Archives of Virology 81, 25—39 (1984)

Archives of Virology

On the Role of Oligosaccharide Trimming in the Maturation of Sindbis and Influenza Virus

By

R. Datema, P. A. Romero, R. Rott, and R. T. Schwarz

Institut für Virologie, Justus-Liebig-Universität Giessen, Giessen, Federal Republic of Germany

With 6 Figures

Accepted January 31, 1984

Summary

The α-glucosidase inhibitor bromoconduritol inhibits the formation of the N-linked, complex-type oligosaccharides of the glycoproteins from influenza viruses (fowl plague virus, influenza virus PR-8) and from sindbis virus. Viral glycoproteins produced in bromoconduritol-treated chicken-embryo and baby-hamster kidney cells are fully glycosylated, but accumulate N-linked, high-mannose oligosaccharides of the composition $Glc_1Man_x(GlcNAc)_2$ (x = 7, 8, and 9). Other α-glucosidase inhibitors (nojirimycin, deoxynojirimycin, acarbose) were not specific inhibitors of oligosaccharide processing under the conditions used in the present investigation.

In bromoconduritol-treated, sindbis virus-infected chicken-embryo and baby-hamster kidney cells, the sindbis glycoproteins are metabolically stable. Specific proteolytic cleavage of the polyprotein precursors to form E_2 and E_1 occurs in bromoconduritol-treated chicken-embryo cells, but cleavage of PE_2 to E_2 is prevented in the infected baby-hamster kidney cells. Yet, release of infectious sindbis virus particles is inhibited in both cell types indicating that the formation of complex oligosaccharides is required for a late step in virus formation.

The release of virus particles from influenza virus PR-8-infected bromoconduritol-treated chicken-embryo cells is not inhibited, and virus with only high-mannose oligosaccharides is formed. In contrast, when chicken-embryo cells were infected with the influenza virus fowl plague virus, release of infectious particles was inhibited. The fowl plague virus hemagglutinin is cleaved in chicken-embryo cells, in contrast to the hemagglutinin of the PR-8 virus. However, the cleavage products HA_1 and HA_2 do not reach the cell surface. In addition, or as a consequence, HA_1

and HA_2 are proteolytically broken down, whereas uncleaved hemagglutinin of PR-8 appeared metabolically stable. These results may explain the decrease in formation of fowl plague virus particles and the lack of effect on PR-8 virus in bromoconduritol-treated cells. This work thus shows different biological roles for oligosaccharide processing.

Introduction

Proteins are glycosylated initially with the oligosaccharide $Glc_3Man_9(GlcNAc)_2$, which is transferred from dolichol-diphosphate to asparagine residues of (nascent) proteins (21, 23, 28, 30). After transfer, processing of the oligosaccharide occurs, and starts with trimming of glucose residues and then mannose residues. Some oligosaccharides are further processed by readdition of GlcNAc, Fuc, NeuNAc (1, 17). The question on the role of trimming became more interesting when it was found that also an oligosaccharide with 5 mannose residues, $Glc_3Man_5(GlcNAc)_2$, rather than 9 mannose residues could be used to glycosylate protein initially (8, 18, 35). We have tried to tackle this problem by the use of glycosidase inhibitors and have recently described (10) the mechanism of inhibition of glucose trimming by the α-glucosidase inhibitor bromoconduritol (19, 20).

For the present investigations we have used cells infected with influenza virus or sindbis virus because these systems have proved to be useful models to study glycosylation and the biological significance of glycosylation. The analysis of phenomena related to roles of glycosylation (such as protection against proteolytic degradation, intracellular transport, specific proteolytic processing of precursor proteins) and the analysis of the oligosaccharide structures and of protein synthesis have been done using techniques described previously (28, 29).

The major glycoprotein synthesized in influenza virus-infected cells is the hemagglutinin, HA. It is glycosylated initially with high-mannose oligosaccharides which are processed during intracellular transport, be it to varying degrees (15). In fowl plague virus-infected, but not PR8-infected (another influenza virus) chicken-embryo cells, the HA is cleaved in the smooth membrane fraction to the subunits HA_1 and HA_2. The precursor proteins to the sindbis virus glycoproteins E_1 and pE_2 is called p 97 or B. Cleavage of B to pE_2 and E_1 is an early event whereas cleavage of pE_2 to E_2 is a late event occuring just before budding of the virus (15). For the present investigation we have analyzed the mode of inhibition of bromoconduritol in different virus-cell systems as an inhibitor of oligosaccharide trimming and have studied the effects of inhibition of oligosaccharide-processing on synthesis, proteolytic precursor-processing, intracellular transport, and metabolic stability of sindbis and influenza virus proteins in chicken-embryo and baby-hamster kidney cells.

Materials and Methods

Chemicals and Enzymes

Bromoconduritol was prepared as described by LEGLER (20). Because of the lability of bromoconduritol in water, bromoconduritol was stored as a powder, quickly dissolved in ice-cold water and used immediately. Bromoconduritol was added at a concentration of 2.4 mM, unless indicated otherwise, at least 1 hour before the addition of radioisotopes. Isotopic precursors were obtained from Amersham, pronase P was from Serva, endo-β-N-acetylglucosaminidase H from Miles, and α-mannosidase (type III from jack beans) was from Sigma. Pronase digestion of glycoproteins, and treatment with endo H and α-mannosidase were as described (9). The α-glucosidase from liver microsomes was prepared, and the enzyme activity assayed according to GRINNA and ROBBINS (12). The assay mixture contained 10 mM EDTA to inhibit residual α-mannosidase activity (10). The oligosaccharide standards labelled in the glucose or mannose moiety were prepared as previously (9, 29), characterized by chromatography on columns of Bio Gel P 4 (7) resistance to α-mannosidase (9) and susceptibility towards α-glucosidase (10). Tunicamycin was from Calbiochem.

Tissue Culture and Labelling Procedures

Confluent monolayer cultures of chicken-embryo cells or baby-hamster kidney cells were used. They were infected with fowl plague virus (Strain Rostock, H_7N_1) or influenza virus PR 8 (H_0N_1) or Sindbis virus. After infection cells were maintained in Earles medium containing 10 mM sodium pyruvate, unless indicated otherwise. Virus quantitation was done by the plaque assay (37). Cells were labelled with isotopic precursors 4 hours post infection, or later as indicated under results, for different times as specified in each described experiment. An operational definition for protein synthesis in intact, infected cells was the amount of radioactive amino acids incorporated into trichloroacetic acid precipitable (10 per cent, ice-cold) products after labelling cells for 15 minutes. After the labelling procedures the cells were rapidly washed with ice-cold phosphate buffered saline, collected and either dispersed in chloroform-methanol (2:1, v/v) (29), dissolved in solutions containing urea and SDS for slab gel electrophoresis (see below) or dissolved in 0.0625 M Tris/Cl, pH 6.8, 3 per cent SDS, 5 per cent mercaptoethanol for all the other purposes.

Separation Techniques

Extractions of lipid-linked oligosaccharides and preparations of viral glycoproteins and glycopeptides were as described previously (29). Bio Gel columns were calibrated as described previously (7). Separation of virus particles on sucrose density gradients has been described in (27). Unlabelled carrier virus was routinely added. Polyacrylamide slab gel electrophoresis of influenza virus proteins was routinely performed in 8.75 per cent gels (29) whereas for the separation of sindbis virus glycoproteins 20 per cent gels as described by (36) were used. Cells were dissolved in a solution containing 4 M urea, 1 per cent SDS and 2.5 per cent mercaptoethanol by boiling for 2 to 4 minutes.

Cell Surface Location

Two procedures were used, namely trypsin treatment (2) of intact monolayers or an extracellular antibody adsorbtions technique (3). Thus, cells infected with influenza virus were labelled with 10 μCi of ^{14}C-labelled amino acids from 4 to 5 hours post infection and then chased in Dulbeccos medium (containing no radioactive isotopes) for 1 hour. Cells were washed with phosphate buffered saline 3 times and then trypsin (25 μg per petri dish) in 0.3 ml of phosphate-buffered saline was added. Control cells

received either phosphate buffered saline and no trypsin, or (to assess whether trypsin enters cells) the cells were treated from 3 to 6 hours post infection with 10 μM of monensine and were incubated with trypsin (see Results). After incubating for 30 minutes at 37° C, 50 μg of trypsin-inhibitor were added per dish, followed by 0.3 ml of a solution containing 8 M urea, 2 per cent SDS and 5 per cent mercaptoethanol.

Alternatively, the antibody precipitation technique described by Schlesinger and Malfer (26) as modified by Bosch *et al.* (3) was used. Monolayers were washed with ice-cold phosphate-buffered saline containing 0.1 per cent BSA kept on ice and 200 μl of antibody in phosphate-buffered saline (15—100 μg per ml of protein) was added. One hour later the cells were washed 3-times with ice-cold buffered saline and the excess of antibody was neutralized with 20 times excess (with respect to the antibody) of unlabelled virus. Controls were: 1. uncleaved hemagglutinin from fowl plague virus-infected chick embryo cells, an intracellular product, was not present in immunoprecipitates; 2. unlabelled, fowl plague virus-infected cells were reacted with anti-fowl plague virus antibody as above and cell lysis performed in the presence of a labelled cell-extract. Under these conditions radiolabelled fowl plague protein was not precipitated.

Results

Tests of Different α-Glucosidase Inhibitors

We have compared the α-glucosidase inhibitors nojirimycin (5-amino-5-deoxy-D-glucopyranose), 1-deoxynojirimycin, acarbose (see 33 for a review on these compounds) and bromoconduritol (6-bromo-3,4,5-trihydroxycyclohex-1-ene, 20) as inhibitors of trimming of glucose residues from the oligosaccharide $Glc_3Man_9GlcNAc$ by a rat liver microsomal glucosidase (12).

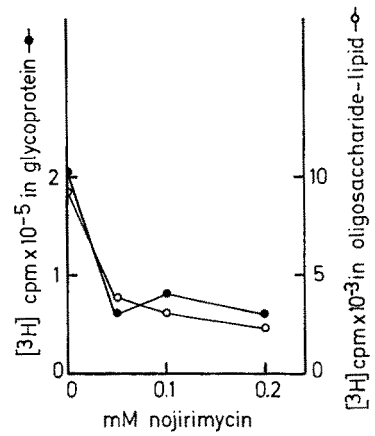

Fig. 1. Incorporation of ^{3}H-mannose into lipid-linked oligosaccharides and glycoproteins as a function of the concentration of nojirimycin. Cells infected with fowl plague virus were treated 2 hours post infection with indicated concentrations of nojirimycin, and after 2 hours ^{3}H-mannose was added without removing nojirimycin. Cells were harvested 1 hour later, and the oligosaccharide lipid and glycoproteins isolated (29)

As shown previously, bromoconduritol inhibits the release of glucose residues (10). After incubation of $(^3H\text{-}Glc)_3Man_9GlcNAc$, the enzyme, and up to 5 mM of acarbose no inhibition of glucose release was noted. On the other hand, 1-deoxynojirimycin and nojirimycin inhibit glucose release completely at 5 μM (results not shown). Also others found 1-deoxynojirimycin to be a potent inhibitor of glucose trimming (13, 25). The possibility to use nojirimycin and deoxynojirimycin as specific inhibitors of glycoprotein processing in intact, virus-infected chicken-embryo cells is, however, limited. As shown in Fig. 1, nojirimycin inhibits the formation of both lipid-linked oligosaccharides and protein-linked oligosaccharides in virus-infected cells maintained in a glucose-containing medium. Also deoxynojirimycin inhibited formation of both lipid- and protein-linked oligosaccharides. Hence, deoxynojirimycin and nojirimycin are not specific inhibitors under the conditions used here. In contrast, bromoconduritol is a specific inhibitor of formation of complex-type oligosaccharides (10).

Synthesis and Glycosylation of Influenza and Sindbis Virus Glycoproteins in Bromodonduritol Treated Cells

The incorporation of ^{14}C-labelled amino acids into trichloroacetic acid-precipitable material in the virus-infected cells, pulse labelled for 10 or 15 minutes was not affected by treating cells with bromoconduritol (2.4 mM) added either 1, 2 or 4 hours before the addition of the isotope. During a subsequent chase in the presence of bromoconduritol for 1 hour, no substantial loss of radioactivity was noted. However, during longer chase periods, or during labelling periods of 4 hours or more, a 40—50 per cent loss of trichloroacetic acid-precipitable radioactivity was observed in fowl plague virus-infected cells. This suggests degradative proteolysis (see also below). The incorporation of ^3H-mannose into lipid-linked oligosaccharides was increased three to five-fold after addition of bromoconduritol. The incorporation of ^3H-mannose into viral glycoproteins was either unaffected by bromoconduritol (in chicken-embryo cells) or slightly increased (BHK-cells).

To analyze the protein-linked oligosaccharides from bromoconduritol treated and non-treated cells sindbis virus-infected BHK-cells were used. Sindbis virus grown in BHK cells has a higher proportion of complex oligosaccharides than virus grown in chicken-embryo cells (5) and an effect of a trimming inhibitor can be more easily made visible in BHK cells. The cells were labelled from 7—8 hours post-infection with ^3H-mannose and then chased for 4 hours. The inhibitor (2.4 mM) was added 2 hours post-infection and was also present in the chase medium. The viral glycoproteins in the lipid-free cellular residue were isolated and digested with pronase to give glycopeptides. Subsequent treatment with endoglycosidase H cleaved high-mannose oligosaccharides from the peptides. Complex-type glyco-

peptides are resistant towards endoglycosidase H. The mixture of oligosaccharides and glycopeptides was then separated on Bio Gel P4 columns (Fig. 2).

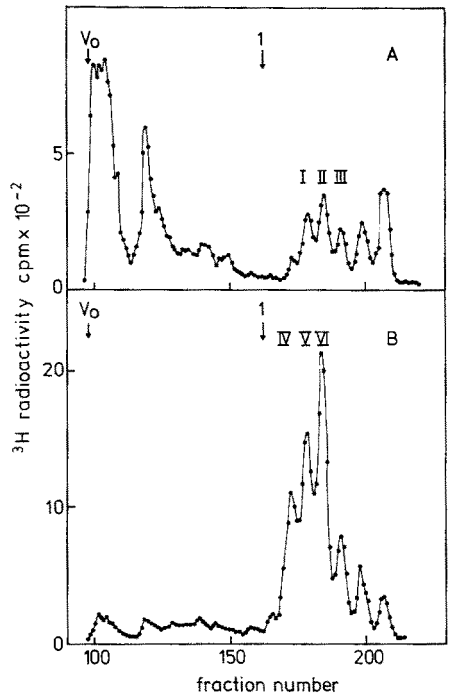

Fig. 2. Bio-Gel P4 chromatography of ^{3}H-labelled oligosaccharides and glycopeptides from non-treated *(A)* or bromoconduritol treated *(B)* cells. Baby-hamster kidney cells infected with sindbis virus were labelled with ^{3}H-mannose from 7 to 8 hours post infection and then chased for 4 hours. The inhibitor was added two hours post infection and was also present in the chase medium *(B)*. The lipid-free residue was digested with pronase and then endo H. Arrows: Arrow 1 indicates elution position of the standard $Glc_3Man_9GlcNAc$, V_0: void volume (bovine serum albumine). For peaks I—VI see text

In bromoconduritol-treated cells the formation of ^{3}H-mannose labelled, complex-glycopeptides (eluted between fraction 98 to 150) was inhibited 73 per cent, and of high-mannose oligosaccharides (eluted after the marker $Glc_3Man_9GlcNAc$) increased almost two-fold (comparison of Figs. 2A and 2B). The fractions under peaks I to VI were pooled separatedly, the oligosaccharides were treated with α-glucosidase, mixed with the ^{14}C-labelled standards $Glc_3Man_9GlcNAc$, $Man_9GlcNAc$ and $Man_8GlcNAc$ and then separated on Bio Gel P4 columns. This analysis, described elsewhere (10) showed peak I to contain $Man_9GlcNAc$, peak II $Man_8GlcNAc$, peak III $Man_7GlcNAc$, peak IV $Glc_1Man_9GlcNAc$ and peak V $Glc_1Man_8GlcNAc$. Material eluting under peak VI contained mainly $Glc_1Man_7GlcNAc$. There-

fore, bromoconduritol not only inhibits the formation of complex-oligosaccharides in chicken-embryo cells (10) but also in (sindbis virus-infected) baby-hamster kidney cells. The high-mannose oligosaccharides accumulating under bromoconduritol in both cell types are identical. Hence, the mechanism of inhibition probably is the same, namely a specific block in glucose trimming.

Effects of Bromoconduritol on Virus Formation

Two criteria were used for virus formation, namely the release into the medium of infectious material (measured by the plaque assay) and of physical particles labelled with radioactive amino acids and analyzed on sucrose density-gradients. The incorporation of radioactive amino acids into sindbis virus particles was inhibited 85 per cent or more by bromoconduritol (2 mM) treatment of either chicken-embryo or baby-hamster kidney cells (Table 1). The release of infectious material was inhibited about 90 per cent (Table 1). Also the release of infectious fowl plague virus particles is inhibited by bromoconduritol (10). In contrast, the release of labelled particles from influenza virus PR8-infected chicken-embryo cells was not inhibited by preventing oligosaccharide processing using bromoconduritol. Complete inhibition of glycosylation by tunicamycin (2 μg/ml), however, inhibits the release of both PR8 virus particles (results not shown) and fowl plague virus particles (27).

Table 1. *Effect of bromoconduritol (BC) on the release into the medium of sindbis virus from infected chicken-embryo and BHK cells*

Cells	Additions	Radioactivity (cpm) of particles labelled with ^{35}S-methionine	Infectivity (PFU/ml)
BHK	None	543,230	1.3×10^9
BHK	2 mM BC	73,336	1.2×10^8
CEF	None	597,101	3.7×10^9
CEF	2 mM BC	97,430	2.6×10^8

Sindbis infected cells were treated with 2 mM bromoconduritol (BC) or not treated. The inhibitor was added two hours post infection and the cells labelled with 50 μCi ^{35}S-methionine from 4 to 8 hours post infection. The virus was pelleted from the medium and then layered on a 15—40 per cent sucrose gradient containing 1 ml of 60 per cent sucrose at the bottom, gradients were centrifuged for two hours at 40,000 rpm (SW 41 rotor). Infectivity was determined before pelleting the virus

To study the oligosaccharides of influenza virus PR8, released from bromoconduritol-treated and non-treated cells, the infected chicken-embryo cells were labelled with ^{3}H-mannose from 4 to 8 hours post-infection. The virus was isolated from the medium by centrifugation through a cushion of 1.5 ml of 25 per cent sucrose in the SW 55 rotor (1 hour, 50,000 rpm)

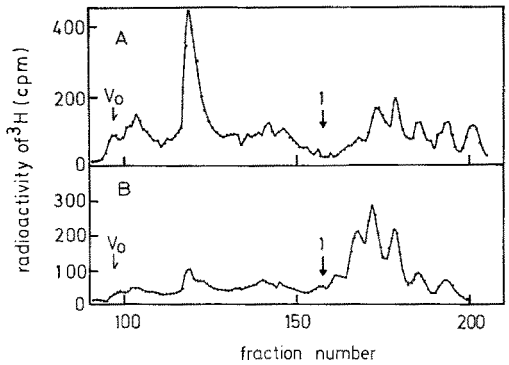

Fig. 3. Bio Gel P4 chromatography of ^{3}H-mannose labelled oligosaccharides and glycopeptides from PR8 virus obtained from bromoconduritol treated *(B)* and non-treated *(A)* cells. Cells infected with PR8 were labelled with ^{3}H-mannose from 4—8 hours post infection in absence (A) or presence (B) of 2 mM bromoconduritol. Virus was isolated and pronase digested. The digest was then treated with endo H. Arrows: V_0 = void volume; 1 is elution position of $Glc_3Man_9GlcNAc$

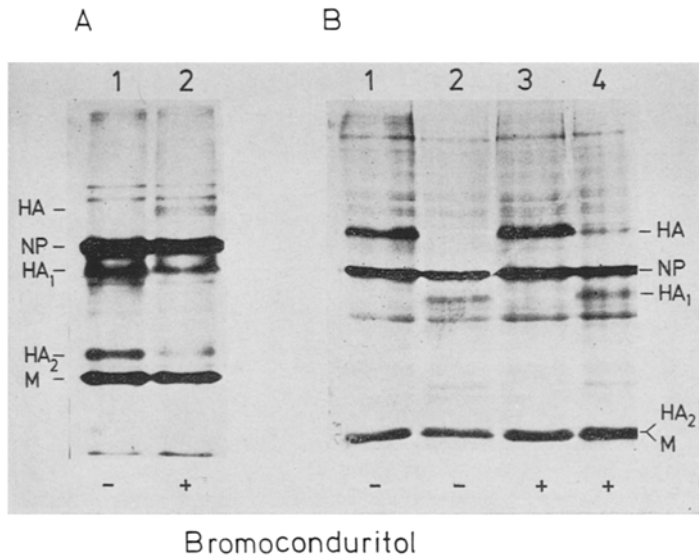

Fig. 4. Polyacrylamide slab gel electrophoresis of fowl plague virus proteins *(A)* and PR8 virus proteins *(B)* from cells labelled with radioactive amino acids. *A* chicken-embryo cells infected with fowl plague virus were labelled with 50 μCi of ^{34}S-methionine from 4 to 8 hours post infection in absence (lane 1) or presence (lane 2) of 2.4 mM bromoconduritol. Cell lysates prepared 8 hours post infection were subjected to electrophoresis. *B* Chicken-embryo cells infected with PR8 virus were labelled for 1 hour with 10 μCi of ^{14}C-amino acids and then chased for 1 hour. The medium contained no (lanes 1 and 2) or 2 mM (lanes 3 and 4) bromoconduritol. After the chase period the monolayers were treated with trypsin (for lanes 2 and 4) or not treated (for lanes 1 and 3). Then cell lysates were prepared for electrophoresis (see Materials and Methods). Migration from top bottom

and then digested exhaustively with pronase. The digest was treated with endoglucosaminidase H and then analyzed on columns of Bio Gel P4 (Fig. 3). Comparison of Figs. 3A and 3B showed that virus from bromoconduritol-treated cells have no complex oligosaccharides (eluted between fraction 95 to 130) but a different set of high-mannose oligosaccharides. The high-mannose oligosaccharides have the elution behaviour of the glucosylated high-mannose oligosaccharides analyzed above. Thus, formation of complex-oligosaccharides is not necessary for the release of influenza virus PR8 particles from infected chicken-embryo cells.

Proteolytic Cleavage of Viral Glycoproteins

Specific proteolytic cleavage of the fowl plague hemagglutinin in chicken-embryo cells takes place in the smooth membrane fraction (15) and indeed this cleavage of hemagglutinin into HA_1 and HA_2 is observed in bromoconduritol treated cells. However, the rate of cleavage may be decreased because uncleaved HA accumulates in bromoconduritol treated cells (Fig. 4A). Fig. 4A shows, in fact, that after prolonged treatment with bromoconduritol (4 hours in this experiment) the amount of radioactivity present in the cleavage product HA_1 and HA_2 is reduced. Degradative proteolysis of glycoproteins is not observed in PR8-infected chicken-embryo cells. This may be related to the fact that the PR8 hemagglutinin is not cleaved into HA_1 and HA_2 in chicken-embryo cells (16).

To investigate specific proteolysis of sindbis polyproteins, pulse-chase experiments in bromoconduritol-treated cells were performed. Chicken-embryo cells pulsed for 15 minutes with ^{35}S-methionine showed radiolabelled B, PE_2 and E_1, whereas during a subsequent chase for 1 hour in the presence of 2 mM methionine also E_2 appeared. Bromoconduritol treatment did not prevent these proteolytic cleavages (Fig. 5), although B accumulated in bromoconduritol-treated chicken cells. Thus, formation of complex oligosaccharides is not necessary for proper proteolytic processing of sindbis proteins in chicken-embryo cells. In contrast, in BHK-cells cleavage of PE_2 to form E_2 was prevented in the bromoconduritol-treated cells (Fig. 5B). Also in BHK-cells B accumulates. This is remarkable because the polyglycoprotein in nontreated cell contains high-mannose oligosaccharides only. In the presence of N-methyl-1-deoxynojirimycin (another glucose-trimming inhibitor) E_2 is also not detected in infected BHK cells, suggesting that lack of E_2 is not the result of an artefact caused by bromoconduritol (Romero *et al.*, submitted).

Plasma Membrane Association of Viral Glycoproteins

As intracellular transport of influenza viral glycoproteins to the smooth membrane fraction is not prevented when inhibiting formation of complex oligosaccharides it was of interest to determine whether the viral glyco-

proteins reach the cell surface to be able to participate in budding. Treatment of intact influenza virus PR8-infected cells with trypsin cleaves the hemagglutinin on the cell surface (2). Thus, PR8-infected bromoconduritol-

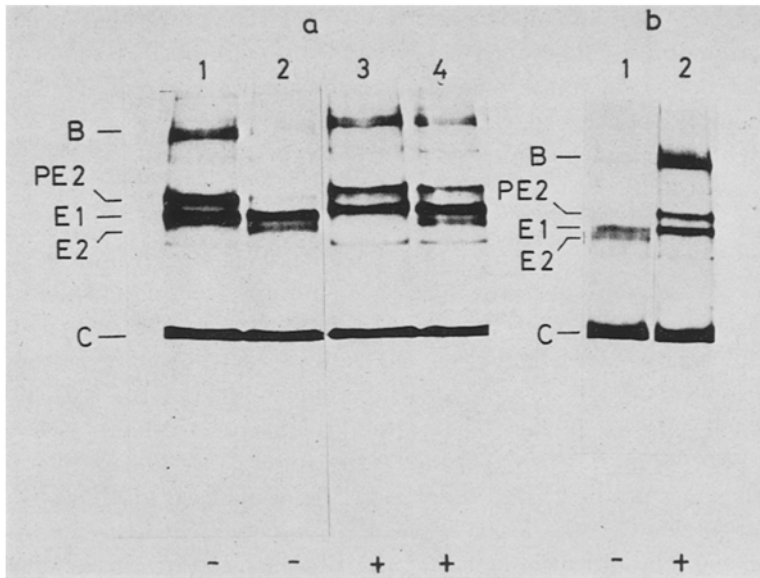

Fig. 5. Polyacrylamide slab gel electrophoresis of sindbis virus proteins from chick embryo *(A)* and BHK *(B)* cells labelled with ^{35}S-methionine in the presence or in the absence of bromoconduritol. Chicken-embryo cells infected with sindbis virus treated with bromoconduritol (lanes 3 and 4) or not treated (lanes 1 and 2) were either labelled for 15 minutes with 50 μCi ^{35}S-methionine (lanes 1 and 3) or labelled and then chased for one hour (lanes 2 and 4). Sindbis virus-infected BHK cells (B) treated (lane 2) or not treated (lane 1) with bromoconduritol were labelled for 20 minutes and then chased for 40 minutes. Bromoconduritol was present in label and chase medium

treated and non-treated cells were labelled with 10 μCi of ^{14}C-amino acids for 60 minutes and then chased for another hour in the presence or absence of bromoconduritol as above. Intact cells were then treated with trypsin and the proteins prepared for electrophoresis. It is shown in Fig. 4B that HA in both untreated (lanes 1 and 2) and bromoconduritol-treated (lanes 3 and 4) cells is cleaved by trypsin. As a control served that the HA of PR8 of cells treated with 10 μM monensin to block intracellular transport (32) was resistant to cleavage by trypsin, indicating that trypsin does not enter the cells (result not shown). The uncleaved hemagglutinin present in fowl plague virus-infected, bromoconduritol-treated cells (Fig. 4A) was resistant to cleavage by trypsin treatment of intact monolayers (not shown), indicating that it has not reached the cell surface.

To assess the cell surface location of the fowl plague glycoproteins HA_1 and HA_2 and of the sindbis virus glycoproteins, the extracellular antibody-absorption technique was used. Thus, pulse-labelled fowl plague virus glycoproteins are barely detectable at the cell surface (Fig. 6, lane 1) but

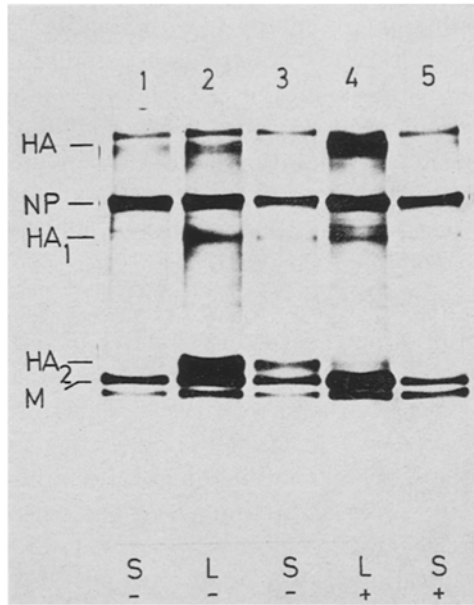

Fig. 6. Polyacrylamide slab gel electrophoresis of ^{35}S-methionine-labelled fowl plague virus proteins present in the total cell lysate (lanes 2 and 4) and on the cell surface (lanes 1, 3, and 5) in the presence (lanes 4, 5) or absence (lanes 1, 2, 3) of bromoconduritol. Cells were infected with fowl plague virus and at three hours post infection bromoconduritol was added to the cells for lanes 4 and 5; 90 minutes later the cells were labelled with 250 μCi of ^{35}S-methionine, after 20 minutes the sample for lane 1 was harvested and the other dishes were chased for forty minutes in the presence of 2 mM methionine, and 2.4 mM bromoconduritol in dishes for lanes 4 and 5. At the end of the chase or label period lysis buffer was added to samples for lanes 2 and 4 and anti-fowl plague virus-antibody to the samples for lane 1, 3, and 5. The samples were then processed as indicated under Material and Methods. The thin band, migrating like HA_1 in, for example lanes 1 and 5, is actin (2)

appear there after a 40 minutes chase (Fig. 6, lane 3). Including bromoconduritol into the medium prevents the migration to the cell surface (Fig. 6, lane 5) although the glycoproteins are present intracellularly (Fig. 6, lane 4).

Bromoconduritol was routinely included in both the pulse and the chase media; omitting bromoconduritol from the chase media did however not lead to different results.

Discussion

By inhibiting the trimming of the innermost glucose residue of the N-linked oligosaccharide Glc $\alpha(1\rightarrow2)$Glc $\alpha(1\rightarrow3)$Glc $\alpha(1\rightarrow3)$$Man_9GlcNAc_2$, an early and obligatory step in the processing of glycoproteins, bromoconduritol impairs the formation of sindbis virions from infected cells. The sindbis virus glycoproteins are equipped with $Glc_1Man_x(GlcNAc)_2$ (x = 7, 8, and 9) oligosaccharides and formation of complex oligosaccharides is inhibited. In infected chicken-embryo cells the polyproteins are cleaved to E_1 and E_2, a late event occurring just before budding of the virus (15). We suggest, that the step of inhibition is virus assembly, probably because proper oligosaccharide processing is required. This may not be valid for BHK cells because cleavage of PE_2 to E_2 is inhibited by inhibition of oligosaccharide processing and this proteolytic cleavage may be an important factor in virus assembly (4).

Bromoconduritol does not prevent the release of influenza virus particles PR8, although glycosylation is affected in the same way and to the same extent as in sindbis virus-infected cells. Therefore, there is no role for oligosaccharide processing in the budding of this virus. This may be related to a role of the M protein in budding of the influenza virus (22, 31). An equivalent protein is absent in sindbis virions where the virus glycoproteins directly interact with the virus cores. Yet, bromoconduritol prevents the release of infectious fowl plague virus particles from the infected cells (10). The reason for this is that after cleavage of HA into HA_1 and HA_2 the cleavage products are broken down, and transport to the plasma membrane is prevented. If cleavage of HA is prevented by using influenza virus PR8, virus budding proceeds as normal.

Delay in the specific proteolytic cleavages of the glycoprotein precursors B, pE_2 and HA is evident from their accumulation. This accumulation is not an artifact of bromoconduritol, because it is also observed in cells treated with another inhibitor of glucose trimming (24) and may be due to retardation of intracellular transport and/or to a reduced activity of certain proteases if these proteases are glycoproteins themselves. This latter explanation may apply to the reduced cleavage rate of B because this protein is also in untreated cells equipped with high mannose oligosaccharides only (14).

Thus, the cleaved HA equipped with high-mannose oligosaccharides $Glc_1Man_x(GlcNAc)_2$ (x = 7, 8, and 9) has a conformation that is susceptible towards degradative proteolysis. If glucose-trimming is inhibited by N-methyl-1-deoxynojirimycin, fowl plague viral glycoproteins contain oligosaccharides of the composition $Glc_3Man_x(GlcNAc)_2$ (x = 7, 8, 9). This treatment does not prevent virus release and the cleaved HA is metabolically stable (24). Uncleaved HA appears metabolically stable independent

of oligosaccharide composition. Apparently, if two glucose residues and two mannose residues are removed, and further processing is inhibited by bromoconduritol, cleavage of HA yields unstable products. If processing is allowed to proceed to the stage that the oligosaccharide has the structure $Man_5GlcNAc_2$ and then inhibited by the α-mannosidase II-inhibitor swainsonine (34), infectious fowl plague virus particles can be formed again with apparently intact cleaved HA (11). Possibly, a specific point of the processing pathway is of importance in determining a stable conformation of HA. Thus, inhibitors of trimming are useful tools, but generalizations concerning the role of oligosaccharide processing could be erroneous.

Acknowledgments

We acknowledge the technical assistance of Kirstin Schallner, Suzanne Fitz and Ulrike Klein. We thank Dr. C. Scholtissek for encouragement. We are indebted to Dr. E. Truscheit, Bayer AG for a gift of acarbose and 1-deoxynojirimycin and Dr. Inouye, Meji Kaisha, Yokohama for a sample of nojirimycin. Antibodies were kindly provided by Dr. G. Kaluza and Dr. H. Becht. We are furthermore grateful to Dr. G. Legler, Universität Köln for his encouragement and valuable advise during the preparation of bromoconduritol. This research was supported by the Deutsche Forschungsgemeinschaft (Sonderforschungsbereich 47 and a Heisenberg Stipendium to R.T.S.) and grants from Stiftung Volkswagenwerk and Justus-Liebig-Universität Giessen. P.A.R. was supported by the Alexander-von-Humboldt-Stiftung and Zentrum für internationale Migration und Entwicklung.

References

1. Beyer, T. A., Sadler, J. E., Rearick, J. I., Paulson, J. C., Hill, R. L.: Glycosyltransferases and their use in assessing oligosaccharide structure and structure-function relationships. Adv. Enzymol. **52**, 23—175 (1981).
2. Bosch, F. X., Garten, W., Klenk, H.-D., Rott, R.: Proteolytic cleavage of influenza virus hemagglutinins: Primary structure of the connecting peptide between HA_1 and HA_2 determines proteolytic cleavability and pathogenicity of avian influenza viruses. Virology **113**, 725—735 (1981).
3. Bosch, J. V., Schwarz, R. T., Ziemiecky, A., Friis, R. R.: Oligosaccharide modifications and the site of processing of gPr 92^{env}, the precursor for the viral glycoprotein of rous sarcoma virus. Virology **119**, 122—132 (1982).
4. Bracha, M., Schlesinger, M. J.: Defects in RNA^+ temperature-sensitive mutants of sindbis virus and evidence for a complex of PE_2 E_1 viral glycoproteins. Virology **74**, 441—445 (1976).
5. Burke, D. J., Keegstra, K.: Purification and composition of the proteins from sindbis virus grown in chick and BHK-cells. J. Virol. **20**, 676—686 (1976).
6. Caliguiri, L. A., Tamm, I.: The role of cytoplasmic membranes in polio virus biosynthesis. Virology **42**, 100—111 (1970).
7. Datema, R., Schwarz, R. T., Jankowski, A. W.: Fluoroglucose-inhibition of protein glycosylation *in vivo*. Inhibition of mannose and glucose incorporation into lipid-linked oligosaccharides. Eur. J. Biochem. **109**, 331—341 (1980a).
8. Datema, R., Schwarz, R. T., Winkler, J.: Glycosylation of influenza virus proteins in the presence of fluoroglucose occurs via a different pathway. Eur. J. Biochem. **110**, 355—366 (1980b).

9. Datema, R., Schwarz, R. T.: Effect of energy depletion on the glycosylation of a viral glycoprotein. J. Biol. Chem. **256**, 11191—11198 (1981).
10. Datema, R., Romero, P. A., Legler, G., Schwarz, R. T.: Inhibition of formation of complex oligosaccharides by the glucosidase inhibitor bromoconduritol. Proc. Natl. Acad. Sci. U.S.A. **79**, 6787—6791 (1982).
11. Elbein, A. D., Dorling, P. R., Vosbeck, K., Horisberger, M.: Swainsonine prevents the processing of the oligosaccharide chains of influenza virus hemagglutinin. J. Biol. Chem. **257**, 1573—1576 (1982).
12. Grinna, L. S., Robbins, P. W.: Glycoprotein biosynthesis. Rat liver microsomal glucosidases which process oligosaccharides. J. Biol. Chem. **254**, 8814—8818 (1979).
13. Hetkamp, H., Bause, E., Legler, G.: Calf liver microsomal glucosidases: characterization and inhibition by various inhibitors. Hoppe-Seyler's Zeitschr. Physiol. Chem. **363**, 1019 (1982).
14. Hakimi, J., Atkinson, P. H.: Glycosylation of intracellular sindbis virus glycoproteins. Biochemistry **21**, 2140—2145 (1982).
15. Klenk, H.-D., Rott, R.: Cotranslational and posttranslational processing of virus glycoproteins. Curr. Top. Microbiol. Immunol. **90**, 19—48 (1980).
16. Klenk, H.-D., Rott, R., Orlich, M., Blödorn, J.: Activation of influenza A viruses by trypsin treatment. Virology **68**, 426—439 (1975).
17. Kornfeld, S.: Oligosaccharide processing during glycoprotein biosynthesis. In: Horowitz, M. I. (ed.), The glycoconjugates, Vol. III. New York: 1982.
18. Kornfeld, S., Gregory, W., Chapman, A.: Class-E-Thy-1-negative mouse lymphona cells utilize an alternate pathway of oligosaccharide processing to synthesize complex-type oligosaccharides. J. Biol. Chem. **259**, 11649—11654 (1979).
19. Legler, G., Lotz, W.: Untersuchungen zum Wirkungsmechanismus glycosidspaltender Enzyme VII. Funktionelle Gruppen am aktiven Zentrum einer α-Glucosidase aus Sacch. cerevisiae. Hoppe-Seyler's Zeitschr. Physiol. Chem. **354**, 243—254 (1973).
20. Legler, G.: Glucosidases. Meth. Enzymol. **46**, 368—381 (1977).
21. Li, E., Tabas, I., Kornfeld, S.: The synthesis of complex-type oligosaccharides. I. Structure of the lipid-linked oligosaccharide precursor of the complex-type oligosaccharides of the vesicular stomatitis virus G protein. J. Biol. Chem. **253**, 7762—7770 (1978).
22. Lohmeyer, J., Talens, L. T., Klenk, H.-D.: Biosynthesis of the influenza virus envelope in abortive infection. J. gen. Virol. **42**, 73—88 (1979).
23. Robbins, P. W., Hubbard, S. C., Turco, S. J., Wirth, D. F.: Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. Cell **12**, 893—900 (1977).
24. Romero, P. A., Datema, R., Schwarz, R. T.: N-Methyl-1-deoxynojirimycin, a novel inhibitor of glycoprotein processing, and its effects on fowl plague virus maturation. Virology **130**, 238—242 (1983).
25. Saunier, B., Kilker, R. D., Tkacz, J. S., Quaroni, A., Herscovics, A.: Inhibition of N-linked complex oligosaccharide formation by 1-deoxy-nojirimycin, an inhibitor of processing glucosidases. J. Biol. Chem. **257**, 14155—14161 (1982).
26. Schlesinger, M. J., Malfer, C.: Cerulenin blocks fatty acid acylation of glycoproteins and inhibits vesicular stomatitis and sindbis virus particle formation. J. Biol. Chem. **257**, 9887—9890 (1982).
27. Schwarz, R. T., Rohrschneider, J. M., Schmidt, M. F. G.: Suppression of glycoprotein formation of semliki forest, influenza, and avian sarcoma viruses by tunicamycin. J. Virol. **19**, 782—791 (1976).

28. Schwarz, R. T., Datema, R.: The lipid pathway of protein glycosylation and its inhibitors. The biological significance of protein-bound carbohydrates. Adv. Carbohyd. Chem. Biochem. **40**, 287—379 (1982a).
29. Schwarz, R. T., Datema, R.: Inhibition of the dolichol pathway of protein glycosylation. Meth. Enzymol. **83**, 432—443 (1982b).
30. Sefton, B. M.: Immediate glycosylation of sindbis virus membrane proteins. Cell **10**, 659—668 (1977).
31. Simons, K., Garoff, H.: The budding mechanisms of enveloped animal viruses. J. gen. Virol. **50**, 1—21 (1980).
32. Tartakoff, A. M.: The Golgi complex: crossroads for vesicular traffic. Int. Rev. Exp. Pathol. **22**, 227—251 (1980).
33. Truscheit, E., Frommer, W., Junge, B., Müller, L., Schmidt, D. D., Wingender, W.: Chemie und Biochemie mikrobieller α-Glucosidase-Inhibitoren. Angewandte Chemie **93**, 738—755 (1981).
34. Tulsiani, D. R. P., Hubbard, S. C., Robbins, P. W., Touster, O.: α-Mannosidases of rat liver Golgi membranes. J. Biol. Chem. **257**, 3660—3668 (1982).
35. Turco, S. J., Pickard, J. L.: Altered G-protein glycosylation in vesicular stomatitis virus-infected, glucose deprived baby hamster kidney cells. J. Biol. Chem. **257**, 8674—8679 (1982).
36. Welch, W. J., Sefton, B. M.: Two small virus specific polypeptides are produced during infection with sindbis virus. J. Virol. **29**, 1186—1195 (1979).
37. Zimmerman, T., Schäfer, W.: Effect of p-fluorophenylalanine on fowl plague virus multiplication. Virology **11**, 676—698 (1960).

Authors' address: Dr. R. T. Schwarz, Institut für Virologie, Justus-Liebig-Universität Giessen, Frankfurter Strasse 107, D-6300 Giessen, Federal Republic of Germany.

Received December 20, 1983