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Proteolytic Processing of the Sindbis Virus Membrane Protein Precursor PE2 Is Nonessential for Growth in Vertebrate Cells but Is Required for Efficient Growth in Invertebrate Cells

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We have shown previously that processing of the Sindbis virus envelope protein precursor PE2 to envelope protein E2 is not required for virus maturation in cultured vertebrate fibroblast cells and that unprocessed PE2 can be incorporated into infectious virus in place of E2 (J. F. Presley and D. T. Brown, J. Virol. 63:1975–1980, 1989; D. L. Russell, J. M. Dalrymple, and R. E. Johnston, J. Virol. 63:1619–1629, 1989). To better understand the role of this processing event in the invertebrate vector portion of the alphavirus life cycle, we have examined the maturation of Sindbis virus mutants defective in PE2 processing in cultured mosquito cells. We found that although substantial amounts of structural proteins PE2, E1, and C were produced in infected mosquito (aedine) cell lines, very little infectious virus was released. When the period of infection was extended, plaque size variants appeared, some of which exhibited a restored ability to grow in mosquito cells. The nucleotide sequences of two such variants were determined. These variants contained point mutations that restored PE2 cleavage, indicating a genetic linkage between failure to cleave PE2 and failure to grow in mosquito cells.

Sindbis virus is a structurally simple enveloped virus (family *Togaviridae*) which is propagated in a natural cycle involving vertebrate hosts (primarily migratory birds) and invertebrate vectors (*Aedes* and *Culex* mosquitoes) (24, 28). The virus contains three structural proteins: a core nucleocapsid protein (C) and two envelope glycoproteins (E1 and E2). The C protein is complexed with the viral RNA to form an icosahedral structure which is situated within an envelope containing the E1 and E2 glycoproteins (5, 29).

The structural proteins of Sindbis virus are encoded in a single large open reading frame which specifies a polyprotein with a molecular size of 130,000 Da. This protein is proteolytically processed to form the C protein and precursors of the envelope proteins PE2 and E1 (1, 10, 30, 31). After translocation into the endoplasmic reticulum, PE2 and E1 are N glycosylated and transported to the cell surface (4, 20, 22, 26, 32); during transport, PE2 is cleaved to yield mature E2 and E3. E3, a small glycopeptide constituting the amino-terminal 64 amino acids of PE2, is discarded into the growth medium by the Sindbis virus but is retained by the Semliki Forest virus particle (11, 15).

Recently, it has been demonstrated that proteolytic processing of PE2 is not required for Sindbis virus maturation in cultured vertebrate cells (17, 21). Monensin treatment of infected baby hamster kidney (BHK) cells blocked PE2 processing and produced virions which had replaced over 75% of their complement of E2 with the envelope protein precursor PE2 (17). A mutant of S.A.AR86, S12, derived from the S.A.AR86 strain by selection for rapid penetration of BHK cells, has a Ser at position 1 of the mature E2 replaced by an Asn, producing a consensus site for N glycosylation (Asn-X-Thr). This site is utilized by cellular glycosylating enzymes with a resulting total block in PE2 processing. Nevertheless, virus growth and viability in BHK cells were not altered, and the progeny virus, which contains PE2 and E1, had a normal particle-to-PFU ratio (21).

The fact that the proteolytic processing of PE2, which is common to all the alphaviruses thus far described, is not required for maturation of Sindbis virus in vertebrate cell cultures raises questions regarding the role of this processing event in the natural vertebrate host-invertebrate vector life cycle of alphaviruses. Evolution would be expected to favor virus capable of replicating efficiently in the invertebrate vector as well as in the vertebrate host.

Since failure to process PE2 is a genetic property of S12, it is possible to use this mutant to analyze the consequences of failure to cleave PE2 in mosquito cells. Virus maturation differs in mosquito cells in that the bulk of maturation occurs in large intracellular vesicles rather than at the cell surface (3, 12). Additionally, N-linked glycans differ significantly from those found in vertebrate cells, particularly in the absence of charged sialic acid residues (3, 16). Because routes for maturation of Sindbis virus differ in invertebrate cells, demands on the processing of virus proteins may also differ.

MATERIALS AND METHODS

Vertebrate and invertebrate cell lines. *Aedes triseriatis* cells were obtained from Barry Miller (Centers for Disease Control, Fort Collins, Colo.). The U4.4 cell line was derived in our laboratory from the original larval isolate of Singh (12, 25). The C6/36 cell line was obtained from Kenneth Ekels (Walter Reed Army Institute of Research, Washington, D.C.) and had originally been isolated by Igarashi (13). The C710 cell line was originally derived from the C6/36 cell line and was provided by V. Stollar (Rutgers Medical School, New Brunswick, N.J.). All cell lines were grown in Eagle minimal essential medium (9) with 10% fetal calf serum (GIBCO) and supplemented with 2 mM glutamine and 10% tryptose phosphate broth (18). BHK cells (cell line BHK 21)

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were routinely maintained at 37°C. All mosquito cell lines were maintained at 28°C.

Virus. S.A.AR86 was obtained from Jordi Casals, Arbovirus Research Unit, Yale University. The isolation of mutant S12 has been described previously (21). Virus stocks were routinely grown on BHK cells as described previously (18). Virus titers were routinely determined by plaque assay on BHK cells (18).

Mosquito cells (C6/36, unless otherwise stated) were infected by allowing virus to adsorb for 90 min at room temperature after the cells were reseeded in serum-free medium for 1 h. C6/36 cells attach firmly after reseeding, preventing cell loss during infection. For plaque assays on mosquito cell monolayers (23), flasks of C6/36 cells were overlaid with a medium-agarose mixture as for BHK cells and incubated at 28°C. Flasks were stained with neutral red after plaques were visible (generally 48 h postinfection). When comparison of mutant and wild-type virus plaque sizes was desired, both sets of flasks were stained simultaneously at a later time (generally 60 h postinfection) to allow visualization of mutant plaques.

Metabolic labeling and polyacrylamide gel electrophoresis. [^{35}S]methionine was routinely used in metabolic labeling experiments at a concentration of 50 μCi/ml. The growth medium in flasks containing mosquito cell monolayers was replaced with a minimal volume of Eagle minimal essential medium depleted of methionine, and radioactive methionine-cysteine (Trans^{35}S-label; ICN) was immediately added to the appropriate concentration. Proteins were immune precipitated with a rabbit serum raised against the Sindbis virus structural proteins as described previously (17) and were resolved by electrophoresis in 11% polyacrylamide gels by the method of Laemmli (14). Fluorography was performed as described previously (2).

RNA sequencing. Sequence data were obtained by the dideoxynucleotide chain termination method (33) directly on intracellular RNA from infected cells by using oligonucleotide E29, 5′-ACGGTTCAGTATGG-3′ (nucleotide 8690 to nucleotide 8703), as the primer. Intracellular RNA was isolated from BHK cell monolayers infected at a multiplicity of infection of 10. At 6 h postinfection, the medium was removed and the cells were washed twice with a hypotonic buffer (3.3 mM NaCl, 3.3 mM Tris-HCl [pH 8.5], 0.5 mM $MgCl_2$). The cells were harvested in 1 ml of buffer by using a rubber policeman and allowed to swell for 10 min before the addition of 0.45% Nonidet P-40. The cells were mixed thoroughly and then centrifuged at 800 × *g* to pellet the nuclei. RNA was isolated by extraction of the supernatants with phenol-chloroform and precipitated at −20°C with ethanol.

Radioimmune quantitation of protein. Equivalent numbers of cells were lysed in 0.5% Nonidet P-40 in phosphate-buffered saline (PBS), and the nuclei were pelleted. Immobilon membranes (Millipore) were prewetted with methanol and transferred to PBS. Cell extract was then slot blotted through a vacuum manifold, and the membrane was air dried. The membrane was placed in a blocking solution (10% Carnation instant Nonfat dry milk in PBS) for 1 h. A 1:100 dilution of the same rabbit serum used in the immune precipitations described above was made in a buffer containing 10% Carnation instant nonfat dry milk and 0.3% Tween 20 in PBS. The membrane was incubated for 1 h at room temperature in this solution. It was then washed three times for 5 min each time in 0.3% Tween 20 in PBS. The membrane was then incubated in incubation solution (as for the antibody) containing 10^5 dpm of iodinated protein A (NEX-146; Du Pont-NEN) for 1 h. The membrane was washed five times in 0.3% Tween 20 in PBS and air dried. The membranes were then sandwiched with X-ray film. After the film was developed, comparative amounts of protein were determined from densitometer measurements of a single exposure. A series of dilutions of virus of known titer were blotted, allowing PFU equivalents to be computed for unknown samples blotted onto the same membrane. Final results were normalized to PFU equivalent protein per 10^6 cells to allow easy comparison.

RESULTS

Replication of PE2 processing-defective virus in mosquito cell monolayers. S12 replicates efficiently in vertebrate tissue culture. However, its ability to grow in the invertebrate host has not been tested previously. The evolutionary conservation of the PE2 processing event suggested that virus defective in PE2 processing would show compromised growth in at least one natural host. We therefore examined infectivity and growth of S12 in insect tissue culture.

Efficiency of infection of mosquito cells (C6/36 and C710) was determined by plaque assay of known titers of virus (as determined on BHK cell monolayers) on monolayers of C6/36 and C710 cells. S12 and S.A.AR86 showed similar infectivities (data not shown). However, S12 formed pinpoint plaques on mosquito cell monolayers, whereas the wild-type parent formed large plaques.

A variation in plaque size may (but need not) indicate different rates of replication in the cell monolayers on which the virus titer is determined. To test this possibility, we infected three independently isolated clones of *Aedes albopictus* cells (the C6/36, C710, and U4.4 cell lines) and an *Aedes triseriatus* cell line with S12 or S.A.AR86 at a multiplicity of infection of 100 and harvested the virus at various time intervals (C6/36 and C710; Fig. 1). Monolayers were washed at 2 h postinfection in order to remove unadsorbed virus. Virus was harvested at the times indicated, and the titer of virus was determined on BHK cell monolayers. After 12 h of infection, S.A.AR86 consistently produced a titer of progeny virus at least 250-fold greater than that of S12 in each mosquito cell line. Similar data were obtained for U4.4 and *A. triseriatus* cells (data not shown). The inhibition of growth in the invertebrate cell was not due to a low-temperature-sensitivity phenotype of the virus, because S12 grew normally in BHK cells at 28°C (data not shown).

At later times postinfection (from 12 to 72 h), S12-infected C6/36 cell cultures demonstrated some increase in virus titers. Most of the virus produced at these later times consisted of large-plaque variants when assayed on either BHK cell or C6/36 monolayers. All plaque isolates that showed renewed ability to form large plaques on mosquito cell monolayers also had regained the ability to process PE2 (Table 1; Fig. 2). Since all of the invertebrate cell lines showed roughly equivalent reductions in their ability to produce S12, the C6/36 line was selected for more-detailed study.

Envelope protein synthesis in mosquito cell monolayers infected with S12. Equivalent monolayers of mosquito cells infected with mutant or wild-type virus were labeled, starting at 12 h postinfection, with [^{35}S]methionine continuously for 3 h and lysed, and viral proteins were analyzed by polyacrylamide gel electrophoresis as described in Materials and Methods. In all four insect cell lines studied (U4.4, C6/36, C710, and *A. triseriatus*), infection with wild-type virus yielded typical protein profiles (E1, E2, and C; Fig. 3).

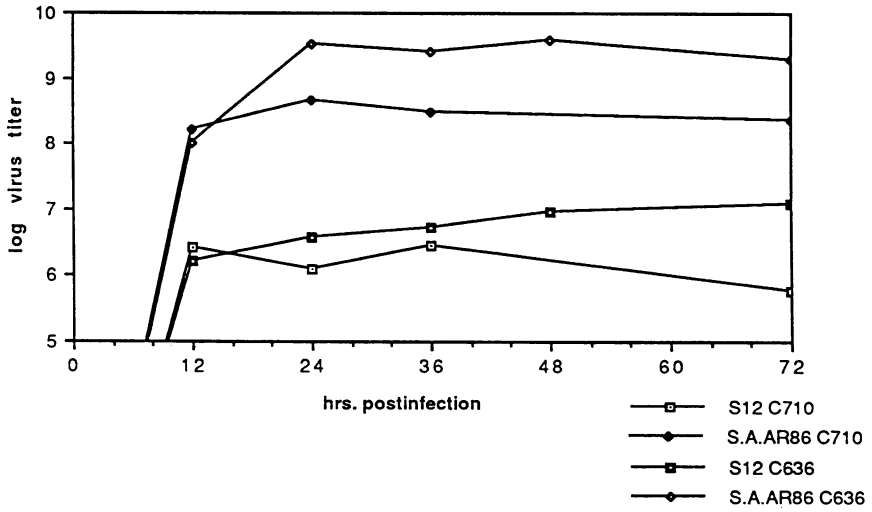

FIG. 1. Growth of mutant and wild-type virus in mosquito cells. Monolayers of the indicated mosquito cell lines grown in 75-cm^2 flasks were infected with mutant or wild-type virus. The medium was removed, and the titers of virus were determined on BHK cells at the indicated time points. The y axis shows the $\log_{10}$ virus titer (PFU per milliliter).

Small amounts of unprocessed PE2 also were seen. Pulse-chase experiments indicated that PE2 processing occurs as rapidly in mosquito cells as it does in vertebrate cells (data not shown). Therefore, the small amount of precursor relative to product seen in this extended labeling period is not surprising. In contrast, no E2 was found in any of the various insect cell lines infected with the mutant S12 (Fig. 3), indicating that the addition of carbohydrate at the E2-E3 junction blocks cleavage in mosquito cells as well as in mammalian cells. Quantitation of viral protein (as described in Materials and Methods) in equivalent cell extracts (C6/36) infected with wild-type or mutant virus showed no significant difference in the total amount of intracellular viral structural proteins (data not shown). Although virus production was greatly restricted in S12-infected insect cells, analysis of the limited amount of virus produced revealed the presence of PE2, E1, and E2, indicating a mixture of mutant and revertant virus proteins (data not shown).

Isolation of revertants with wild-type growth characteristics in mosquito cells. As shown above, progeny virus isolated after prolonged growth of S12 in mosquito cells had an altered plaque size when concentrations of virus were determined on either BHK or C6/36 cells. Most experiments showed the production of some large-plaque-producing virus by 72 h postinfection. There was a correlation between the appearance of these large plaques and an increase in virus production in the cells infected with S12. Since S12 normally produces plaques which are much smaller than S.A.AR86 plaques on BHK cells, we considered the possibility that the appearance of large-plaque virus represented a reversion to the wild-type phenotype. Stocks of virus were produced from large and small plaques isolated from the assay described above. A number of these plaque-purified variants were examined with respect to their ability to grow in vertebrate and invertebrate cells and to process PE2 in C6/36 cells (Table 1; Fig. 2). We found that all plaque isolates which grew to a titer of 1×10^8 or more in mosquito cells also proteolytically processed PE2 to E2 in mosquito cells.

TABLE 1. Characteristics of virus isolated after prolonged infection of mosquito cells

Isolate	Plaque size	Titer on:		PE2 processing
		C6/36 cells	BHK cells	
1	Small	1.55×10^6	3.6×10^8	No
8	Medium	1.2×10^6	1.0×10^9	No
9	Medium	1.75×10^8	1.1×10^7	Yes
13	Large	1.2×10^9	5.9×10^9	Yes
14	Large	1.6×10^9	4.1×10^9	Yes
15	Large	1.25×10^9	4.9×10^9	Yes
16	Medium	9.2×10^5	7.05×10^8	No
17	Medium	2.1×10^8	3.0×10^7	Yes
18	Small	9.4×10^5	6.8×10^8	No
19	Small	1.1×10^6	2.7×10^9	No

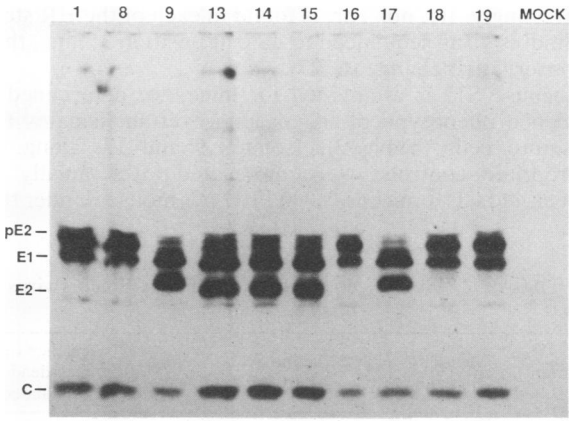

FIG. 2. Mutant and revertant structural proteins present in mosquito cells. Stocks originating from plaque-purified isolates obtained late in S12 infection of mosquito cell monolayers were used to infect BHK cell monolayers in the presence of dactinomycin. Cells were labeled for 3 h with [^{35}S]methionine beginning at 8 h postinfection and lysed, and viral proteins were immunoprecipitated with anti-Sindbis virus rabbit serum. Proteins were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized by fluorography.

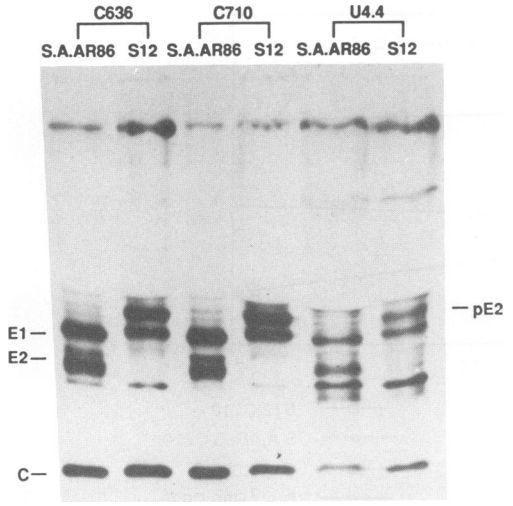

FIG. 3. Composition of virus envelope proteins in infected mosquito cell monolayers. The indicated viruses were used to infect mosquito cell lines. At 12 h postinfection, [^{35}S]methionine was added to the medium (50 μCi/ml), and viral proteins were labeled continuously for 3 h. Afterward, monolayers were lysed in 0.5% Nonidet P-40 and virus proteins were immunoprecipitated with anti-Sindbis virus rabbit serum. Virus proteins were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and fluorography. The band running slightly faster than E2 is a host protein and appears in mock-infected controls.

For two such isolates (9 and 17), the nucleotide sequence of viral RNA in the region encoding the E2-E3 cleavage site was determined. These isolates retained the original S12 Asn substitution for Ser at position 1 of E2 but contained an Ile-for-Thr substitution at position 3 (Table 2). This substitution disrupted the N-linked glycosylation site (Asn-X-Thr) present in S12, preventing addition of carbohydrate and therefore allowing PE2 processing. The retention of Asn at position 1 (Ser in S.A.AR86) showed that these isolates were pseudorevertants rather than true revertants or wild-type contaminants in the S12 stock used to initiate the infection. Interestingly, Ile, not Thr, is found in each of the HR strains of Sindbis virus sequenced to date at position 3; thus, this is a conservative change (6, 27).

Because S12 is attenuated for mice, we determined the virulence phenotype of the pseudorevertant isolates from mosquito cells (Table 2). Isolates 9 and 17, along with appropriate controls, were inoculated intracranially into 4-week-old CD-1 mice at 1,000 PFU per mouse. Under these conditions, S.A.AR86 infection results in a uniformly fatal encephalitis, whereas infection with S12 is generally nonfatal (21). We found that isolate 18 (no PE2 cleavage) failed to kill infected mice, whereas isolates 9 and 17 (revertant with respect to PE2 cleavage; Ile at position 3 in E2) proved uniformly fatal, each killing five of five infected mice (Table 2). Thus, neurovirulence in mice was also correlated with the ability to process PE2.

TABLE 2. Phenotypic comparisons among Sindbis virus plaque variants

Variant	Mosquito cell plaque size	E2 amino acids 1 to 3[a]	PE2 cleavage	Virulence (no. of mice dead/no. inoculated)[b]
S.A.AR86	Large	Ser-Val-Thr	Yes	4/4
1 (S12)	Small	Asn-Val-Thr	No	0/5
9	Large	Asn-Val-Ile	Yes	5/5
17	Large	Asn-Val-Ile	Yes	5/5
18	Small	Asn-Val-Thr	No	0/5

[a] Amino acid sequence as deduced from direct RNA sequence analysis (see Results).

[b] Virulence after intracranial inoculation of 1,000 PFU into 4-week-old CD-1 mice. Animals were observed for a period of 14 days postinoculation.

Recently, the phenotype of S12 has been reproduced by site-directed mutagenesis using a full-length cDNA clone of Sindbis virus, pTR4000, capable of producing infectious RNA transcripts (14a, 19). This mutant, TR4001-N, contains the same glycosylation signal as the S12 mutant, exhibits near-normal growth in BHK cells, fails to process PE2, and produces virions containing PE2 instead of E2. We have found that this mutant has growth properties and PE2 processing similar to those of S12 in mosquito cells, indicating that the phenotype of S12 described above is due to the alteration at the E3-E2 cleavage site and not to some other alteration in the virus structural or nonstructural proteins.

DISCUSSION

S12 is a mutant of Sindbis virus in which PE2 contains an additional N-linked glycosylation site at the precise location of the E2-E3 cleavage. This additional oligosaccharide blocks proteolytic cleavage of this protein (21), an event previously believed to be absolutely essential for virus assembly. The processing of PE2 to E2 is conserved among the alphaviruses, which require both vertebrate and invertebrate hosts for their survival in nature. Nevertheless, the mutant S12 grows with normal kinetics in BHK cells, and infectious virions containing PE2 in place of E2 are produced. The resolution of this dichotomy may be that PE2 processing is, in fact, required for virus replication in the invertebrate portion of the natural virus replication cycle.

S12 grew extremely poorly in the four mosquito cell lines tested (three *A. albopictus* and one *A. triseriatus*). These data suggest a failure of intracellular S12-encoded envelope protein to assemble into infectious virions in mosquito cells. In contrast to its parent, S.A.AR86, S12 is also avirulent upon intracranial inoculation of adult mice, suggesting that it has only a limited ability to replicate in critical differentiated cells of the central nervous system. That these in vivo and in vitro phenotypes are attributable to the defect in PE2 processing is strongly suggested by analysis of second-site virulent revertants of S12 which are PE2 processing competent. Therefore, the permissiveness of an undifferentiated fibroblast cell line such as BHK for S12 replication simply may not accurately reflect requirements exhibited by critical differentiated target cells in vivo or by cultured aedine cell lines. This question is currently under investigation.

Failure of virus to mature in mosquito cells could be the result of a direct failure of uncleaved PE2 to transport correctly in the infected cell or to make the correct protein-protein contacts required for virus assembly. Since sites of maturation of virus differ between mosquito and BHK cells and the major site of PE2 processing may be earlier in the transport pathway in mosquito cells than in BHK cells (7, 16), it is possible that cleavage of PE2 to E2 unmasks transport signals on the protein or makes possible the appropriate formation of oligomers. We have acquired data suggesting that virus proteins (E1 and PE2) are transported to the surface of S12-infected C6/36 cells but fail to assemble into virions (17a).

Because S12 PE2 contains an extra N-linked oligosaccha-

ride compared with wild-type PE2, we must be cautious about concluding that PE2 cleavage is required for or coupled to virus maturation in mosquito cells. Durbin and Stollar (8) have shown that some glycosylation mutants of Sindbis virus are also host range mutants able to grow in invertebrate tissue culture but not vertebrate tissue culture. The Sindbis virus mutants examined in this study also demonstrate the potential utility of mutations in and around the PE2 cleavage site in the development of recombinant alphavirus vaccines. Such mutations not only could attenuate virulence in rodent hosts but also could prevent uncontrolled transmission of the recombinant viruses by the mosquito vectors.

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