NOTES

Effects of Tunicamycin on Rotavirus Morphogenesis and Infectivity

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The functions of the two rotavirus glycoproteins were investigated by using tunicamycin and a variant of SA11 rotavirus having nonglycosylated VP7. Results showed that glycosylation of VP7 is not required for normal viral morphogenesis and infectivity and suggested that the nonstructural glycoprotein is involved in assembly of the outer capsid.

Glycoproteins are major components of the outer membrane of enveloped viruses. They participate in specific viral interactions with cellular receptors, such as adsorption to host cells, hemagglutination, and induction of cell fusion (reviewed in reference 5), and play a role in the budding of virions from the surface of infected cells (21, 30). Tunicamycin (TM), a specific inhibitor of *N*-linked glycosylation (12), has been used extensively to probe viral glycoprotein function (4, 11, 15, 18, 22-24, 26, 28, 29, 32).

Among the nonenveloped viruses, structural glycoproteins are rare (14, 17). Rotaviruses, however, have a major outer capsid glycoprotein, VP7 (6, 19, 27), which is the type-specific, neutralization antigen of the virus (3, 16). A second, smaller glycoprotein is also present in rotavirus-infected cells (2, 7, 8, 20). This glycoprotein is probably nonstructural (2, 8), but it may be a minor component of the virion (7, 20). The glycosylation of both rotavirus glycoproteins can be inhibited by TM (8, 10, 28).

Rotavirus morphogenesis is also different from that of other nonenveloped viruses. Particles assemble in cytoplasmic inclusions (viroplasm) and then bud through the membranes of the rough endoplasmic reticulum (1, 13, 25). The envelope acquired in this process appears to be lost as the particles move toward the interior of the endoplasmic reticulum cisternae, and it is absent from purified virions (25). The work presented in this paper concerns the role of the two glycoproteins in rotavirus morphogenesis and infectivity.

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TM was used to study the replication of the simian rotavirus SA11 under conditions where neither VP7 (molecular weight, 38,000 [38K]) nor the nonstructural (28K) glycoprotein was glycosylated. The results were compared with those from experiments using a recently isolated variant of SA11 (clone 28) (10), which contains the nonglycosylated (35.5K) protein moiety of VP7, although the 28K protein is glycosylated normally. We were thus able to examine the roles of the carbohydrate moieties of the two rotavirus glycoproteins separately.

When monkey kidney (MA104) cells were infected with either wild-type SA11 or clone 28 in the presence of TM, the production of infectious progeny virus was reduced by as much as 99.9% (Fig. 1). The magnitude of inhibition was dependent upon the concentration of TM, up to a level of 1.0 µg/ml, and was greater at a multiplicity of infection (MOI) of less than 1 PFU/cell than at higher MOIs (Fig. 1). In all cases, a drop in the total number of virus particles produced paralleled the decrease in infectivity induced by TM (Fig. 1A), indicating that reduced particle formation is the primary mechanism of TM inhibition. The proportion of noninfectious virus particles was also increased two- to sixfold in lysates of TM-treated cultures (Fig. 1A). Growth curves of SA11 in the presence of TM showed that little or no progeny virus was produced after the first round of virus replication (Fig. 1B). This could contribute to the greater TM sensitivity observed at low MOI, where only a fraction of the cells was initially infected. In addition, al-

FIG. 1. Inhibition of SA11 replication by TM. (A) Effect of TM concentration. Symbols: Δ , total virus particles, wild-type SA11 (MOI = 10 PFU/cell); $\blacktriangle$, infectious virus, wild-type SA11 (MOI = 10 PFU/cell); $\bullet$, infectious virus, wild-type SA11 (MOI = 0.1 PFU/cell); $\blacksquare$, infectious virus, clone 28 (MOI = 20 PFU/cell); $\blacklozenge$, infectious virus, clone 28 (MOI = 0.2 PFU/cell). (B) Growth curves of wild-type SA11 in the presence or absence of 0.5 μg of TM per ml. Symbols: $\bullet$, TM present (MOI = 0.1 PFU/cell); $\blacktriangle$, no TM (MOI = 0.1 PFU/cell); $\circ$, TM present (MOI = 10 PFU/cell); Δ , no TM (MOI = 10 PFU/cell).

FIG. 2. Inhibition of SA11 protein glycosylation by TM. Proteins were analyzed on 12.5% polyacrylamide gels as previously described (19). Lanes a and b show Coomassie blue-stained proteins from purified double-shelled virus grown in the absence (lane a) or presence (lane b) of 1.0 μg of TM per ml. The viral polypeptides are numbered as previously described (9), with the 5 major structural proteins (VP1, 2, 3, 6, and 7) present. VP9 was not visible in this preparation. In lane b, an arrowhead marks the 35.5K precursor to VP7, and a dot indicates a related 37K protein induced by TM (8). The extra bands between VP3 and VP6 are assumed to be proteolytic degradation products (9) and were not further investigated. Lanes c, d, and e show an autoradiogram of [^{35}S]methionine-labeled proteins from

though TM is a specific inhibitor of glycosylation, incorporation of [^{35}S]methionine into acid-precipitable material (protein synthesis) over a 24-h infection period in cultures treated with TM (0.25 to 4.0 $\mu\text{g/ml}$) was reduced in proportion to TM concentration by as much as 79% at an MOI of 0.1 PFU/cell, 61% at an MOI of 10 PFU/cell, and 68% in mock-infected cells. Thus, the inhibition of SA11 replication by TM may be partially indirect, due to effects of the drug on host cell metabolism.

TM concentrations ≥ 0.25 $\mu\text{g/ml}$ blocked the glycosylation of both VP7 and the 28K protein and allowed the accumulation of their 35.5K and 20K, respectively, precursor proteins in SA11-infected cells (Fig. 2). Those double-capsid virus particles that were assembled in the presence of TM (0.5 or 1.0 $\mu\text{g/ml}$) during infection at high MOI contained only the nonglycosylated form of VP7 (Fig. 2). The specific infectivity (50 to 100 particles per PFU), hemagglutination activity, and stability to proteolytic enzymes and extremes of pH were found to be similar for purified double-shelled TM-grown virus, clone

SA11-infected cell lysates. Label was present throughout infection (20 h). Lane c, no TM; lane d, 0.25 μg of TM per ml; lane e, 1.0 μg of TM per ml. Arrowheads mark the 38K (VP7) and 28K glycoproteins in lane c, and their 35.5K and 20K nonglycosylated counterparts in lanes d and e. The dots indicate the 37K, TM-induced protein. Molecular weights (MW, in thousands) are shown at the right.

FIG. 3. Effects of TM on rotavirus morphogenesis. Electron microscopy was performed as previously described (1, 25). (A) Cytoplasm of cell infected with wild-type SA11 in the presence of $1.0\text{ }\mu\text{g}$ of TM per ml. All of the particles are enveloped. (B) Similar area in untreated, SA11-infected cell. Enveloped particles (arrowheads) are located near the periphery of the endoplasmic reticulum cisternum. (C) Enveloped particles in cell infected with clone 28 virus in the presence of $0.25\text{ }\mu\text{g}$ of TM per ml. (D) Virus particles in clone 28 virus-infected cell grown without TM. Only a few particles (arrowheads) are enveloped. Abbreviations: vi, viroplasmic inclusion; er, endoplasmic reticulum. Magnification bar = $0.5\text{ }\mu\text{m}$.

28 virus, and SA11 control virus (data not shown).

Morphogenesis of SA11 in the presence of TM was studied by thin-section electron microscop-

py. Formation of viroplasmic inclusions and budding of particles into the endoplasmic reticulum occurred normally. The most striking effect of the drug was that 80 to 100% of the virus

particles within cisternae of the endoplasmic reticulum were enveloped (Fig. 3A), whereas only about 10% of the particles were enveloped in SA11-infected cells not exposed to TM (Fig. 3B). In contrast to the inhibition of infectivity and protein synthesis, the increase in enveloped particles did not vary with TM concentration (0.06 to 2.0 $\mu\text{g/ml}$) or MOI (0.1 to 100 PFU/cell). However, both the amount of virus per cell and the number of infected cells was reduced at low MOI. At TM concentrations of 0.5 to 2.0 $\mu\text{g/ml}$, the number of virus particles per infected cell was also reduced.

When the morphogenesis of clone 28 virus was compared with that of standard virus in both the presence and absence of TM, no differences were observed. Like the wild-type virus, less than 10% of the particles were enveloped under normal growth conditions (Fig. 3D), indicating that the accumulation of enveloped particles was not due to lack of glycosylated VP7. The percentage of enveloped particles increased to 90% in the presence of 0.25 or 1.0 μg of TM per ml (Fig. 3C). These results suggest that the accumulation of enveloped virus in TM-treated cells resulted from failure to glycosylate the nonstructural (28K) glycoprotein.

The data presented in this paper agree generally with the findings of Sabara et al. (28) on TM inhibition of bovine rotavirus replication except that we did not observe a large increase in incomplete (single capsid) particles in lysates from TM-treated cells. This difference could be due to differences in virus strain or batches of TM, or it could be due to increased fragility to TM-grown virus during the long centrifugation in cesium chloride used by those workers. The existence of the variant clone 28, which replicates to high titers even though VP7 is not glycosylated, and the production of infectious SA11 in TM-treated cells infected at high MOI demonstrate that glycosylation is not an absolute requirement for assembly of SA11 virions.

Although the function of the envelope in rotavirus maturation is not yet known, the localization of VP7 by electron microscopic immunocytochemistry in that portion of the endoplasmic reticulum through which particles bud suggests that it is important in assembly of the outer capsid layer (25a). Biochemical evidence (B. L. Ericson, D. Y. Graham, B. B. Mason, H. H. Hanssen, and M. K. Estes, *Virology*, in press) and data from subcellular fractionation of SA11-infected cells (31) support the location of VP7 in the endoplasmic reticulum and indicate that the 28K protein is present in membranes as well. A possible function of the 28K protein would be that of a scaffolding protein, structurally important during outer capsid assembly, but later lost along with the lipid bilayer.

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