Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 422-428

STREPTOVIRUDIN INHIBITS GLYCOSYLATION AND MULTIPLICATION OF VESICULAR STOMATITIS VIRUS

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Received February 3, 1981

Abstract

Streptovirudin inhibits the growth of vesicular stomatitis virus in BHK cells. At 1.0 µg of streptovirudin per ml, the replication of virus is inhibited 98%. Noninfectious particles were not detected in the presence of streptovirudin. Within 2-4 hrs after the addition of antibiotic, [^{3}H] glucosamine incorporation into glycoproteins was inhibited 70%, whereas [^{14}C] leucine incorporation was unaffected. By 6-8 hrs after streptovirudin addition, glucosamine incorporation had fallen to 5% of control value, with leucine incorporation being 78% of normal, suggesting that streptovirudin is not a general inhibitor of protein synthesis. These data demonstrate that streptovirudin specifically inhibits glycosylation of viral glycoproteins.

Introduction

Streptovirudin (St.) has been isolated from cultures of Streptomyces (1). We had previously shown that streptovirudin acts analogously to tunicamycin (TM) (2,3) in that it blocks the formation of N-acetylglucosaminyl-pyrophosphoryldolichol in cell free extracts of pig aorta. Recently, Eckardt et al. (4) have shown by the use of gel chromatography, high performance liquid chromatography and hydrolysis that tunicamycin and streptovirudin are not identical antibiotic complexes. The antibiotic tunicamycin was proven to be a useful inhibitor of the synthesis of N-acetylglucosaminyl lipid in prokaryotes, eukaryotes and viruses.

0006-291X/81/060422-07$01.00/0

Vesicular stomatitus virus (VSV) is an enveloped RNA virus that is assembled by budding from cellular membranes during virus maturation. VSV contains five viral polypeptides, the only membrane glycoprotein (gp70) is found on the outer surface of the virus. No additional viral proteins are detected in VSV-infected cells and there is no evidence for the existence of a precursor for the glycoprotein (5-8). Previous studies using 2-deoxy-D-glucose and D-glucosamine, compounds that inhibit glycosylation of glycoproteins, have suggested that oligosaccharide units of the viral glycoproteins are necessary for the formation of infectious virus (9-15). However, since both of these compounds affect cellular processes other than glycosylation, the data obtained do not allow unequivocal interpretations (16-18). We have used St. to study the role of oligosaccharide of VSV glycoproteins in viral replication and particle formation. Our results demonstrate that St. specifically inhibits protein glycosylation and that the carbohydrate moieties of viral glycoproteins are necessary for virion formation.

Materials and Methods

[^{3}H] glucosamine (10-30 Ci/mmol) and [^{14}C] leucine (>300 mCi/mmol) were from New England Nuclear Co., Boston, MA. BHK and VSV were gifts from Dr. Robert A. Lazzarini, NIH, Bethesda, MD. Streptovirudin was obtained from Dr. Alan D. Elbein, University of Texas Health Science Center, San Antonio, TX, and from Dr. K. Eckardt, Zentralinstitut Fur Mikrobiology and Experimentalle Therapie, Germany. Tunicamycin was obtained from Calbiochem-Behring Corp., San Diego, CA. Minimal essential medium (MEM), fetal bovine serum, glutamine, PSN antibiotic mixture and BME vitamin solution were all purchased from GIBCO Laboratories, Grand Island, NY.

Cells were grown as monolayers (19) in MEM supplemented with 6% fetal bovine serum, 2 mM glutamine, 1X of vitamins and 1X of PSN antibiotics. Nearly confluent monolayers of cells growing in 75 cm^{2} flasks or 35 cm^{2} plastic tissue culture petri dishes were infected with VSV at a multiplicity of infection of 20-30 PFU per cell. The virus was allowed to absorb to cells for 1 hr at 37°C in 0.5 to 1.0 ml of medium, followed by removal of unadsorbed inoculum and the addition of 5-10 ml of fresh medium. Virus was harvested 16-20 hrs after infection. Unless otherwise indicated, St. at 1.0 µg/ml was added to VSV infected cells at 1 hr after the infection. To label the virus with [^{14}C] leucine the MEM added after removal of unadsorbed virus lacked amino acids. The MEM was not supplemented with PSN antibiotic mixture whenever St. was used. To label the cells, infected cells were incubated with complete medium containing 5-15 µCi of [^{14}C] leucine or 10-20 µCi of [^{3}H] glucosamine. The time of addition of label is indicated for each experiment. For double labelled experiments, 20 µCi of [^{3}H] glucosamine and 5 µCi of [^{14}C] leucine were added per plate at the indicated times.

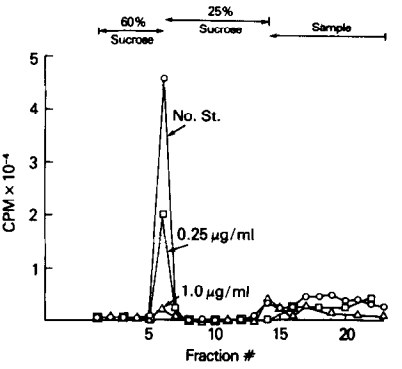


Figure 1.

Purification of [^{14}C] leucine-labelled particles released from VSV-infected cells by centrifugation in a sucrose density interphase. The indicated amount of St. was added to the cells 1 hr post-infection. [^{14}C] leucine was added to the cells 4 hr post-infection and the medium was harvested 20 hrs post-infection. A portion from each fraction was precipitated with trichloroacetic acid and counted.

Virus was purified by discontinuous sucrose gradients (20) or sucrose density interphase gradients. A 5 ml portion of medium was layered onto 3 ml of 60% sucrose and 4 ml of 25% sucrose. The interphase was separated at 100,000xg for 90 min at 4°C. PFU was determined either on virus purified in sucrose interphase or medium harvested by centrifugation at 5,000 rpm for 10 min at 4°C. For PFU determination diluted media were applied onto 50% confluent BHK monolayers in six well limbo plates. The unadsorbed virus was removed and 0.75% methylcellulose in 2 ml MEM supplemented as above was layered. The plaques were read 20-24 hrs post-infection after washing the cells with 10% formaldehyde in PBS and staining with 0.1% crystal violet in 20% ethanol for 10 minutes.

Results

Figure 1 shows that 0.25 µg/ml St. inhibits the release of radioactivity by more than 50% into the sucrose density interphase peak. With 1.0 µg/ml of St. radioactivity present in the peak is inhibited by more than 90%. Similar results were obtained with sucrose gradients where virions sediment around 40% sucrose concentration (data not shown). St. is a potent inhibitor of VSV multiplication, producing more than 90% inhibition in the yield of virus at 1.0 µg/ml (Table 1). The inhibition of infectious particle formation (PFU) correlates very closely with the inhibition of particle formation, indicating that nonfectious particles are not formed in the presence of St. Similar results were obtained when TM was used to inhibit VSV multiplication.

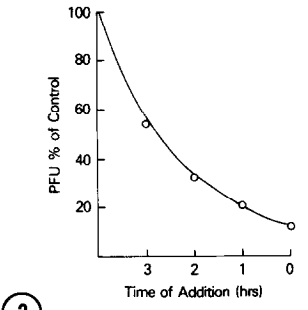
Table I INHIBITION OF THE MULTIPLICATION OF VSV AS A FUNCTION OF ANTIBIOTIC CONCENTRATION.

St. (μg/ml)	PFU/ml ($\times 10^{-8}$)	% Inhibition of PFU	CPM/50 λ of Interphase	% Inhibition of CPM
0.0	3.5	0.0	4422	0.0
0.05	3.2	8.6	4288	3.1
0.1	1.2	65.7	2615	40.9
0.2	1.4	60.0	1620	63.4
0.4	0.5	85.7	1408	68.2
0.6	—	—	1032	76.7
1.0	0.07	98.0	445	90.0
2.0	0.06	98.3	354	92.0
5.0	0.02	99.5	280	93.7
TM (μg/ml)	PFU/ml ($\times 10^{-8}$)	% Inhibition of PFU	CPM/50 λ of Interphase	% Inhibition of CPM
0.0	3.5	0.0	4422	0.0
0.05	1.4	60.0	2325	47.5
0.10	1.3	62.9	1325	70.1
0.25	1.0	71.5	781	82.3
0.50	0.06	98.3	606	86.3
1.00	0.03	99.2	166	96.3

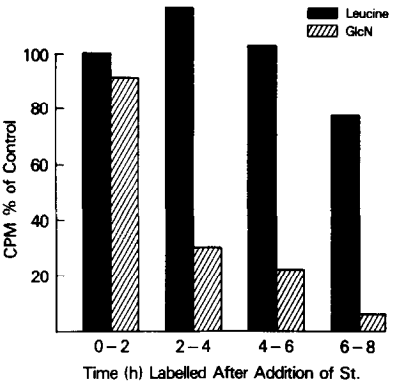
The supernatant fluids from [^{14}C] leucine-labelled particles released from VSV-infected cells were harvested and stored at 4°C until used the next day. The indicated amount of antibiotic (St. or TM) was added to the cells 1 hr post-infection. [^{14}C] leucine was added to the cells 4 hr post-infection and the medium was harvested 20 hrs post-infection by centrifugation at 5,000 rpm for 10 min. A portion of medium from each concentration of antibiotic used was taken to determine PFU, and the remainder was sedimented in sucrose density interphase. To determine the counts per minute of radioactivity from sucrose density interphase, a 50 λ portion from each 0.5 ml fractions was precipitated with trichloracetic acid, millipored and counted in Biofluor.

However, these two antibiotics cannot be compared directly in terms of the amount required for inhibition, since their purity may differ. The addition of St. to VSV-infected BHK cells within the first hour appears to inhibit the virus production maximally (Fig. 2). However, St. added as late as 3 hrs after infection still caused more than 50% inhibition. St. does not seem to effect the adsorption of VSV to BHK cells, since we see no significant difference in inhibition when the antibiotic is added along with the infecting medium or 1 hr post-infection. For this reason, St. was routinely added 1 hr post-infection.

In order to obtain evidence that St. selectively blocks glycosylation of glycoproteins, we studied the effect of 1.0 μg/ml St. on the relative uptake of [^{14}C] leucine and [^{3}H] glucosamine into VSV-infected cells. In 2-4 hrs, the incorporation of [^{3}H] glucosamine into trichloroacetic acid



(2)



(3)

Figure 2

Inhibition of the formation of VSV as a function of the time of addition of St. The cells were infected as described before and St. 1.0 μg/ml was added at the indicated times after the infection. At 16 hrs post-infection, media was harvested and titered to determine the yield of infectious virus.

Figure 3.

Relative rates of incorporation of [^{14}C] leucine and [^{3}H] glucosamine into VSV-infected cells in the presence of St. The cells were infected with VSV and St. (1.0 μg/ml) was added at 1 hr postinfection. Single plates were double labelled with [^{14}C] leucine and [^{3}H] glucosamine for the indicated time intervals. At the end of labelling period, monolayers were solubilized in 5% SDS and trichloroacetic acid-precipitable radioactivity determined.

(TCA) precipitable material was inhibited by 70%; whereas, [^{14}C] leucine incorporation was unaffected (Fig. 3). Although at 6-8 hrs, we observed a decrease in [^{14}C] leucine incorporation into TCA-precipitable material, the inhibition of protein synthesis was only 22% as compared to 95% decrease in glucosamine incorporation. Since the inhibition of protein synthesis occurs after the decrease in glucosamine incorporation, and the former effect is not as pronounced as the latter (Fig. 3), this suggests that the observed decrease in protein synthesis is secondary to the derangement in glycoprotein synthesis.

We wish to determine whether inhibition of virus formation correlates with glycoprotein synthesis. We found that progressive decrease in glycoprotein synthesis in VSV-infected cells correlated very well with the inhibition of

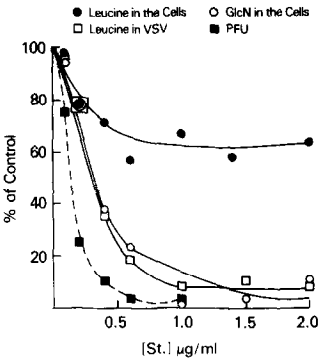


Figure 4

Relative rates of protein and glycoprotein synthesis and inhibition of VSV formation at various concentrations of St. BHK cells were infected with VSV as described before and St. was added 1 hr later. [^{14}C] leucine and [^{3}H] glucosamine were added 4 hr post-infection and cells and medium were harvested 4 hrs later. The monolayers were solubilized in 5% SDS and trichloacetic acid-precipitable radioactivity determined. The media was stored at -100°C for several days and then titered to determine the yield of infectious virus. To determine the radioactivity in the medium, a portion of the supernatant medium was precipitated with trichloacetic acid and counted.

virus formation (Fig. 4). In contrast, the observed inhibition of protein synthesis did not correlate with the inhibition of virus formation. The level of inhibition of protein synthesis observed at 0.4 µg/ml of St. was not increased at higher concentrations of the drug; whereas, yield of PFU, glucosamine incorporation and particle formation as indicated by leucine incorporation into the medium all three parameters decreased significantly between 0.4 and 2.0 µg of St. per ml (Fig. 4). Again, the good correlation observed between glucosamine incorporation into infected cells, particle formation and PFU suggests that glycosylation of viral glycoproteins is necessary for the virion formation and that noninfectious particles are not formed.

Antibiotics which inhibit the synthesis of oligosaccharide chain of glycoproteins should be useful in studies of the function of the carbohydrate moiety of the molecule as well as in studies on the mechanism of assembly of glycoproteins. The results described here show that St. specifically blocks

glycosylation and multiplication of VSV. The evidence presented in the present studies, as well as studies from other laboratories (19), indicate that the carbohydrate moieties of viral glycoproteins are necessary for virion formation.

Acknowledgements

The authors wish to thank Drs. George J. Todaro and Ernest J. Plata of NCI/NIH for their critical evaluation of the manuscript and to Mrs. Barbara Eader for typing the manuscript.

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