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Modification of Sindbis Virus Glycoprotein by Host-Specified Glycosyl Transferases

W. J. GRIMES AND B. W. BURGE

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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The amino acid sequence of the membrane glycoprotein of Sindbis virus is specified by the viral genome, but it has not been determined whether the carbohydrate portion of this molecule is specified by the cell or by the virus. We have examined two of the enzyme activities which catalyze transfer of monosaccharides to glycoprotein (sialyl and fucosyl transferases). Comparison of particulate enzyme preparations from infected and uninfected cells showed no difference in either the specific activity or acceptor specificity of these enzymes. This is impressive in view of the fact that the Sindbis membrane glycoprotein is the only glycoprotein synthesized in the infected cell. It was also determined that sialyl transferase from uninfected cells is capable of transferring (^{3}H) sialic acid to acceptor prepared from Sindbis membrane glycoprotein. These results imply that at least some of the carbohydrate of the virus glycoprotein can arise by host modification.

Glycoproteins are known to be a constituent of the lipoprotein envelope of all enveloped viruses yet examined, including influenza virus, arbovirus, vesicular stomatitis virus, herpesvirus, and vaccinia virus (2, 4, 7, 8, 10; J. A. Holowczak, Bacteriol. Proc., p. 189, 1970).

The peptide portion of the glycoprotein of these viruses is specified by the virus genome. In contrast, the available evidence indicates that the carbohydrate portion of the virus glycoprotein is determined by the host. It should be emphasized, however, that the evidence for host determination is indirect. The principal arguments are (i) that the relative amounts of various monosaccharides found in virus glycoprotein are very similar to the relative amounts of these same monosaccharides found in the membrane of the uninfected host cell and (ii) that the carbohydrate composition of large and small glycopeptides from Sindbis virus is the same as that found in the two principal glycopeptides of serum glycoprotein (3).

To obtain more direct information on this question we have examined two enzyme activities (sialyl and fucosyl transferases) responsible for the addition of monosaccharides to protein in animal cells. We have asked two questions. (i) Are the activities of glycosyl transferases increased or altered in acceptor specificity on infection of the cell by Sindbis virus, and (ii) are the transferases of uninfected cells capable of transferring monosaccharide to virus glycoprotein? Our results show that glycosyl transferase activities and specificities are unchanged by Sindbis virus infection and that host glycosyl transferases are capable of catalyzing carbohydrate transfer to viral glycoproteins. This provides direct evidence that addition of carbohydrate to virus protein is, at least in part, a host-controlled function.

MATERIALS AND METHODS

Cells and viruses. The preparation of chick embryo fibroblast cultures and growth of Sindbis virus in these cultures have been described (10). Virus was purified by an adaptation of the polyethylene glycol phase-separation technique of Yamamoto et al. (13) which was suggested by Ansel David of Columbia Univ. In this technique, virus is first concentrated by centrifugation at 16,000 × *g* from a solution of 0.5 M NaCl and 10% (w/v) polyethylene glycol-6000. The virus-containing pellet is then suspended in TNE buffer [0.05 M tris(hydroxymethyl)aminomethane, *p*H 7.5, 0.1 M NaCl, 0.001 M Na_2 ethylenediaminetetraacetic acid (EDTA)] and further purified by zonal and equilibrium sedimentation, as described previously (10). The polyethylene glycol modification results in almost complete recovery of both virus particles and infectivity and is thus preferable to the $(NH_4)_2SO_4$ precipitation previously used.

Polyacrylamide gel electrophoresis. The sodium dodecyl sulfate-phosphate buffer system was used, as modified by Strauss et al. (10).

Preparation of glycosyl transferases. Medium was changed on two confluent monolayers of chick embryo fibroblasts in Bellco roller bottles. At the same time, 0.001 plaque-forming units/cell of wild-type Sindbis virus was added to one of the monolayers. Fourteen

hours later, when virus-directed synthetic activity in the cells was at a maximum, medium was harvested for titration of virus. Particulate glycosyl transferases were then prepared from the confluent monolayers by procedures previously described for preparation of glycosyl transferases from monolayers of 3T3 fibroblasts (5). Briefly, cell monolayers were washed with 50 ml of Dulbecco's phosphate-buffered saline (0.8% NaCl, 0.05% KCl, and 0.001 M KPO_4, *p*H 7.4) containing 10^{-3} M EDTA (buffer I). The cells were removed in 25 ml of buffer I with the aid of a rubber policeman. The cell suspension was centrifuged at 1,000 × *g* for 5 min and the supernatant solution was decanted. The cell pellet was suspended in a volume of 0.01 M KPO_4 (*p*H 6.5) to give 0.6 ml of packed cells per 10 ml of buffer. After standing for 10 min, the cells were disrupted by equilibration for 20 min at 800 psi in a nitrogen pressure homogenizer followed by rapid return of the cell suspension to atmospheric pressure. After 10 min at 0 C, unbroken nuclei were removed by centrifugation for 10 min at 600 × *g*. The supernatant solution was removed and the pellet was suspended in 10 ml of 0.01 M KPO_4 (*p*H 6.5) and centrifuged again. The wash and supernatant solution were pooled and centrifuged at 100,000 × *g* in a Spinco SW40 rotor for 1 hr. The resulting pellet was suspended by homogenization in 1 ml of 0.01 M KPO_4 (*p*H 6.5) containing 10^{-3} M $MgCl_2$ and 0.1% Triton X-100 detergent. This procedure yields a particulate enzyme preparation containing about 8 mg of protein from a monolayer of 50 mg of protein mass.

Preparation of glycoprotein acceptors. Purified virus was used as acceptor without modification. Attempts to desialyate virus by hydrolysis at *p*H 1.0 so decreased the acceptor activity that, with the limited amount of virus available, it was impossible to measure transferase activities. Since Sindbis virus grown in chick cells has only one-half the sialic acid content of virus grown in hamster cells (11), there is reason to think that many acceptor sites for sialic acid are available in unmodified chick cell-grown Sindbis virus.

Bovine submaxillary mucin (BSM) was prepared from bovine submaxillary glands as described previously (5). Fetuin was purchased from Grand Island Biological Co., Grand Island, N.Y. Sialic acid was removed from BSM and fetuin by hydrolysis at *p*H 1.0 for 1 hr at 85 C. Sialic acid hydrolyzed from the glycoprotein was determined by the thiobarbituric acid method of Warren (12). After hydrolysis, the solutions were neutralized, exhaustively dialyzed against distilled water, and lyophilyzed. Acid hydrolysis removed 0.22 μmoles of sialic acid per 0.5 mg of BSM and 0.14 μmoles per 0.5 mg of fetuin. The desialized acceptors are designated BSM h and Fet h, respectively. Galactose was removed from Fet h by treatment with β-galactosidase from *Escherichia coli*. The chromatographically purified enzyme (Worthington Biochemical Corp., Freehold, N.J.) showed no protease activity when incubated with Azocoll (Calbiochem, Los Angeles, Calif.). In a typical preparation, 100 mg of desialized fetuin was dissolved in 40 ml of 0.01 M potassium phosphate, *p*H 7.0. β-Galactosidase (2,000 units) was added along with several drops of toluene, and the mixture was incubated at 37 C for 200 hr. The release of free galactose was determined by the anthrone procedure after precipitation of protein with 10% trichloroacetic acid and subsequent removal of trichloroacetic acid by ether extraction. The procedure removed about 70% of the theoretical amount of galactose in fetuin. β-Galactosidase was precipitated from the reaction by the addition of perchloric acid to a final concentration of 0.34 N. After removal of β-galactosidase by centrifugation, the supernatant solution was neutralized by the addition of 0.5 N KOH, dialyzed, and lyophilyzed; the dry powder obtained was stored at −20 C. Desialized, β-galactosidase-treated fetuin is referred to as Fet e. β-Galactosidase-treated Fet h had 0.10 μmole of acceptor sites per 0.5 mg of protein as determined by the amount of galactose removed from Fet h during enzymatic hydrolysis. It was previously shown that fetuin, after removal of galactose and sialic acid, serves as a fucosyl acceptor in reactions catalyzed by enzyme preparations from 3T3 cells. (1, 5). Preparation of radiolabeled sugar nucleotides cytidine monophosphate (CMP)-^{3}H-sialic acid and guanosine diphosphate (GDP)-^{3}H-fucose was as described previously (5).

Measurement of ^{3}H-sialic acid and ^{3}H-fucose transfer to glycoproteins. Incorporation of ^{3}H-sialic acid or ^{3}H-fucose into glycoprotein was measured as described previously by Grimes (5). Reactions were stopped by addition of 1 ml of cold 0.5 N HCl containing 1% phosphotungstic acid. Protein was collected by centrifugation and washed three times with 1 ml of cold 5% trichloroacetic acid. The trichloroacetic acid-washed pellets were suspended in 1 ml of ether-95% ethanol (1:1) and subjected to centrifugation. The supernatant solutions were removed and placed in a scintillation vial. The radioactivity extracted by ether-ethanol is most probably present as glycolipid, but no effort was made to characterize this fraction. Material insoluble in ether-ethanol (presumably glycoprotein) was dissolved in 1 N NH_4OH and placed in a scintillation vial for determination of counts. A 10-ml amount of the scintillation fluid described by Patterson and Green (9) containing 33% Triton X-100 was added to each vial, and ^{3}H counts were measured in a three-channel scintillation spectrometer.

RESULTS

Comparison of transferases from infected and uninfected cells. Most glycosyl transferases from animal cells are insoluble in aqueous buffers and remain particulate, even when mild detergents are used. We have been unable, thus far, to prepare purified soluble transferases, but particulate preparations allow many important parameters to be measured in vitro, including acceptor specificity and specific activity.

Incubation of particulate enzyme preparations from uninfected and Sindbis-infected chick embryo fibroblasts with CMP-^{3}H-sialic acid results in the incorporation of ^{3}H into trichloroacetic acid-precipitable protein and into a product

TABLE 1. *Incorporation of ^{3}H-sialic acid into acid-precipitable products*[a]

Enzyme from	Acceptor[b]	Glycoprotein		Ether-ethanol extractable (counts/min)
		Counts/min	Counts per min per mg of enzyme protein	
Uninfected cells	None	536	344	305
Uninfected cells	BSM h	3,638	2,320	
Uninfected cells	Fet h	5,305	3,410	
Uninfected cells	Virus	1,750	1,120	892
Sindbis-infected cells	None	451	284	712
Sindbis-infected cells	BSM h	4,330	2,770	
Sindbis-infected cells	Fet h	5,890	3,760	
Sindbis-infected cells	Virus	2,250	1,440	922

[a] Reaction mixtures contained 0.1 ml of enzyme (1.56 mg of protein) in buffer composed of 0.01 M KPO_4 (*p*H 6.5), 0.1% Triton X-100, and 10^{-3} M $MgCl_2$ (buffer I); 50 μliters of acceptor, 0.5 mg of desialized bovine submixillary mucin (BSM h), 0.5 mg of desialized fetuin (Fet h.), or 1.0 mg of Sindbis virus protein in buffer I; and 50 μliters of CMP-^{3}H-sialic acid (10^5 counts/min at 25 mCi/mmole). In enzyme preparations from 3T3 cells, these reaction conditions have been shown to contain excess substrate and acceptor and, therefore, are limited only by the amount of the sialyl transferase. After incubation at 37 C for 2 hr, ^{3}H counts incorporated into glycoprotein and ether-ethanol-extractable material were determined as described in the text.

[b] BSM h, bovine submaxillary mucin desialized acceptor; Fet h, fetuin desialized acceptor.

which was extracted by ether-ethanol (1:1; Table 1). It is likely, but not certain, that ether-ethanol-soluble product is a glycolipid. Addition of desialized BSM and fetuin acceptors to reaction mixtures resulted in a 5- to 10-fold increase in acid-precipitable radioactivity. The particulate enzyme preparations were also capable of catalyzing transfer of ^{3}H-sialic acid to acid-precipitable glycoprotein when Sindbis virus was added as acceptor. The ability of particulate preparations to catalyze transfer of ^{3}H-sialic acid to endogenous acceptor, desialized bovine submaxillary mucin, fetuin, glycolipid, or Sindbis virus was the same (within a factor of 20%) regardless of whether the enzyme was prepared from infected or uninfected cells. The three glycoproteins used here were approximately equal in their ability to serve as acceptors, in terms of counts per minute of ^{3}H-sialic acid transferred per milligram of acceptor protein. It is not known whether these reactions are catalyzed by one or several enzymes. In a second experiment, the ability of enzyme preparations from uninfected and Sindbis-infected cells to catalyze both ^{3}H-sialic acid and ^{3}H-fucose transfer to glycoprotein was measured (Table 2). The specific activities of both sialic acid and fucosyl transferases were similar in preparations from infected and uninfected cells.

Transfer of sialic acid to Sindbis glycoprotein. Two proteins are present in the purified Sindbis virus used as acceptor: a membrane glycoprotein and a core protein which is not a glycoprotein. Since the enzyme reaction is carried out in the

TABLE 2. *Incorporation of ^{3}H-sialic acid and ^{3}H-fucose into acid-precipitable products*[a]

Enzyme from	Substrate	Acceptor	Glycoprotein	
			Counts/min	Counts per min per mg of enzyme protein
Uninfected cells	CMP-S	None	169	393
Uninfected cells	CMP-S	BSM h	4,175	9,700
Uninfected cells	CMP-S	Fet h	7,396	17,800
Uninfected cells	GDP-F	None	850	1,970
Uninfected cells	GDP-F	Fet e	26,409	58,700
Sindbis-infected cells	CMP-S	None	132	322
Sindbis-infected cells	CMP-S	BSM h	3,388	8,270
Sindbis-infected cells	CMP-S	Fet h	4,901	12,000
Sindbis-infected cells	GDP-F	None	1,063	2,595
Sindbis-infected cells	GDP-F	Fet h	25,633	62,500

[a] Reaction mixtures contained 50 μliters of enzyme (0.41 mg of protein) in buffer composed of 0.01 M KPO_4 (pH 6.5), 0.1% Triton X-100, and 10^{-3} M $MgCl_2$ (buffer I); 50 μliters of acceptor, 0.5 mg of desialized bovine submaxillary mucin (BSM h), 0.5 mg of desialized fetuin (Fet h) or 0.5 mg of desialized β-galactosidase-treated fetuin (Fet e) in buffer I; and 5 μliters of cytidine monophosphate (CMP)-^{3}H-sialic acid (CMP-S, 10^5 counts/min at 25 mCi/mmole) or guanosine diphosphate (GDP)-^{3}H-fucose (GDP-F, 5×10^4 counts/min at 4 Ci/mmole). After incubation at 37 C for 2 hr, ^{3}H counts incorporated into glycoprotein were determined as described in the text.

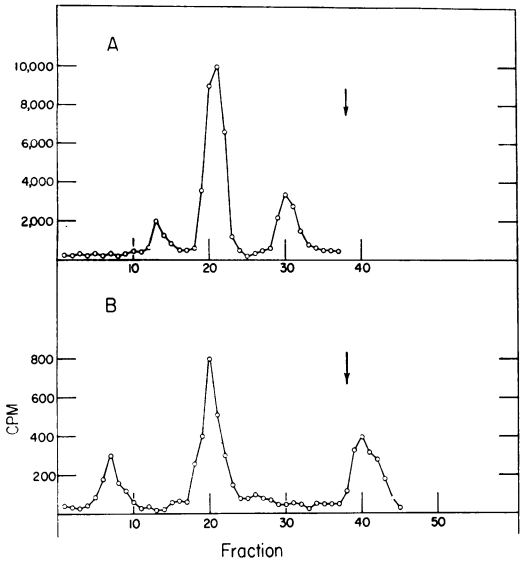

FIG. 1. *Gel electrophoresis of ^{14}C-labeled Sindbis proteins and ^{3}H-sialic acid-labeled reaction products. (A) ^{14}C-amino acid-labeled Sindbis virus from chick embryo fibroblasts was disrupted with sodium dodecyl sulfate in a reducing environment and subjected to acrylamide gel electrophoresis. The electrophoresis conditions and gel fractionation have been described (11). The peak at fraction 22 is the membrane protein, whereas that near fraction 30 is the core protein (11). (B) Electrophoresis of the products of a reaction mixture containing particulate enzyme from uninfected cells, Sindbis virus as acceptor, and CMP-^{3}H-sialic acid. Conditions of the reaction were described in the legend of Table 1. After incubation, the reaction mixture was disrupted with 2% sodium dodecyl sulfate and dialyzed entensively before electrophoresis. The arrow (↓) represents the position where the bromophenol blue dye marker migrated in the electrophoresis. Migration in all cases was from left (cathode) to right (anode).*

presence of Triton X-100, the virus was disrupted and both virus proteins might conceivably have served as acceptor. There was also the possibility that virus protein might have caused a stimulation of incorporation into endogenous acceptor. Therefore, we examined the products of the reaction by disrupting with 2% sodium dodecyl sulfate and electrophoresing on polyacrylamide gels (Fig. 1). In one experiment, ^{14}C-amino acid-labeled Sindbis virus was electrophoresed to show the positions of migration of the viral proteins (Fig. 1A). This pattern can be compared to that of polypeptide-bound ^{3}H-sialic acid from a reaction consisting of particulate extract from Sindbis virus as acceptor and CMP-^{3}H-sialic acid (Fig. 1B). The ^{3}H-sialic acid is found in three regions of the gel: (i) a slow moving product of high molecular weight which probably represents the attachment of sialic acid to endogenous acceptor, (ii) a second peak which migrates at the same rate as the envelope glycoprotein from Sindbis virus (Fig. 1A), and (iii) a final labeled product which migrates with the dye marker, where glycolipid is also found to migrate. Virtually no counts were found in the position of the virus core protein.

A comparison of Table 1 with Fig. 1B demonstrates that most of the increase in sialic acid incorporation on addition of virus acceptor is accounted for in the peak at fractions 18 through 22 in Fig. 1B. In another experiment (*not shown*), this peak was found to comigrate with marker virus glycoprotein. We take this as evidence that sialic acid is transferred to the Sindbis membrane protein by formation of a covalent bond.

DISCUSSION

Five monosaccharides are found in Sindbis membrane glycoprotein: glucosamine, mannose, galactose, fucose, and sialic acid. To determine whether any new or altered sugar transferases arise in virus-infected cells, one would ideally like to measure all five transferase activities with a number of different acceptor glycoproteins. Because of the prohibitive difficulty of preparing so many labeled substrates and acceptors, we have limited our study to transferase activities specific for two of the five relevant sugars—fucose and sialic acid. Although our study is in this sense incomplete, the experiments reported here are in agreement with the hypothesis that the carbohydrate portion of viral glycoproteins is host specified.

During the later part of the Sindbis virus infection cycle, the virus membrane protein accounts for more than 25% of the protein synthesis of the cell (12) and for the majority of glycoprotein synthesis (Burge, *unpublished data*). Although this glycoprotein is completely foreign to the cell, we find that the specific activity and acceptor specificity of cell sialic acid transferases are not changed. Moreover, there is no evidence that infection by Sindbis virus leads to any new sialic acid or fucosyl transferases in host cells or to any modification of the specificity of host cell transferase for virus glycoprotein acceptor.

An important limitation in these experiments was the lack of sufficient virus to prepare desialyzed acceptor. Although the unmodified virus served as an efficient acceptor of sialic acid (Table 1), it may be argued that a virus-specified sialyl transferase has been overlooked because of the lack of a proper acceptor. Despite this caveat, it is clear that viral glycoprotein can be modified by the direct transfer of monosaccharides catalyzed by glycosyl transferases of uninfected host cells.

The acceptor sites to which sialic acid is attached are uncertain. Since Sindbis virus grown in chick embryo fibroblasts contains less sialic acid per molecule of envelope glycoprotein than does Sindbis virus grown in hamster cells (1), it is likely that the Sindbis virus used as acceptor in these experiments had available sites for the attachment of sialic acid. It is not clear why sialic acid is not transferred to these sites during maturation of Sindbis virus since the present results indicate that chick sialyl transferases can utilize Sindbis virus glycoprotein as acceptor.

At least two questions remain to be resolved in this system. First, is all of the virus carbohydrate structure a result of the activity of normal host glycosyl transferases, or only a part? If the host determines the entire structure, then the virus-specified proteins must contain an amino acid sequence recognizable as acceptor by host glycosyl transferases. The second question, of great intrinsic interest, is that of the biological significance of the host modification of virus envelope proteins.

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