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Two Small Virus-Specific Polypeptides Are Produced During Infection with Sindbis Virus

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We have identified and characterized two small virus-specific polypeptides which are produced during infection of cells with Sindbis virus, but which are not incorporated into the mature virion. The larger of these is a glycoprotein with an approximate molecular weight of 9,800 and is found predominantly in the medium of infected cells. Three independent lines of evidence demonstrate conclusively that this 9,800-dalton glycoprotein is produced during the proteolytic conversion of the precursor polypeptide, PE2, to the virion glycoprotein E2. This small glycoprotein is therefore analogous to the virion glycoprotein E3 of the very closely related alphavirus, Semliki Forest virus. The 9,800-dalton glycoprotein of Sindbis virus, unlike the E3 glycoprotein of Semliki Forest virus, is not, however, present in the viral particle. The other virus-specific polypeptide is 4,200 daltons in size, does not appear to be a glycoprotein, and is neither incorporated into the mature virus nor released into the culture medium. The gene for this small polypeptide is present in the viral 26S mRNA (the mRNA which encodes all the viral structural polypeptides) and appears to be located in the portion of the mRNA which encodes the two viral glycoproteins. The possibility that this 4,200-dalton polypeptide functions as a signal peptide during the synthesis of the viral membrane glycoproteins is discussed.

Sindbis virus and Semliki Forest virus (SFV) are two very closely related alphaviruses. Both virions consist of an icosahedral nucleocapsid, which contains the 42S RNA genome, and a lipoprotein envelope. The SFV contains four structural polypeptides: two large envelope glycoproteins E1 and E2, a small envelope glycoprotein E3, and a nonglycosylated nucleocapsid protein, C (11). In contrast, the Sindbis virion is reported to contain only three structural polypeptides: two large envelope glycoproteins E1 and E2, and a nonglycosylated nucleocapsid protein, C (17). The structural polypeptides E1, E2, and C of the two viruses appear to be very similar in both size and function. The only obvious difference between the polypeptide composition of the two viruses is the absence of a third small glycoprotein in the Sindbis virion.

All the structural proteins of both SFV and Sindbis virus are encoded by a single subgenomic polycistronic 26S mRNA (10, 20). In the case of SFV, the genes for the structural proteins are arranged on the 26S message in the order of (5'→3') C, E2, E1, (9). It is clear that the gene encoding the nucleocapsid protein of Sindbis virus is located nearest the 5' end of the 26S mRNA, and that the genes encoding the envelope glycoproteins adjoin each other near the 3' end of the message (18). The exact order of the two genes encoding E1 and E2 of Sindbis virus is not, however, known. Translation of the 26S mRNA of both viruses is initiated at a single site, and production of the individual proteins appears to occur by way of a series of proteolytic cleavages of the nascent polypeptide chain (8, 10). Evidence of this is the fact that cells infected with a certain class of temperature-sensitive mutants produce, at the nonpermissive temperature, no viral structural proteins, but instead, produce a large 130,000-dalton polypeptide (referred to as the ts-2 protein) which contains the tryptic peptides of all the viral structural proteins (16). This 130,000-dalton polypeptide appears to be the complete unprocessed translate of the 26S mRNA.

Even during infection with wild-type virus, cleavage of the nascent chain, which results in production of the individual viral glycoproteins, sometimes fails to occur. As a result, variable amounts of a large nonstructural polypeptide, referred to as the B protein, accumulates in cells infected with either wild-type SFV or Sindbis virus. This protein, which has an approximate molecular weight of 100,000, contains within it both E1 and E2 (16). The structure of the B protein is direct evidence that the two genes encoding E1 and E2 adjoin one another on the viral mRNA.

Two of the SFV glycoproteins, E2 (approximate molecular weight, 52,000) and E3 (approximate molecular weight, 10,000), are released from the polysome in the form of a single precursor polypeptide PE2 (approximate molecular weight, 68,000) (11, 21). Cleavage of this precursor results in the simultaneous appearance of E2 and E3. The Sindbis virion glycoprotein E2 (approximate molecular weight, 49,500) is similarly derived from a precursor polypeptide PE2 (approximate molecular weight, 59,500) (18). Here, however, the small polypeptide corresponding to that portion of PE2 not found in E2 (which should be approximately 10,000 daltons in size) has not been identified. The studies reported here, which were initially aimed at detecting the small polypeptide derived from PE2, have allowed us to identify not one, but two small virus-specific polypeptides.

MATERIALS AND METHODS

Cells. Secondary chicken embryo fibroblast cells or BHK-21 (clone 13) cells were used for all experiments. Chick cells were seeded at a density of 8×10^5 cells per 35-mm plastic petri dish (Falcon) in Dulbecco modified Eagle medium (DME) supplemented with 2% tryptose phosphate broth (Difco) and 1% calf serum (Colorado Serum Co.). BHK-21 cells were seeded at a density of 5×10^5 cells per 35-mm plastic petri dish in DME supplemented with 10% tryptose phosphate broth and 10% calf serum. In all experiments, cells were infected with virus 18 to 24 h after subculturing. Except in the experiments with temperature-sensitive mutants, the cells were grown at 37°C.

Labeling of infected cells. BHK or chick cells were infected at a high multiplicity (300 to 500 PFU per cell) with either wild-type Sindbis virus or SFV. The virus was diluted with DME supplemented with 1% calf serum and actinomycin D (3 μg/ml, Calbiochem) and allowed to absorb in 0.25 ml for 60 min at 37°C. Two milliliters of DME supplemented with 1% calf serum and actinomycin D (3 μg/ml) was then added to the infected cells. All labeling was done 8 to 10 h after infection. The cells were labeled with [^{35}S]methionine (Amersham/Searle, >500 Ci/mmol) in methionine-free DME supplemented with 0.2% calf serum and actinomycin D (3 μg/ml). Cells were labeled with D-[2-^{3}H]mannose (Amersham/Searle, 2 Ci/mmol) in DME containing glucose at a concentration of 100 μg/ml (equal to 10% that of the normal concentration of glucose), 0.2% calf serum, 5 mM sodium pyruvate, nonessential amino acids, and actinomycin D (3 μg/ml). Cells were labeled with a mixture of essential ^{14}C-amino acids in DME lacking these same essential amino acids and supplemented with 0.2% calf serum and actinomycin D (3 μg/ml). In all experiments, a total of 0.3 ml of labeling media was used per 35-mm plate. Labeling was terminated by the addition of 100 μl of concentrated electrophoresis sample buffer to the culture medium. The concentrated electrophoresis sample buffer consisted of 8% sodium dodecyl sulfate (SDS) (Bio-Rad and BDH), 4% β-mercaptoethanol, 40% glycerol, 20 mM sodium phosphate (pH 7.0), and 0.4 M dithiothreitol. The dissolved samples were passed through a 1/2-inch 27-gauge needle three times, boiled for 5 min, and frozen at −70°C until analyzed.

Sindbis temperature-sensitive mutants. Sindbis temperature-sensitive mutants, ts-2, ts-5, ts-10, and ts-20, were obtained from Boyce Burge (7). Chick cells were infected at a multiplicity of approximately 800 to 1,200 PFU per cell in DME supplemented with 1% calf serum and actinomycin D (3 μg/ml) at 37°C. One hour after infection, 2.0 ml of DME supplemented with 2% tryptose phosphate broth, 1% calf serum, and actinomycin D (3 μg/ml) was added, and the infection was allowed to proceed at 30°C for 10 h. The infected cells were then shifted to 41°C (the nonpermissive temperature) for approximately 2 h. Labeling was then performed at 41°C as described above.

Pulse-chase experiments. BHK cells infected with wild-type Sindbis virus were grown for 7.5 h in normal DME supplemented with 1% calf serum and actinomycin D (3 μg/ml). The infected cells were then depleted of methionine by incubation in 2.0 ml of methionine-free DME supplemented with 1% calf serum and actinomycin D (3 μg/ml) for 30 min. This medium was then removed, and 0.3 ml of methionine-free DME supplemented with 0.2% calf serum and 100 μCi of [^{35}S]methionine was added to the infected cells. After a 10-min labeling period, 100 μl of concentrated electrophoresis sample buffer was added directly to the culture medium of one dish, and the sample was sheared, boiled, and stored as described above. The culture medium was removed from the remaining dishes, the cells were washed three times with DME and then incubated in 2.0 ml of DME supplemented with 10% tryptose phosphate broth, 0.5% calf serum, and nonradioactive methionine (150 μg/ml, which is equal to five times the normal concentration in DME). At appropriate intervals, 100 μl of concentrated electrophoresis sample buffer was added directly to the culture medium, and the samples were treated as described above.

Virus purification. So as to properly compare the structural proteins of SFV and Sindbis virus, the two viruses were purified by exactly the same procedure. Briefly, BHK cells were infected with wild-type SFV, and chick cells were infected with wild-type Sindbis virus, both at a multiplicity of 50 PFU/cell, in 1.0 ml of DME supplemented with 1% calf serum. After 2.5 h of incubation at 37°C, the infected cells were labeled for 10 h with [^{35}S]methionine (10 μCi/ml) in methionine-free DME supplemented with 2% calf serum. The culture medium was then removed, and the cells were washed with 1 to 2 ml of Ca^{2+}- and Mg^{2+}-free Tris-buffered saline containing an additional 0.1 M NaCl. This wash was combined with the culture medium, and the resulting mixture was chilled on ice and clarified by centrifugation for 5 min at 18,000 × *g* at 4°C. The virus was then pelleted by centrifugation for 60 min at 176,000 × *g* at 4°C in a Titanium 60 rotor. The virus pellet was resuspended in 1.0 ml of 0.2 M NaCl, 0.05 M Tris-hydrochloride (pH 7.4 at 4°C), and 0.001 M EDTA. The resuspended virus was subjected to sonic oscillation for 30 s to disrupt viral aggregates and then clarified for 10 min at 18,000 × *g* at 4°C. The supernatant was layered on top of a continuous 15 to

30% (wt/wt) sucrose gradient which contained 0.05 M NaCl, 0.05 M Tris-hydrochloride (pH 7.0 at 4°C), and 0.001 M EDTA and then centrifuged for 85 min at 155,000 × *g* at 4°C in an SW-41 rotor. The purified virus was removed from the gradient and stored in sucrose at −70°C until analyzed.

SDS-polyacrylamide gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed by using 1-mm thick, 13.5-cm long polyacrylamide slab gels essentially as described by Laemmli (14). The resolving gel contained 20% acrylamide (Bio-Rad, recrystallized from chloroform), 0.065% bisacrylamide (Eastman, recrystallized from acetone), 0.1% SDS (BDH or Bio-Rad), and 0.375 M Tris-hydrochloride (pH 8.82) (Sigma). The running buffer consisted of 0.192 M glycine (Sigma), 0.025 M Trisma base (Sigma), and 0.1% SDS and had a final pH of 8.3 to 8.4. The protein samples were dissolved in 2% SDS, 0.1 M dithiothreitol, 5 mM sodium phosphate (pH 7.0), 15% glycerol (vol/vol), 1% mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride, and boiled for 2 min immediately before application to the gel. Ten-microliter portions were loaded on the gel. Variable amounts of both the Sindbis 9,800-dalton (9.8K) polypeptide and the E3 of SFV were found in our preliminary experiments. We discovered that this was due to the elution of the proteins from the gel during fixation (and staining) in 25% isopropanol, 10% acetic acid, and Coomassie brilliant blue (0.33 g/liter) and destaining in 10% isopropanol–10% acetic acid. Therefore, all gels were immediately impregnated with diphenyloxazole (4) and dried as quickly as possible. Fluorography was done as described by Laskey and Mills (15) by using prefogged Kodak XR-5 film.

Apparent molecular weights were obtained by electrophoresis in parallel with suitable markers on a 20% acrylamide–0.065% bisacrylamide gel as described above. These gels were able to resolve proteins with molecular weights as low as 3,000. The proteins used as markers and the apparent molecular weights assumed here were: bovine serum albumin (68,000), ovalbumin (43,000), trypsin inhibitor (6,100), glucagon (3,400), synthetic adrenocorticotrophic hormone, amino acids 1 to 24 (2,900), and synthetic B-endorphin (3,400). Synthetic adrenocorticotrophic hormone and B-endorphin were kindly provided by J. Rivier, The Salk Institute. Staining of molecular weight markers was done by using 25% isopropanol, 10% acetic acid, and Coomassie brilliant blue (0.33 g/liter). Destaining solution consisted of 10% acetic acid and 10% isopropanol.

Two-dimensional tryptic peptide maps. Two-dimensional tryptic peptide maps were done as described by Gibson (12) and Beemon and Hunter (1). Briefly, protein bands were excised from a dried 20% polyacrylamide gel, homogenized, and extracted with 0.05 M NH_4HCO_3 in 0.1% SDS. The polyacrylamide was removed by centrifugation (10 min, 18,000 × *g*). Proteins were concentrated by precipitation with 20% trichloroacetic acid and washed successively with absolute ethanol and absolute ethanol–ethyl ether (1:1). They were then oxidized with performic acid and digested with tosyl-L-phenylalanyl chloromethane-treated trypsin (Worthington) in 0.05 M NH_4HCO_3 for 24 h at room temperature. The resulting peptides were separated on cellulose thin-layer plates (Merck) by electrophoresis in one dimension in butanol, pyridine, acetic acid, and water (2:1:1:36, vol/vol, pH 4.7) and in the second dimension by ascending chromatography in butanol, pyridine, acetic acid, and water (97:75:15:60, vol/vol, pH 5.3). After drying, the plates were briefly immersed in 0.4% diphenyloxazole in 2-methylnaphthalene (45°C) and exposed to prefogged Kodak XR-5 film for 2 to 4 weeks at −70°C (4).

The above procedure was less than ideal for the preparation of the Sindbis 9.8K polypeptide (and that of E3 of SFV). Precipitation of these two small glycoproteins with 20% trichloroacetic acid was quite inefficient. As a result, trichloroacetic acid precipitation caused some enrichment of contaminating polypeptides in our preparation of the 9.8K protein. This is most obvious in the map of the 9.8K protein labeled with a mixture of ^{14}C-amino acids. Here, contamination with peptides derived from the C protein is detectable.

RESULTS

Polypeptide composition of Sindbis virus and SFV virions. SFV virions, labeled with [^{35}S]methionine and purified by our usual procedure, contained four structural polypeptides (Fig. 1, lane A): two large envelope glycoproteins, E1 and E2, a small envelope glycoprotein, E3, and a nucleocapsid protein, C, as has been described before (11). The Sindbis virion, when purified in exactly the same way as SFV, contained only three structural polypeptides (Fig. 1, lanes B and G): two large envelope glycoproteins, E1 and E2, and a nucleocapsid protein, C (17). Because both these viruses were purified and analyzed in exactly the same manner, these results demonstrate unequivocally that Sindbis virions differ from SFV in that they do not contain a fourth small polypeptide analogous to E3 of SFV.

Polypeptides produced in Sindbis virus-infected cells. Since the Sindbis virus glycoprotein, E2, like glycoprotein E2 of SFV, is produced by proteolytic cleavage of a precursor polypeptide PE2, we felt that a small polypeptide analogous to the E3 of SFV must be produced during infection, but not be incorporated into the mature Sindbis virion. We therefore examined all the [^{35}S]methionine-labeled polypeptides produced in cultures of Sindbis-infected cells by adding a concentrated SDS solution directly to the culture medium and separating the labeled polypeptides by SDS-polyacrylamide gel electrophoresis on a 20% gel (Fig. 1, lane D). We found that Sindbis-infected BHK cells produced not one, but two small polypeptides not found in the purified virus. The larger of these migrated on a 20% SDS-polyacrylamide gel with an apparent molecular weight of approximately 9,800 and will be referred to as the

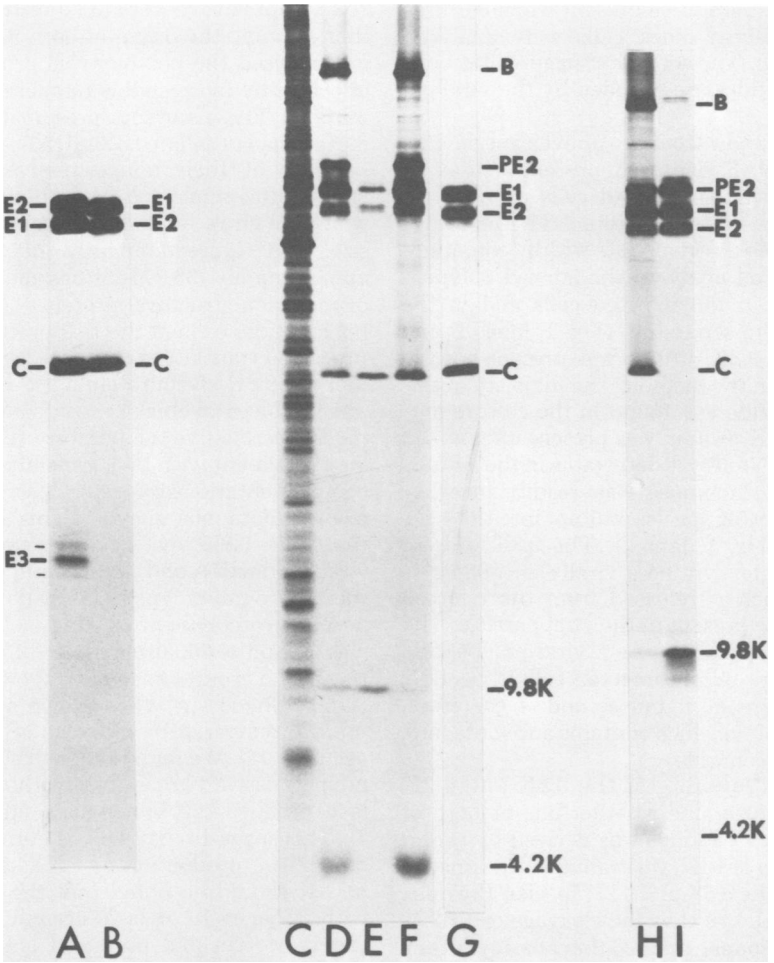

FIG. 1. *Polypeptide composition of the SFV virion, the Sindbis virion, and cells infected with Sindbis virus. SFV and Sindbis virus were labeled with [^{35}S]methionine and purified as described in Materials and Methods. BHK cells, infected with Sindbis virus, were labeled with [^{35}S]methionine or [2-^{3}H]mannose for 2 h, 8 h after infection at 37°C. Samples of combined cells and medium were prepared by the addition of a concentrated SDS solution directly to the medium. Cells and cell debris were removed from the sample of the culture medium by centrifugation at 18,000 × g for 20 min at 4°C. The polypeptides present in the medium were then solubilized by the addition of a small volume of concentrated SDS solution. The cellular sample was prepared by washing the monolayer once with Tris-buffered saline and then dissolving the cells in a solution of culture medium containing 2% SDS, 0.1 M dithiothreitol, 5 mM sodium phosphate (pH 7.0), 15% glycerol, 1% mercaptoethanol, and 2 mM phenylmethylsulfonyl fluoride. Equal fractions of the cellular sample and the sample of medium were applied to the gel. The figure is a composite of three fluorograms of three different gels. (A) and (B) are from one gel; (C), (D), (E), (F), and (G) are from a second gel, and (H) and (I) are from a third gel. (A) SFV virions labeled with [^{35}S]methionine. (B) Sindbis virus virions labeled with [^{35}S]-methionine. (C) Uninfected BHK cells labeled with [^{35}S]methionine for 2 h at 37°C. (D) Sindbis-infected BHK cells and their culture medium labeled with [^{35}S]methionine. (E) Medium from Sindbis-infected BHK cells labeled with [^{35}S]methionine. (F) Sindbis-infected BHK cells labeled with [^{35}S]methionine. (G) Sindbis virus virions labeled with [^{35}S]methionine. (H) Sindbis-infected BHK cells and their culture medium labeled with [^{35}S]methionine. (I) Sindbis-infected BHK cells and their culture medium labeled with [2-^{3}H]mannose.*

9.8K protein. This polypeptide is heterodisperse. It often migrates during SDS-polyacrylamide gel electrophoresis as a triplet (data not shown). The smaller polypeptide migrated as a single diffuse band with an apparent molecular weight of approximately 4,200 and will be termed the 4.2K protein. Neither small polypeptide was found in cultures of uninfected BHK cells (Fig.

1, lane C), but both were present when cultures of Sindbis-infected chick cells were analyzed (Fig. 3, lane E). These results suggest that both small polypeptides are encoded by the viral genome.

We did not know from the above experiments whether the 9.8K and 4.2K proteins remained associated with the infected cells or were released into the culture medium. Therefore, we labeled Sindbis-infected cells with [^{35}S]methionine for 2 h and analyzed the labeled polypeptides present in the infected cells and in the culture medium separately (Fig. 1, lanes E and F). While the 9.8K protein was present both in the cells and in the medium, the majority of this small polypeptide was found in the culture medium. The 4.2K protein was present only in the infected cells. None was detectable in the culture medium. [2-^{3}H]mannose was readily incorporated into the 9.8K species, but not into the 4.2K polypeptide (Fig. 1, lane I). The 9.8K protein appears, therefore, to be a virally encoded glycoprotein which is released from the infected cells, but is not present in the viral particle. The 4.2K protein appears to be a viral polypeptide which is found only in infected cells. The 4.2K protein contains no mannose and is, therefore, not a glycoprotein which contains an asparagine-linked oligosaccharide.

Kinetics of labeling of the 9.8K and 4.2K proteins. To examine whether one or both of these small polypeptides was derived by proteolytic cleavage of PE2, we examined the kinetics of labeling of the 9.8K and 4.2K species. Previous studies have shown that the cleavage of PE2 to E2 occurs no sooner than 20 min after synthesis of the precursor (18, 19). The appearance of a labeled polypeptide corresponding to that portion of PE2 not found in E2 should, therefore, coincide with the appearance of labeled E2. The results of a pulse-chase experiment, in which cells were labeled for 10 min with [^{35}S]methionine and then were incubated further in the absence of label, are shown in Fig. 2. After 10 min no labeled E2 or 9.8K could be detected while considerable amounts of labeled 4.2K were clearly evident (Fig. 2, lane B). Only after a chase of 20 min could labeled E2 and 9.8K be detected (Fig. 2, lane D). These results demonstrate that the 9.8K glycoprotein displays the same delay in incorporation of radiolabel as does E2 and suggests that both 9.8K and E2 are derived by proteolytic cleavage of the precursor PE2. Labeled 4.2K was detected at all time points examined. This suggests that the synthesis of the 4.2K protein is not dependent upon processing of PE2.

Polypeptides synthesized by Sindbis virus temperature-sensitive mutants. To further examine the origin of both 9.8K and 4.2K, we analyzed the polypeptides produced during infection by four Sindbis temperature-sensitive mutants. These mutants, presumably containing mutations in the late 26S mRNA, produce polypeptides at their nonpermissive temperature which differ somewhat from those produced by wild-type virus. ts-2-infected chick cells synthesize, at 41°C, predominantly one polypeptide of approximately 130,000 daltons and produce little or no virion structural proteins (22). This large polypeptide, termed the ts-2 protein, is the unprocessed translate of the viral 26S mRNA. Neither the 9.8K glycoprotein nor the 4.2K protein was produced in chick cells infected with ts-2 at the nonpermissive temperature (Fig. 3). A similar experiment with ts-5, a member of the same complementation group as ts-2, yielded the same results (data not shown). This demonstrated that both 9.8K and 4.2K are encoded by the viral 26S mRNA and that their appearance, like the other viral polypeptides, is dependent upon normal proteolytic processing.

ts-10 and ts-20, although in different complementation groups, have been shown to exhibit a similar phenotype when grown at the nonpermissive temperature; cleavage of PE2 does not occur (6, 13). We found that cells infected at the nonpermissive temperature with either ts-10 or ts-20 produce 4.2K but do not produce 9.8K (Fig. 3). This suggests that 9.8K is derived from PE2 during the production of E2 and that 4.2K is not. It should be noted that the cells infected with either ts-10 or ts-20 accumulated more of the B protein and produced less of the 4.2K protein than did cells infected with wild-type virus.

Relationship of the viral polypeptides as determined by two-dimensional tryptic peptide maps. To examine the relationship of 4.2K and 9.8K to the larger polypeptides produced in Sindbis-infected cells, two-dimensional tryptic peptide maps of all the [^{35}S]methionine-labeled viral polypeptides present in cells infected with wild-type virus and the large polypeptide present in cells infected with ts-2 were prepared (Fig. 4). The map of the ts-2 protein can be seen to consist of those methionine-containing peptides present in PE2, E1, and C, confirming the earlier results of Schlesinger and Schlesinger (16). The B protein can be seen to contain those methionine-containing peptides present in PE2 and E1 as was also shown by Schlesinger and Schlesinger (16).

The 4.2K protein was found to have two methionine-containing tryptic peptides which appeared to be present in both the ts-2 protein and

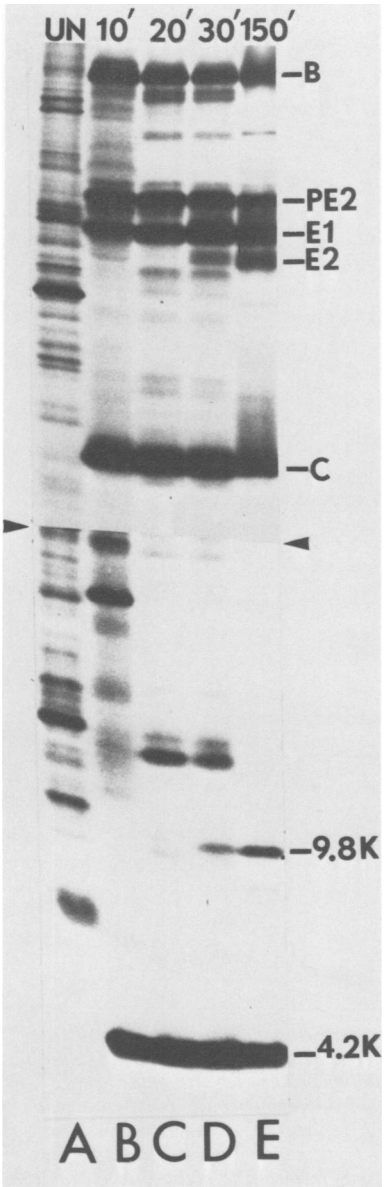

FIG. 2. *The kinetics of labeling of 9.8K and 4.2K. Sindbis-infected BHK cells were labeled for 10 min with [^{35}S]methionine and then chased for 10 and 20 min, 8 h after infection. The samples were prepared by adding concentrated sample buffer solution directly to the culture medium. The labeled polypeptides were separated on a 20% SDS-polyacrylamide gel. The figure shown is a composite of two different exposures of the same gel. Such a composite allows the low-molecular-weight polypeptides to be seen clearly without overexposing the high-molecular-weight polypeptides. The portion of the figure above the arrowheads was exposed for 5 days, while the portion below the arrowheads was exposed for 19 days. (A) Uninfected BHK cells labeled for 150 min. (B) Infected cells labeled for 10 min. (C) Infected cells labeled for 10 min and chased for 10 min. (D) Infected cells labeled for 10 min and chased for 20 min. (E) Infected cells labeled for 150 min.*

the B protein, and possibly even in PE2 and E1 (Fig. 4). To determine whether this was indeed the case, the 4.2K peptides were mixed with those of the other viral polypeptides, and the mixtures were analyzed by two-dimensional mapping (Fig. 5). The ts-2 protein and the B protein clearly contained both the peptides present in 4.2K. Both of the 4.2K-specific peptides were reproducibly somewhat fainter than the major methionine-containing peptides of the ts-2 protein and the B protein. Why this is so is not yet clear. Small amounts of one of the 4.2K methionine-containing tryptic peptides appeared to be present in these preparations of PE2 and E1. However, we are not sure that this is significant. The presence of the 4.2K-specific peptides in the B protein is consistent with our earlier observations that cells infected with ts-10 and ts-20 produce, at the nonpermissive temperature, somewhat more B protein and less 4.2K than cells infected with wild-type virus (Fig. 3).

The kinetics of labeling of 9.8K and the analysis of the polypeptides produced by Sindbis-temperature-sensitive mutants at the nonpermissive temperature strongly suggested that 9.8K was derived by proteolytic cleavage of PE2. We expected our two-dimensional tryptic peptide maps to substantiate these earlier observations. However, our procedure produced essentially no resolved methionine-containing tryptic peptides from the 9.8K protein (Fig. 4). Instead, most of the methionine-labeled material remained at or near the origin of the map. Therefore, two-dimensional tryptic peptide maps of PE2, E2 and 9.8K, labeled instead with a mixture of ^{14}C-amino acids, were prepared. In contrast to the results using [^{35}S]methionine, 9.8K labeled with ^{14}C-amino acids was found to contain two well resolved tryptic peptides (Fig. 6). The maps of PE2, E2, and 9.8K and that of a mixture of the peptides of PE2 and 9.8K clearly demonstrated that the two predominant ^{14}C-amino acid-labeled 9.8K tryptic peptides (indicated by arrows in Fig. 6) were contained in the precursor PE2, but were absent from E2. The map of 9.8K in Fig. 6 also contained a few minor tryptic peptides. These minor peptides appear as predominant peptides in the two-dimensional map of ^{14}C-amino acid-labeled C protein (not shown) and result from partial contamination of our 9.8K preparations (see Materials and Methods).

The reason the two-dimensional tryptic peptide map of 9.8K, labeled with [^{35}S]methionine, failed to reveal any resolvable methionine-con-

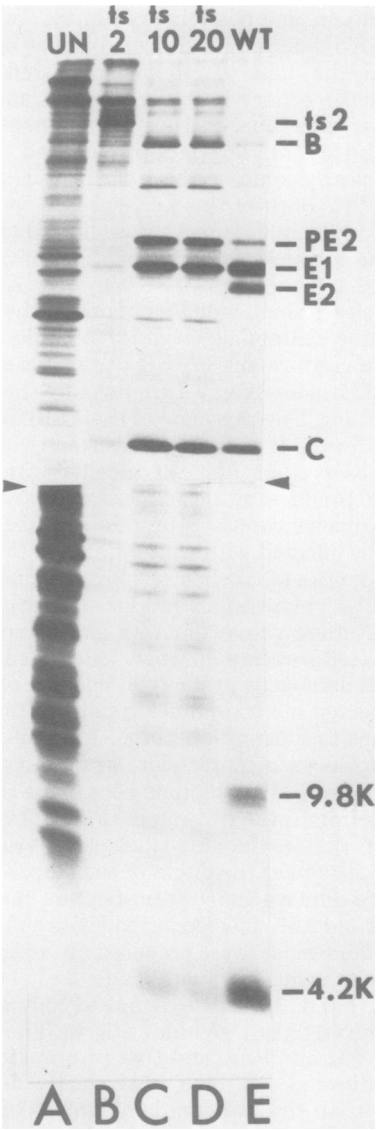

FIG. 3. *The polypeptides synthesized by Sindbis temperature-sensitive mutants at the nonpermissive temperature. Chick cells infected with ts-2, ts-10, and ts-20 were labeled at the nonpermissive temperature (41°C) with [^{35}S]methionine approximately 10 h after infection. Labeling was allowed to proceed for approximately 2 h, at which time concentrated sample buffer was added directly to the culture medium. Uninfected chick cells and wild-type Sindbis virus-infected cells were labeled for 150 min at 37°C. The labeled polypeptides were analyzed on a 20% SDS-polyacrylamide gel. The figure shown is a composite of two different exposures of the same gel, as described in the legend to Fig. 2. The portion of the figure above the arrowheads was exposed for 3 days, while the portion below the arrowheads was exposed for 8 days. (A) Uninfected chick cells. (B) ts-2-infected chick cells. (C) ts-10-infected chick cells. (D) ts-20-infected chick cells. (E) Wild-type Sindbis virus-infected chick cells.*

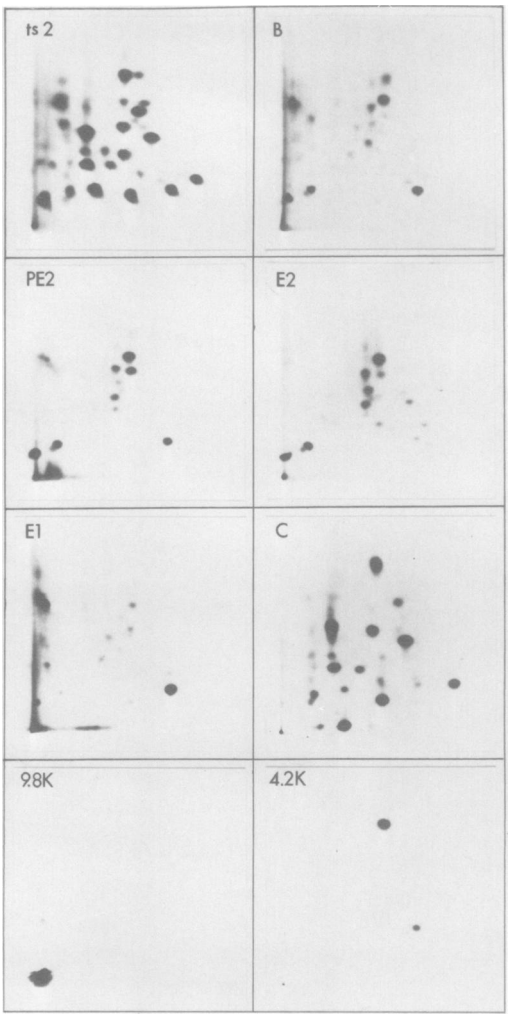

FIG. 4. *Two-dimensional analysis of the [^{35}S]methionine-containing tryptic peptides of Sindbis virus-specific polypeptides. Tryptic peptides of [^{35}S]methionine-labeled virus-specific polypeptides were prepared and separated as described in Materials and Methods. In this and all subsequent figures, electrophoresis was from left to right, and chromatography was from bottom to top.*

taining peptides may be due to the fact that 9.8K is a glycoprotein. It is possible that the presence of large amounts of carbohydrate in the 9.8K protein results in inefficient digestion by the trypsin of that portion of the molecule which contains methionine. Alternatively, the digestion may go to completion, but the methionine-labeled peptides so generated may fail to migrate in the buffers used here.

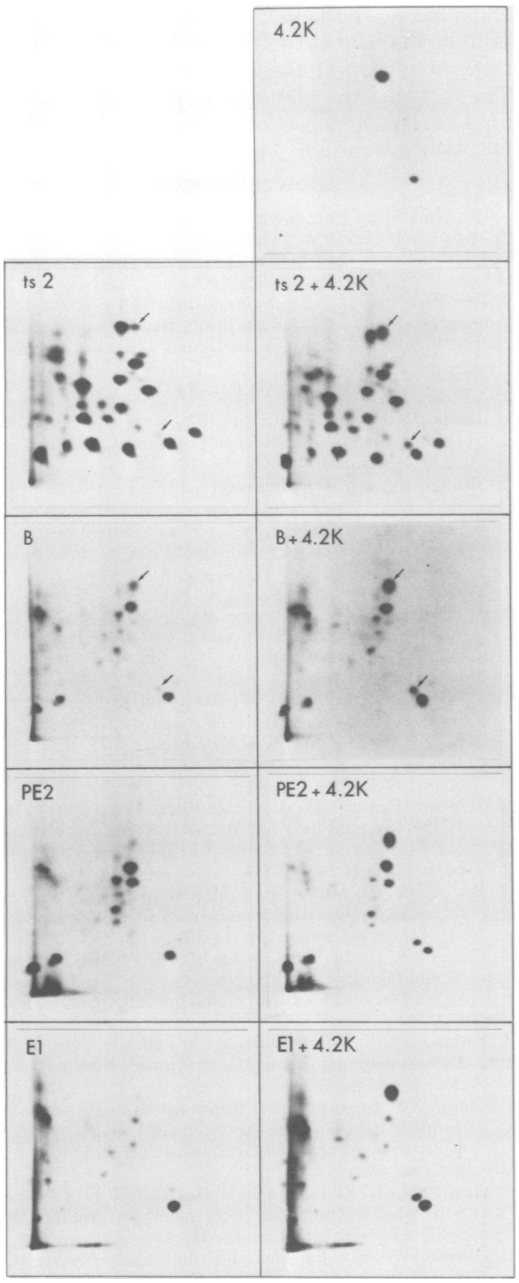

FIG. 5. *Comparison of the methionine-containing tryptic peptides of 4.2K with those of the ts-2 protein, the B protein, PE2, and E1. [^{35}S]methionine-labeled peptides were prepared and separated as described in Materials and Methods. Mixtures of the peptides were prepared just before electrophoresis. The arrows indicate the 4.2K peptides.*

DISCUSSION

We have described here two small virally encoded polypeptides which are present in cultures of Sindbis-infected BHK and chick cells, but which are not found in the virion itself. The larger of these, 9.8K, is a glycoprotein and migrates with an average apparent molecular weight of approximately 9,800 during SDS-polyacrylamide gel electrophoresis. Only a small amount of this glycoprotein is found associated with the infected cell. The majority of 9.8K is found in the culture medium. The smaller polypeptide, 4.2K, migrates with an apparent molecular weight of approximatelv 4,200, contains no mannose, and hence is probably not a glycoprotein which contains an asparagine-linked oligosaccharide. Unlike the 9.8K glycoprotein, all of 4.2K remains associated with the infected cell. Several lines of evidence indicate that 9.8K is produced during the proteolytic conversion of the precursor PE2 to the virion glycoprotein E2. Peptide mapping and experiments with temperature-sensitive mutants make it clear that the 4.2K protein, like the viral structural polypeptides, is encoded by the viral 26S mRNA. The function of this small polypeptide during virus infection is not, however, apparent.

We have performed three types of experiments which demonstrate that 9.8K is derived from PE2. First, pulse-chase experiments with radioactive amino acids demonstrated that both PE2 and 9.8K become labeled only after a lag of 20 to 30 min. Second, we found that two temperature-sensitive mutants, ts-10 and ts-20, both of which fail to cleave the precursor PE2 at the nonpermissive temperature, produce neither E2 nor 9.8K. Finally, two-dimensional tryptic pep-

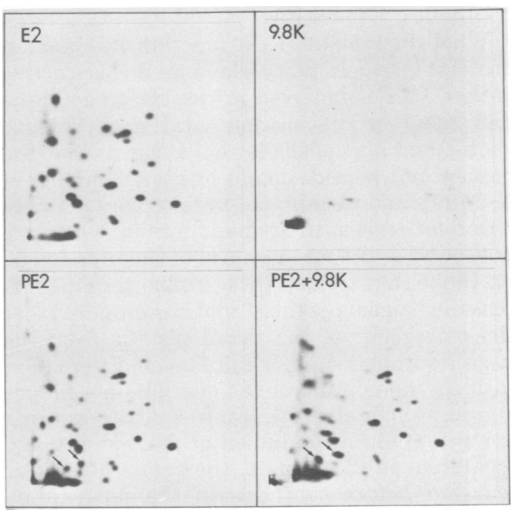

FIG. 6. *Comparison of the ^{14}C-amino acid-labeled tryptic peptides of PE2, E2, and 9.8K. The tryptic peptides of PE2, E2, and 9.8K, labeled with ^{14}C-amino acids, were prepared and separated as described in Materials and Methods. The arrows indicate the peptides from 9.8K.*

tide maps of ^{14}C-amino acid-labeled PE2, E2, and 9.8K revealed that the tryptic peptides present in 9.8K are found in PE2 but are absent from E2. The Sindbis 9.8K glycoprotein is therefore analogous to the E3 glycoprotein, present in the SFV virion, in that both are small glycoproteins which are produced during the proteolytic conversion of PE2 to E2. Our results have shown, however, that the Sindbis 9.8K polypeptide, unlike the E3 glycoprotein of SFV, does not become permanently associated with the mature virion, but instead accumulates in the medium of infected cells. Similar observations have been made by J. T. Mayne, C. Rice, and J. Strauss (manuscript in preparation) and by D. Brown (personal communication). It is possible, however, that the 9.8K glycoprotein is, in fact, incorporated into the mature Sindbis virion, but is lost soon after release of the virus from the host cell.

The role of the 4.2K protein during virus infection is unclear at this time. We know that this small polypeptide is encoded by the same mRNA which directs the synthesis of the viral structural polypeptides. This is demonstrated by the fact that the tryptic peptides of 4.2K are present in both the 130,000-dalton polypeptide, which is produced in ts-2-infected cells, and the 100,000-dalton B polypeptide, which accumulates during infection with wild-type virus. The fact that 4.2K is present in the B protein allows us to conclude that the sequences which encode 4.2K are located between the 3′ end of the gene for the C protein and the 3′ end of the 26S mRNA, as are the genes for the two viral glycoproteins, E1 and E2.

What then might be the function of this small protein? One possibility which we find attractive is that 4.2K is involved in the transmembrane movement of the nascent viral glycoproteins. Blobel and his colleagues have shown that the nascent polypeptide chains of a large number of secretory and membrane proteins contain 20 to 30 amino acids at their amino termini which are not found in the mature form of the proteins (2, 3). Blobel has termed these amino terminal extensions "signal peptides" and has proposed that the emergence of the signal peptide from the large ribosomal subunit initiates an interaction with the membrane of the endoplasmic reticulum and facilitates the transfer of the growing polypeptide into the lumen of the endoplasmic reticulum. In most cases, the signal peptide is removed before synthesis of the polypeptide chain is complete. It seems reasonable that the Sindbis glycoproteins, like most other membrane and secretory proteins, require a signal peptide to facilitate their transfer across the endoplasmic reticulum membrane. The 4.2K protein may function, during the synthesis of the viral glycoproteins, as such a signal peptide. The fact that the apparent location of the gene for 4.2K is near those for PE2 and E1 is consistent with this notion.

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