normal for 40 h phosphatidylserine deprivation (Fig. 2) suggests that the immediate cessation of Sindbis virus production does not reflect general defects in cellular activities of PSA-3 cells.

Binding and internalization of [^{35}S]methionine-labeled Sindbis virus

To determine which step(s) of virus production is defective in PSA-3 cells grown without phosphatidylserine, we first examined the binding and internalization of Sindbis virus in PSA-3 and CHO-K1 cells grown in the medium not supplemented with phosphatidylserine for 24 h, by using [^{35}S]methionine-labeled virus. As shown in Fig. 5, the kinetics of virus association with cells at 0 or 37°C were similar in the two strains. At 0°C, virtually no acid-soluble radioactivity was found in the medium. At 37°C, degraded viral protein became detectable after 30 min incubation, and the acid-soluble radioactivity in the medium of PSA-3 cells was about 2-fold less than that in the case of CHO-K1 cells. Internalization of viral protein was determined by treating cells with proteinase K to remove surface-bound virions [11], followed by measurement of the remaining cell-associated radioactivity. The rate of Sindbis virus internalization in PSA-3 cells was a little slower, 30%, than that in CHO-K1 cells but the difference was not very significant (Fig. 6). Thus, the striking decrease in Sindbis virus production in PSA-3 cells appeared not to be caused by a defect in virus binding or internalization.

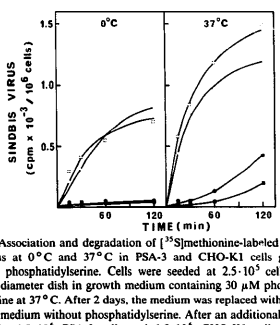

Fig. 5. Association and degradation of [^{35}S]methionine-labeled Sindbis virus at 0°C and 37°C in PSA-3 and CHO-K1 cells grown without phosphatidylserine. Cells were seeded at 2.5·10^5 cells per 60-mm diameter dish in growth medium containing 30 μM phosphatidylserine at 37°C. After 2 days, the medium was replaced with fresh growth medium without phosphatidylserine. After an additional 23 h, the cells (1.2·10^6 PSA-3 cells and 1.8·10^6 CHO-K1 cells) were incubated with [^{35}S]methionine-labeled Sindbis virus (9200 cpm, 17 cpm/1000 pfu) in F-medium as described under Materials and Methods. The acid-precipitable radioactivities in the cells (□, ○) and acid-soluble radioactivities in the medium (■, ●) were determined after incubation at 0°C or 37°C for different periods of time, as described under Materials and Methods. PSA-3: □, ■; CHO-K1: ○, ●.

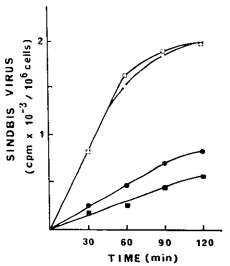

Fig. 6. Association and internalization of Sindbis virus by PSA-3 and CHO-K1 cells. Cells were grown and then infected with [^{35}S]methionine-labeled Sindbis virus as described in the legend to Fig. 5. After incubation at 37°C for different periods of time, the cells were washed three times with 1 ml of cold F-medium and then incubated at 0°C with 1 ml of PBS containing 0.5 mg/ml bovine serum albumin or 0.5 mg/ml proteinase K, for determination of total cell-associated virions (□, ○) or internalized virions (■, ●), respectively [11]. After 45 min at 0°C, 1 ml of PBS containing 30 mg bovine serum albumin and 1 mM PMSF was added to each dish. The cells were harvested with a rubber policeman and then each dish was washed twice with 1 ml of PBS containing 0.2% bovine serum albumin. After centrifugation (5 min, 1000 rpm, 4°C), the precipitated cells were solubilized with 1 ml of 0.5 M NaOH, neutralized with 5 M HCl and then assayed for radioactivity using ACS II scintillation fluid. PSA-3: □, ■; KHO-K1: ○, ●.

Production of Sindbis virus RNA

We next examined whether penetration of the internalized virus into the cytoplasm was normal or not, by measuring the synthesis of Sindbis virus RNA, which was monitored as the incorporation of [^{3}H]uridine in the presence of actinomycin D. As shown in Fig. 7, the production of viral RNA in PSA-3 cells grown in the presence of phosphatidylserine was similar to that in CHO-K1 cells grown in both its absence and presence. However, when PSA-3 cells were grown without phosphatidylserine for 24 h, viral RNA synthesis greatly decreased in the mutant cells, compared with in the parental cells. These results suggest that in PSA-3 cells grown without phosphatidylserine the nucleocapsids of internalized Sindbis virus are not normally released into the cytoplasm. Alternatively, phosphatidylserine and/or phosphatidylethanolamine might be required for Sindbis virus RNA synthesis; however, it is believed that phospholipids hardly exist in cytoplasm where viral RNA is synthesized.

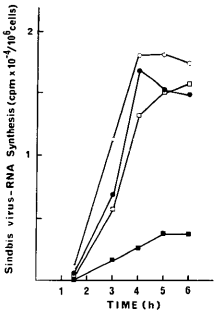

Fig. 7. Production of Sinbis virus RNA by PSA-3 and CHO-K1 cells. Cells were seeded at $5 \cdot 10^5$ cells per 60-mm diameter dish and then grown in growth medium supplemented with 30 μM phosphatidylserine. After 2 days, the medium in half of the dishes of each strain (■, ●) was replaced with growth medium without exogenous phosphatidylserine. After 24 h, the cells were exposed to the virus at a moi of approx. 10 for 1 h at 37 °C, and then the medium was replaced with growth medium containing 2 μg/ml actinomycin D. After additional incubation for 30 min at 37 °C, the latter was replaced with growth medium containing 2 μg/ml actinomycin D, 15 mM NH_4Cl and [^{3}H]uridine (2 μCi/ml). After the indicated times the dishes were put on ice, and then the cells were rinsed twice with 2 ml of PBS plus 10 mM uridine and scraped off with a rubber policeman. The released cells were collected on strips of Whatman GF/C filter, and then washed twice with 5 ml of cold 5% TCA and then with 2 ml of cold ethanol. The strips were dried and counted. The values were corrected for the incorporation in uninfected cells. PSA-3: □, ■; CHO-K1: ○, ●

Sensitivity to toxins

Recently, several mammalian cell mutants resistant to both diphtheria toxin and enveloped RNA viruses have been shown to be defective in acidification of endosomes [9,12,14,15], which, together with other findings (for a review, see Ref. 16), provides strong evidence that acidification of endosomes is required for the release of the active subunit of diphtheria toxin and viral nucleocapsids from endosomes into the cytoplasm. We thus examined the sensitivity of PSA-3 cells to the diphtheria toxin, by assaying the inhibition of protein synthesis in the mutant and parental cells by various concentrations of the toxin. As shown in Fig. 8, there was no significant difference in the sensitivity to the toxin between PSA-3 and CHO-K1 cells grown in the presence or absence of phosphatidylserine. These results suggest that the functioning of apparatuses for the adsorptive endocytosis of diphtheria toxin, including acidification of endosomes, is normal even in PSA-3 cells grown without phosphatidylserine; therefore, the decreased production of Sindbis virus in the mutant is probably not caused by defective acidification of endosomes.

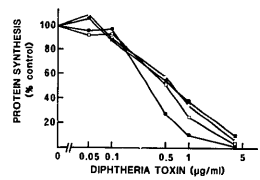

Fig. 8. Effect of diphtheria toxin on protein synthesis in PSA-3 and CHO-K1 cells. Cells were seeded at $1.5 \cdot 10^5$ cells per 60-mm diameter dish in growth medium containing 30 μM phosphatidylserine at 37 °C. After 3 days, the medium in half of the dishes of each strain (■, ●) was replaced with growth medium without phosphatidylserine. After 24 h, the indicated amounts of diphtheria toxin were added to the dishes and then the cells were incubated for 3 h at 37 °C. Protein synthesis during 1 h was measured, as described under Materials and Methods. PSA-3: □, ■; CHO-K1: ○, ●.

Effects of low pH treatment on production of Sindbis virus and its RNA synthesis

It is known that the production of Sindbis virus from CHO-K1 cells proceeds almost normally even in the presence of chloroquine, which raises the endosomal pH, if, following incubation with the virus, the cells are briefly shifted to medium of pH 5.5 (Ref. 11 and Table I). Brief exposure to low pH is known to induce the penetration of nucleocapsids into the cytoplasm through fusion of the viruses with the plasma membranes of host cells [11]. We examined whether or not the decreased

TABLE I

Effects of chloroquine and low-pH treatment on the production of mature Sindbis virus virions

Cells were seeded at $2.5 \cdot 10^5$ cells per 60-mm diameter dish in growth medium containing 30 μM phosphatidylserine at 37 °C. After 2 days, the medium was replaced with growth medium without phosphatidylserine. After additional incubation for 24 h, the cells ($6 \cdot 10^5$ cells per 60-mm diameter dish) were adsorbed with Sindbis virus for 15 min at 0 °C and then washed twice with Ham's F-12. The cells were then incubated for 3 min in medium containing 10 mM 2-morpholinoethanesulfonic acid and 2 mg/ml BSA, at pH 7.5 or pH 5.5 [9]. The medium was replaced with growth medium with or without 0.1 mM chlrooquine and then the incubation was continued at 37 °C overnight. The Sindbis virus titer in the medium was measured as described under Materials and Methods.

pH	Chloroquine (0.1 mM)	PFU per cell	
		CHO-K1	PSA-3
7.5	–	$1.0 \cdot 10$	$2.8 \cdot 10^{-2}$
7.5	+	$7.3 \cdot 10^{-2}$	$1.7 \cdot 10^{-2}$
5.5	–	$1.2 \cdot 10$	$3.3 \cdot 10^{-1}$
5.5	+	6.8	$2.6 \cdot 10^{-1}$

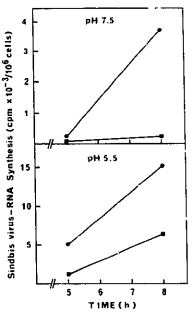

Fig. 9. Effect of low-pH treatment on the production of Sindbis virus RNA. Cells were seeded at $2.5 \cdot 10^5$ cells per 60-mm diameter dish in growth medium containing 30 μM phosphatidylserine at 37 °C. After 2 days, the medium was replaced with growth medium without phosphatidylserine. After additional incubation for 24 h, the cells were washed twice with 2 ml of cold Ham's F-12 medium and then incubated with Sindbis virus at approx. 10 pfu/cell for 15 min at 0 °C. The cells were washed twice with 2 ml of cold Ham's F-12 medium and then incubated for 3 min at 37 °C in 1 ml of Ham's F-12 medium containing 10 mM 2-morpholineethanesulfonic acid and 2 mg/ml BSA, at pH 7.5 or pH 5.5 [9]. Viral RNA synthesis was assayed as described in the legend to Fig. 7. PSA-3: ■; CHO-K1: ●.

production and RNA synthesis of Sindbis virus in PSA-3 cells grown for 24 h without phosphatidylserine are restored on brief exposure to low pH (Table I). There was no significant difference in production of the Sindbis virus between CHO-K1 cells exposed to pH 5.5 and pH 7.5. On the other hand, the low pH treatment increased the yield of the virus 10-fold in PSA-3 cells. However, the level of virus production in PSA-3 cells treated at pH 5.5 was still 50-fold lower than that in parental cells. Viral RNA synthesis in PSA-3 cells was also stimulated about 7-fold by low pH treatment (Fig. 9). Again, however, the level of RNA synthesis in PSA-3 cells treated at pH 5.5 was 1/4–1/3 of that in parental cells. These results, taken together, suggest that the release of viral nucleocapsids into the cytoplasm through the plasma membrane induced by acid-treatment is impaired in PSA-3 cells. The incomplete restoration of virus production and RNA synthesis could be due to the inefficient fusion of the Sindbis virus with the plasma membrane deficient in phosphatidylserine and phosphatidylethanolamine (see Discussion).

Discussion

We previously described the isolation of a CHO cell mutant, called PSA-3, that required exogenously added phosphatidylserine for cell growth [2]. This phosphatidylserine-requiring mutant is defective in serine-exchange enzyme I and lacks the ability to synthesize phosphatidylserine. The objective of the present study was to determine the roles of specific membrane phospholipids in host cells as to infection by enveloped animal viruses, employing mutant cells defective in phosphatidylserine biogenesis. We reported here that the maturation of the Sindbis virus, an enveloped RNA virus, was greatly impaired in PSA-3 cells when the cellular contents of phosphatidylserine and also phosphatidylethanolamine produced through decarboxylation of phosphatidylserine were reduced by growing the mutant cells in medium not supplemented with phosphatidylserine. This is the first demonstration of the requirement of membrane phospholipids, such as phosphatidylserine and/or phosphatidylethanolamine, in host cells membranes for animal virus infection.

The binding of animal viruses to the host cell surface is mediated by the interaction of viral surface proteins with specific receptors on the host cell membranes. However, few virus receptors have been purified or identified. Recently, Schlegel et al. [17] found that phosphatidylserine directly binds to the vesicular stomatitus virus (VSV) and inhibits VSV attachment and infectivity, and suggested that phosphatidylserine could function as a binding site or a portion of a binding site for VSV. In this study, we found that the binding and internalization of Sindbis virus in PSA-3 cells grown without phosphatidylserine was almost normal. These results suggest that phosphatidylserine is not a cell surface binding site for Sindbis virus, though the possibility that residual phosphatidylserine in the plasma membrane of PSA-3 cells grown in the absence of phosphatidylserine may be enough to serve as the virus binding site cannot be completely ruled out. With respect to the receptor for the Semliki Forest virus (SFV), which is an α-virus like the Sindbis virus, Helenius et al. [18] reported that the receptor apparently includes histocompatibility antigens; whereas Oldstone et al. [19] argued that major histocompatibility complex antigens are not specific receptors for this virus.

Although PSA-3 cells grown without phosphatidylserine for 24 h were able to bind and internalize Sindbis virus, viral RNA synthesis was greatly reduced in the cells. These results indicate that nucleocapsids of the internalized Sindbis virus are not normally released into the cytoplasm. Acidification of endocytic vesicles is known to be essential for the release of ligands, such as toxins and viruses, into the cytoplasm [16]. One of the facts supporting this idea is that several mammalian cell mutants resistant to diphtheria toxin have been shown to be cross-resistant to the virus and to have a defect in endosomal acidification [9,12,14,15,20]. Unlike these mutants defective in endosomal acidification, PSA-3 cells grown without phosphatidylserine were not re-

sistant to the diphtheria toxin. These findings suggest that the reduced production of Sindbis virus in PSA-3 cells is unrelated to the defect in the acidification of endocytic vesicles. However, to demonstrate the normal acidification of endosomes in PSA-3 cells, the direct measurement of endosomal pH both in vivo and in vitro is needed.

Ono et al. [21] reported that a monensin-resistant mouse Balb/3T3 cell mutant is cross-resistant to VSV and that its endosomal acidification is normal, as in the case of PSA-3 cells; however, the biochemical defect in the mutant remains to be identified.

The rate of degradation of viral protein in PSA-3 cells grown without phosphatidylserine was found to be about 50% of that in the case of CHO-K1 cells (Fig. 5), when cells were incubated with [^{35}S]methionine-labeled Sindbis virus at 37°C. This may be due to either that viral proteins reach the lysosomes at a slower rate in phosphatidylserine-starved cells than in the parent cells, or that the lysosomal function is impaired in the mutant cells. It appears unlikely, however, that the Sindbis virus, which is internalized into PSA-3 cells but not released into the cytoplasm, is resistant to degradation enzyme(s), because Helenius et al. [11] have shown that chloroquine-treated cells internalize and degrade SFV normally.

Finally, on the basis of our finding that the abortive production of Sindbis virus and reduced RNA synthesis were not completely overcome on low-pH treatment of PSA-3 cells, we speculate that membrane fusion of the Sindbis virus with the plasma membrane may be impaired in the mutant cells deficient in phosphatidylserine and phosphatidylethanolamine. If this is true, fusion of the viral membrane with the endosomal membrane should be impaired as well, because the phospholipid composition of the endosomal membrane is similar to those of the plasma membrane [22]. Cullis and de Kruijff [23] argued that certain phospholipids, such as phosphatidylethanolamine and cardiolipin, which preferentially adopt the non-bilayer hexagonal (H_{II}) phase under certain conditions, may be directly involved in fusion events. In mixed lipid systems, phosphatidylserine can stabilize the bilayer structure in the same manner as phosphatidylcholine, inducing a bilayer structure of egg phosphatidylethanolamine at about 20 mol% [24], the subsequent addition of Ca^{2+}, however, results in triggering of H_{II} phase formation of phosphatidylethanolamine and eventually induces membrane fusion [25,26]. Envelope protein E1 of the Sindbis virus, like Ca^{2+}, may bind to phosphatidylserine in the endosomal membrane of host cells and trigger the formation of a non-bilayer structure of phosphatidylethanolamine, resulting in membrane fusion of the virus with endosomes. Conversely, the deficiency of phosphatidylserine and/or phosphatidylethanolamine in host cells may suppress the fusion of the viral membrane with the endosomal membrane. As in the case of endosomal acidification, however, definite proof of this possibility will depend upon the successful direct measurement of the membrane fusion in mutant cells.

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