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NOTES

Alterations in Sindbis Viral Envelope Proteins by Treating BHK cells with Glucosamine

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Addition of D-glucosamine to BHK cells infected with Sindbis virus inhibited the formation of the E-2 viral envelope from its precursor PE-2. Release of virus was blocked, and two new viral protein bands replaced the normal envelope protein bands detected in SDS-gel electropherograms of infected cell extracts.

Sindbis virus, a member of the group A togavirus class, is composed of three structural proteins; two of these are part of the virion envelope and are glycoproteins (15). In the process of virion formation in infected BHK or chicken embryo cells, the viral-specific envelope proteins pass through a chain of glycosylation reactions leading to the addition of a variety of carbohydrate residues and providing a pattern of glycosyl residues that is host cell dependent (4, 5). In the final stages of maturation of the virus—before the actual budding of the virion from the cell—there is a proteolytic cleavage of one of the envelope proteins (called PE2) that produces the virion protein E2 (13). In this conversion of PE2 to E2, additional carbohydrate residues appear to be added, whereas others may be lost from the precursor (16).

These latter modifications most likely occur on the host cell plasma membrane, where some glycosyl transferases are known to be localized (11) and where the actual budding process for Sindbis virus takes place (1, 3, 9). It is not known, however, whether the proteolytic cleavage is an essential part of the budding process or whether it can occur in the absence of glycosylation. We attempted to answer the latter question by examining formation of virus and viral-specific proteins in BHK cells that have been blocked in the glycosylation reaction. We used relatively high concentrations of glucosamine (20 mM) as an inhibitor of glycosylation (2, 8), adding it 2 h postinfection and 3 h before labeling the infected BHK cells with [^{35}S]methionine. Previous experiments with cells infected with Semliki Forest virus, fowl plaque virus, and Sindbis virus have shown that the presence of glucosamine prevents the appearance of the virus but does not significantly alter protein synthesis (7).

In our experiments, monolayers of labeled cells were treated with 2% SDS and proteins were prepared for electrophoresis according to our published procedures, which involve a reduction with mercaptoethanol, alkylation with iodoacetamide, and dialysis (15). Infected cells labeled for 10 min but not treated with glucosamine showed four viral-specific proteins (sample 1, Fig. 1); the capsid, two envelope proteins (E-1 and PE-2), and a larger protein (B-1) that contained the amino acid sequences of the smaller envelope proteins (13). Cells treated with glucosamine also contained four labeled proteins. Two of these had mobilities identical to the B-1 and capsid proteins, but two were distinct from the envelope proteins detected in untreated cells (sample 2, Fig. 1). We believe that these latter are under-glycosylated forms of the two Sindbis virus envelope proteins. They were precipitated by anti-Sindbis antibodies essentially to the same extent as the glycoproteins PE-2 and E-1 (Table 1), and they also shared a number of [^{35}S]methionine tryptic peptides in common with the glycosylated forms of these proteins (Fig. 2). The lack of complete identity in both the immunological and biochemical tests suggests that the glycosyl residues influence both the antigenicity of the SDS-denatured polypeptides and the electrophoretic mobilities of some of the methionine-containing tryptic peptides. The molecular weights of these putative virus envelope pro-

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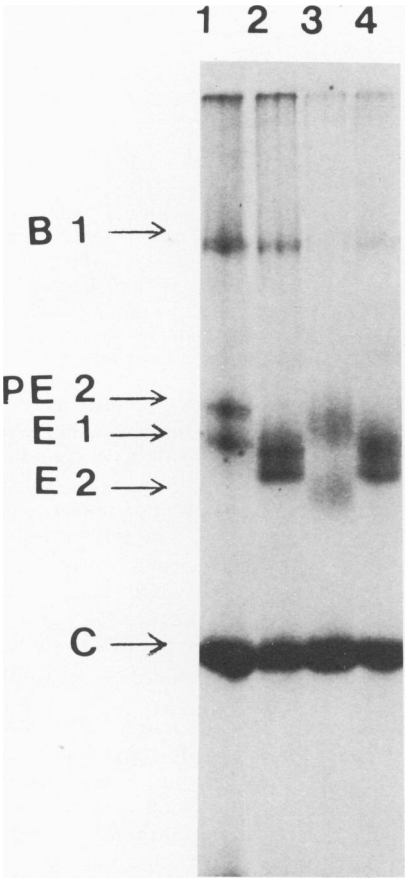

FIG. 1. *SDS-polyacrylamide slab gel analysis of [^{35}S]methionine-labeled, Sindbis-specific proteins from BHK cells treated with D-glucosamine. Monolayers of BHK-21 cells were infected with Sindbis (multiplicity of infection = 100) and labeled for 10 min with 10 μCi of [^{35}S]methionine (~100 Ci/mmol; New England Nuclear Corp) 5 h postinfection. Cells were either harvested or the medium was removed and replaced with a solution of minimal Eagle medium containing a fourfold excess of amino acids and 100 μg of cycloheximide per ml. After an additional 60 min, cells were harvested. Preparation of proteins for electrophoresis and the details of electrophoresis in slab gels were exactly as described (15). When used, glucosamine was added 2 h before the radioactive label. (1) No glucosamine, 10-min pulse; (2) glucosamine, 10-min pulse; (3) no glucosamine, 60-min chase; (4) glucosamine, 60-min chase. Samples had 10,000 to 20,000 counts/min loaded into gel slots; samples contained the same amount of cell extract. In general, electrophoresis was at 120 V for 2 h in the apparatus described by Studier (17).*

teins were estimated to be about 50,000 and 54,000 based on their mobilities relative to B-1 (molecular weight = 112,000) and the capsid (molecular weight = 30,000). The sum of their molecular weights was close to that of the B-1 protein, and the latter has been shown to contain the polypeptide sequences of the virion envelope proteins (13).

The two new polypeptides were metabolically rather stable. In a pulse-chase experiment, the PE-2 and E-1 proteins of Sindbis-infected, untreated BHK cells (sample 1, Fig. 1) was converted to the glycosylated forms of E-1 and E-2 noted in sample 3, Fig. 1, but the protein pattern of sample 2, Fig. 1, obtained from glucosamine-treated cells remained unchanged after the chase in the presence of glucosamine, except for the disappearance of the B-1 protein (sample 4, Fig. 1).

In addition to the altered pattern of intracellular viral-specific proteins, the production of extracellular virus and radioactive particles in the medium of viral-infected cells treated with glucosamine was decreased some 10-fold (Table 2). Thus, both the cleavage of PE-2 to E-2 and the budding of virus appear to require appropriate glycosylation of the envelope proteins. The processing of the B-1 protein, which in our experiment is not glycosylated extensively if at

TABLE 1. *Antigenic similarity between envelope proteins formed in presence and absence of glucosamine*[a]

Sample	Antiserum	[^{35}S]protein in precipitin (%)
Untreated cells	Anti-Sindbis	70
	Anti-BγG	11
Glucosamine-treated cells	Anti-Sindbis	63
	Anti-BγG	11

[a] ^{35}S-labeled protein was prepared as described in the legend to Fig. 1. After electrophoresis in slab gels and preparation of autoradiograms, the region of the gels containing only the envelope proteins (PE-2, E-1) was cut out and eluted with water. Samples of 200 μliters (5,760 counts/min for the untreated and 12,390 counts/min for the glucosamine-treated cells) were added to 5 μliters of rabbit serum containing either anti-Sindbis antibodies or anti-trinitrophenyl bovine gamma globulin antibodies (BγG). Phosphate-buffered saline was added to a final volume of 0.5 ml. After 30 min at 37 C, 25 μliters of a preparation of goat anti-rabbit gamma globulin (Gateway Immunosera Co., Cahokia, Ill.) was added, and samples were incubated an additional 120 min at 37 C. Precipitates were collected by centrifugation, washed three times with cold saline solution, and collected on membrane filters (Millipore Corp., Bedford, Mass.). The anti-Sindbis serum was prepared by injecting rabbits with purified virus mixed in complete Freund adjuvant; it blocks Sindbis infection and was used here under conditions of antibody excess.

FIG. 2. *Electrophoretic pattern of tryptic-digested Sindbis envelope proteins. Proteins were isolated from infected cells by the procedures described in Fig. 1 and Table 1. Preparation of the tryptic peptides from proteins eluted from gels has been published (13). Electrophoresis was at pH 3.5 for 2 h at 3.5 kV. (Left sample) Envelope proteins from glucosamine-treated cells, 29,000 counts/min added; (right sample) envelope proteins from untreated cells, 18,000 counts/min added. Site of application of the samples is at the bottom of the figure.*

TABLE 2. *Virus formation by cells treated with glucosamine*[a]

Treatment	Yield of Virus (PFU)	[^{35}S]virus (counts/min)
With glucosamine	1.2×10^8	150
No glucosamine	2×10^9	3,300

[a] Two hours after infection of 10^7 BHK cells, 20 mM glucosamine was added, and 3 hours later, [^{35}S]methionine was added (10 μCi of 100 Ci/mmol). The medium was collected 1 h later and a sample was assayed for plaque-forming units on chicken embryo fibroblast cells (10). The balance of material was layered on a composite sucrose gradient and centrifuged to equilibrium according to the procedure of Scheele and Pfefferkorn (12). The ^{35}S-labeled virus counts per minute represents that amount of radioactivity banding in the region of virus for this gradient.

all with glucosamine (data not shown), is not inhibited by glucosamine. The B-1 protein may be cleaved to the two enveloped proteins or may be degraded nonspecifically. Our earlier data on this point suggested the former (13), but more recent experiments using cycloheximide during the chase experiment suggest a degradative pathway.

These experiments offer some data implicating an essential role for the conversion of PE-2 to E-2 in Sindbis virus budding. This result supports a recently published observation that one of the defects in a temperature-sensitive mutant of Sindbis is an inability to convert PE-2 to E-2 (6). The under-glycosylated envelope proteins of Sindbis virus show mobilities in acrylamide gel faster than that observed when they are in their glycosylated form.

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LITERATURE CITED

1. Birdwell, C. R., E. G. Strauss, and J. H. Strauss. 1973. Replication of Sindbis virus. III. An electron microscope study of virus maturation using the surface replica technique. Virology **56**:429-438.
2. Bosmann, H. B. 1971. Inhibition of protein, glycoprotein, ribonucleic acid, and deoxyribonucleic acid synthesis by D-glucosamine and other sugars in mouse leukemic cells L51784 and selective inhibition in SV-3T3 compared with 3TC cells. Biochim. Biophys. Acta **240**:74-93.
3. Brown, D. T., M. R. F. Waite, and E. R. Pfefferkorn. 1972. Morphology and morphogenesis of Sindbis virus as seen with freeze-etching techniques. J. Virol. **10**:524-536.
4. Burge, B. W., and A. S. Huang. 1970. Comparison of membrane protein glycopeptides of Sindbis virus and vesicular stomatitis virus. J. Virol. **6**:176-182.
5. Grimes, W. J., and B. W. Burge. 1971. Modification of Sindbis virus by host-specified glycosyl transferases. J. Virol. **7**:309-319.
6. Jones, K. J., M. R. F. Waite, and H. R. Bose. 1974.

Cleavage of a viral envelope precursor during the morphogenesis of Sindbis virus. J. Virol. **13**:809–817.
7. Kaluza, G., C. Scholtissek, and R. Rott. 1972. Inhibition of the multiplication of enveloped viruses by glucosamine and 2-deoxy-D-glucose. J. Gen. Virol. **14**:251–259.
8. Klenk, H. D., C. Scholtissek, and R. Rott. 1972. Inhibition of glycoprotein biosynthesis of influenza virus by D-glucosamine and- 2-deoxy-D-glucose. Virology **49**:723–734.
9. Pedersen, C. E., Jr., and B. P. Sagik. 1973. Sindbis virus maturation. J. Gen. Virol. **18**:375–379.
10. Pfefferkorn, E. R., and H. J. Hunter. 1963. Purification and partial chemical analysis of Sindbis virus. Virology **20**:433–445.
11. Roth, S., E. McGuire, and S. Roseman. 1971. Evidence for cell-surface glycosyl transferases. Their potential role in cellular recognition. J. Cell. Biol. **51**:536–547.
12. Scheele, C. M., and E. R. Pfefferkorn. 1969. Kinetics of incorporation of structural proteins into Sindbis virions. J. Virol. **3**:369–375.
13. Schlesinger, S., and M. J. Schlesinger. 1972. Formation of Sindbis virus proteins: identification of a precursor for one of the envelope proteins. J. Virol. **10**:925–932.
14. Schlesinger, M. J., and S. Schlesinger. 1973. Large-molecular-weight procursors of Sindbis virus proteins. J. Virol. **11**:1013–1016.
15. Schlesinger, M. J., S. Schlesinger, and B. Burge. 1972. Identification of a second glycoprotein in Sindbis virus. Virology **47**:539–541.
16. Sefton, B. M., and B. W. Burge. 1973. Biosynthesis of the Sindbis virus carbohydrates. J. Virol. **12**:1366–1374.
17. Studier, W. F. 1973. Analysis of bacteriophate T7 early RNAs and proteins on slab gels. J. Mol. Biol. **79**:237–248.