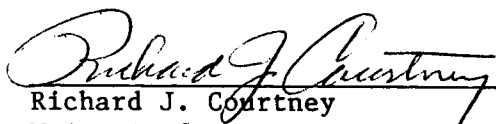
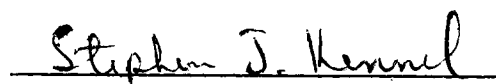


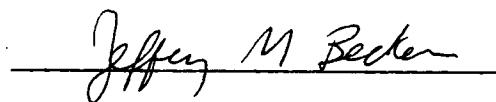
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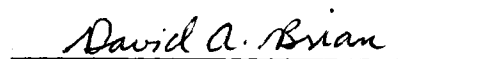
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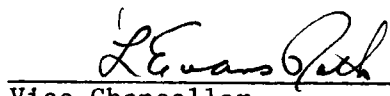
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SYNTHESIS AND PROCESSING OF HERPES SIMPLEX VIRUS TYPE 1

GLYCOPROTEINS gB and gC

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Elizabeth Ann Wenske

December 1982

3065022

DEDICATION

This dissertation is dedicated to my family;
especially to my parents,
for their love, laughter and chocolate chip cookies.

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I wish to express my sincere appreciation to my committee members: Drs. Jeffrey Becker, Steve Kennel and David Brian for their suggestions and encouragement. I would also like to thank Dr. Richard Eberle for his assistance as well as helpful discussions.

In addition, I want to thank my laboratory companions for their support, friendship, and especially for enduring my classical music. I will always remember the "Sam and Andy's" group and the feeling of family which we developed as we shared our research frustrations and successes.

Above all, to Dr. Richard J. Courtney, my major advisor, mentor, critic, and friend, thank you.

ABSTRACT

The glycoproteins of herpes simplex virus type 1 (HSV-1) range in molecular weight from 44,000 to 130,000. The objective of this research was to define the sequence of events involved in the synthesis of the high molecular HSV-1 glycoproteins gB (120,000 MW) and gC (130,000 MW). The studies focused on: (a) the identification and characterization of the partially-glycosylated precursors to gB and gC; (b) the manner in which the precursors were processed to the mature glycoproteins; and (c) the identification of the nonglycosylated forms of gB and gC.

The enzyme endo- β -N-acetylglucosaminidase H was used to identify the presence of mannose-rich oligosaccharides on the mature glycoproteins and their precursors. The gB glycoprotein contained both mannose-rich and complex-type oligosaccharides, whereas its proposed precursor, gA, contained only mannose-rich N-glycosidically linked oligosaccharides. The gB glycoprotein synthesized in tunicamycin- or deoxyglucose-treated cells had an approximate molecular weight of 92,000 and represented the nonglycosylated form of gB.

Only N-linked oligosaccharides of the complex-type were detected on the gC glycoprotein. In addition, the presence of O-linked oligosaccharides was indicated by the incorporation of isotopically-labeled glucosamine and galactose into a gC-related glycoprotein synthesized in the presence of tunicamycin and the susceptibility of gC associated oligosaccharides to alkaline hydrolysis. Only mannose-rich N-glycosidically linked oligosaccharides were associated with the

precursor of gC, designated pgC₍₁₀₅₎ (105,000 MW). Endo H digestion of pgC₍₁₀₅₎ demonstrated that the nonglycosylated form of gC had a molecular weight of 75,000. In addition, a 75,000 and a 92,000 molecular weight protein were synthesized in the presence of tunicamycin which suggests the posttranslational addition of O-linked oligosaccharides to the 75,000 MW protein. In the absence of tunicamycin, it is proposed that the O-linked oligosaccharides are added during the processing of pgC₍₁₀₅₎ to gC.

The incorporation of [³H]mannose into gB and gC glycoproteins synthesized in the presence of deoxyglucose was also examined. Glycoproteins synthesized in the presence of deoxyglucose contained both endo H sensitive and resistant oligosaccharides.

Based on this research a biosynthetic pathway for glycoproteins gB and gC is proposed.

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It is the glory of God to conceal things,
but the glory of kings is to search things out.

Proverbs 25:2

INTRODUCTION

Herpes simplex virus type 1 (HSV-1) virions contain four known structural glycoproteins designed gB, gC, gD, and gE which are associated with the viral envelope. Approximately 12 viral glycoproteins can be found in an infected cell extract; however, many of these have been identified as precursors to the glycoproteins found in the virion. At least one, and probably several of the HSV-1 glycoproteins are necessary for viral penetration at the surface of infected cells and for inducing cell fusion. The viral glycoproteins are also responsible for stimulating an immune response during the course of an HSV-1 infection. In order to understand the involvement of the glycoproteins in viral pathogenesis, latency and/or transformation it is necessary to investigate the biosynthesis and function of the herpes simplex glycoproteins.

The ability to follow the maturation of the HSV-1 virions at the cellular level will be dependent upon a means to identify the various stages of viral development. The nucleocapsid assembly takes place in the nucleus and the nucleocapsid becomes enveloped by budding through the nuclear membrane modified by viral glycoproteins. It is proposed that the viral glycoproteins inserted at the nuclear membrane are primarily underglycosylated precursors and further processing occurs after their incorporation into the virion envelope while the virion is in transit through the Golgi apparatus. It is therefore necessary to

further characterize the HSV-1 glycoproteins and their intermediates in order to use these as specific markers for identifying intracellular locations of viral maturation. For example, development of monoclonal antibodies which will selectively detect precursors or mature glycoproteins could be used as specific probes to identify sites of viral maturation within the infected cell. This becomes important in comparing virus replication within a productively infected cell and during reactivation of a latently-infected cell. Probes which discriminate between related glycoproteins will ameliorate our knowledge about their synthesis and expression in transformed cells.

This project was undertaken to define the biosynthetic pathway of the herpes simplex virus type 1 high molecular weight glycoproteins, gB and gC. The specific aims of this project were to:

1. Identify the nonglycosylated polypeptide backbone of the glycoproteins gB and gC.
2. Describe the type of glycosidic linkage in gB and gC and their precursors.
3. Determine whether the N-glycosidically linked oligosaccharide side chains of gB and gC are of the simple or complex type.
4. Determine the sequence of events in the synthesis and processing of gB and gC.

PART I

REVIEW OF THE LITERATURE

I. INTRODUCTION

Carbohydrates were at one time considered "impurities" in protein samples and much effort was spent in attempting to remove the sugar impurities until it was finally acknowledged that many proteins exist with covalently linked sugars. In the last two decades more emphasis has been placed on studying these ubiquitous macromolecules because of the crucial roles they play in cellular differentiation, growth and recognition. Glycoproteins are found in both plants and animals and may function as enzymes, hormones, immunoglobulins or structural proteins.

Biosynthetic studies have indicated that glycoproteins have in common covalently linked carbohydrate units, and share a similar biosynthetic pathway for the attachment of the sugar residues to the completed peptide chain. That the carbohydrate moiety is necessary for biological activity of the glycoproteins is evidenced by the cessation of these activities when glycosylation is inhibited.

One group of glycosylated proteins which has been studied extensively in the past decade is the virus specified glycoproteins. Viral glycoproteins are of extreme interest because of the essential roles they play in viral pathogenesis, morphogenesis, and the host's immune response to the viral infection. Much information has accumulated since the early 1970's on the biosynthesis and function of virus-specific glycoproteins associated with the enveloped RNA-containing viruses. In fact, the vesicular stomatitis virus G glycoprotein served as one of the models for defining the biosynthetic pathway for N-glycosidically linked oligosaccharides (117). The study

of viral glycoproteins also provides insight into the glycosylation mechanisms of the host cell since it is assumed that the virus uses the cell's machinery for the glycosylation of its proteins.

II. GLYCOPROTEIN BIOSYNTHESIS

The carbohydrate-peptide linkage of glycoproteins was first reported by Neuberger in 1928 at University College Hospital Medical School in London (136). Nearly 20 years later Neuberger and his associates demonstrated that the linkage of the oligosaccharide chain of hen ovalbumin was N-glycosidic between the anomeric carbon of N-acetylglucosamine and the nitrogen of the amide group. The characterization of the N-acetylglucosamyl-asparagine linking group by Neuberger and others was followed by the identification of several types of O-glycosidic linkages between N-acetylgalactosamine and the hydroxyl group of serine and threonine and between galactose and the hydroxyl group of hydroxylysine (136).

Until the early 1970's characterization of the oligosaccharides was limited to simple carbohydrate units. Since that time, the development of new techniques has allowed the structural analysis of glycoproteins from many sources. The emphasis of the following discussion is to summarize the common features which have become known for the structures and biosynthesis of glycoproteins in general.

N-Glycosidically Linked Glycoproteins

The N-linked oligosaccharides of glycoproteins consist mainly of two types: (1) mannose-rich or simple-type oligosaccharides and (2) complex-type oligosaccharides. The mannose-rich, simple-type oligosaccharides contain only mannose and N-acetylglucosamine, whereas, complex type oligosaccharides contain in addition to mannose and

N-acetylglucosamine, galactose, fucose, and terminal sialic acid residues (59, 78) (Figure 1B, 1C). A third type of N-glycosidically linked oligosaccharides has been identified which is a hybrid of complex- and simple-type oligosaccharides (Figure 1D). One branch of the hybrid oligosaccharide side chain may be mannose-rich while the other branch may contain N-acetylglucosamine, galactose, and sialic acid (62, 165). The basic common core of N-linked oligosaccharides is a mannosyl di-N-acetylchitobiose structure ($\text{Man} \rightarrow \text{GlcNAc} \rightarrow \text{GlcNAc} \rightarrow \text{Asn}$) (Figure 1A) (14) with the N-acetylglucosamine being attached to an asparagine residue found in the triamino acid structure of asparaginy-X-serine/threonine, the X standing for any other amino acid (59, 78). Not all asparagine residues found in this sequence within the polypeptide are glycosylated.

A general scheme for assembly of these oligosaccharides in animal cells has been determined based on the study of such glycoproteins as membrane proteins, secretory proteins, enzymes, immunoglobulins and viral proteins. The synthesis of the oligosaccharides begins by assembly of pyrophosphate-lipid-linked oligosaccharides. The carrier lipid involved is a dolichol phosphate (Dol-P) (106, 124) which serves as an acceptor for sugar donors GDP-mannose (GDP-Man) and UDP-glucose (GDP-Glc) to yield Man-P-Dol and Glc-P-Dol (106, 147). Therefore, the availability of Dol-P can serve as a means of regulation in synthesis of Dol-P-monosaccharides, such as Man-P-Dol and the oligosaccharides in general (44, 50, 59).

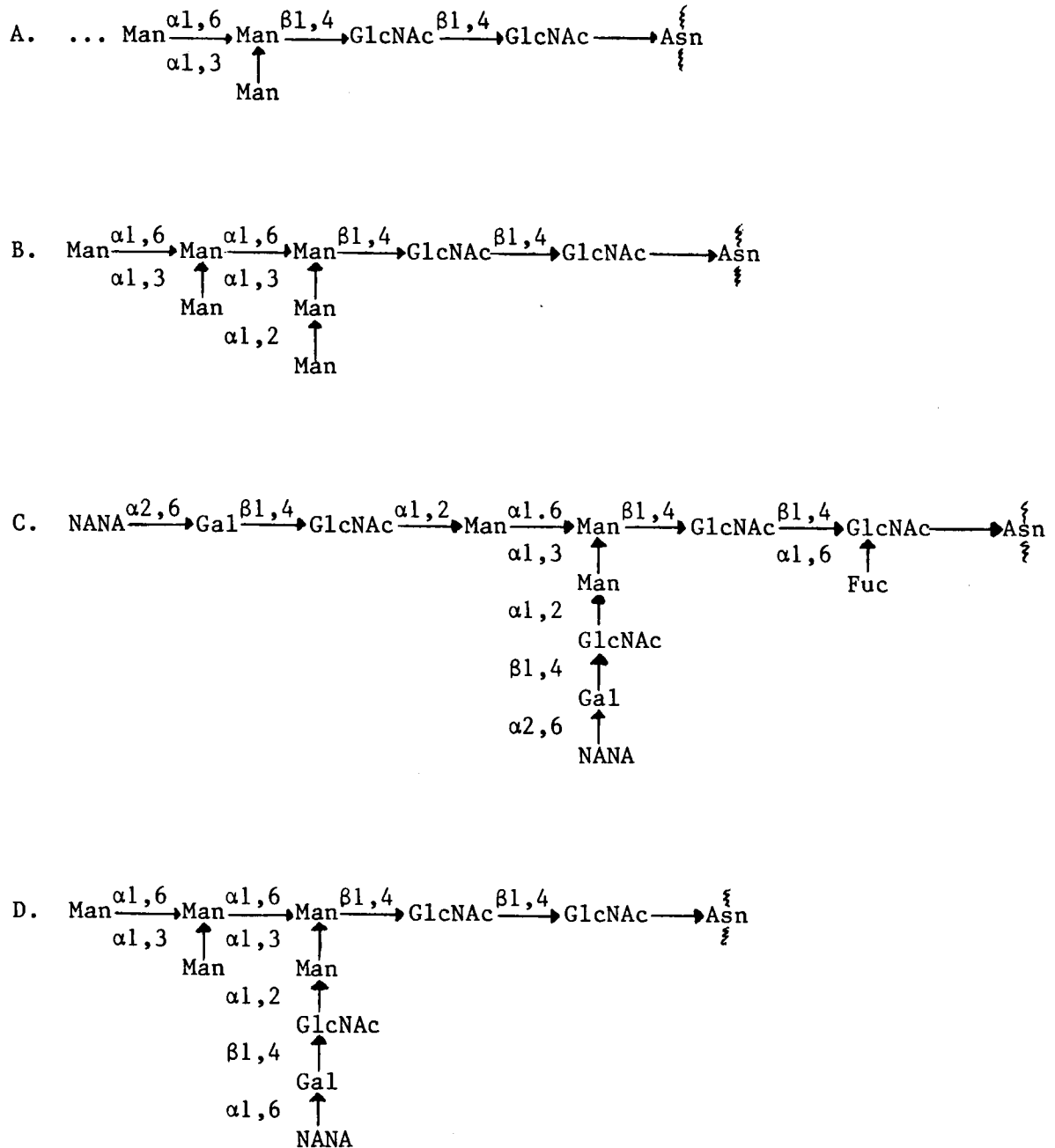


Figure 1. Basic structural features of N-glycosidically linked oligosaccharides. The common core structure (A); mannose-rich, simple-type (B); complex-type (C) (modified from Struck and Lennarz, 1980); hybrid-type (D) (modified from Hunt, 1981).

The sequence of reactions by which N-linked oligosaccharides are formed begins with the initial transfer of GlcNAc-1-PO₄ (106) (Figure 2). This is followed by the addition of a second GlcNAc from UDP-GlcNAc (156, 159). The next step is the addition of a β -mannosyl-residue donated by GDP-Man (12). The next four α -mannosyl residues can be donated by Man-P-Dol (10, 83) or GDP-Man (14, 35). However, Man-P-Dol is required for the addition of the subsequent outer mannosyl residues (10, 12). It is assumed that intermediates exist representing the addition of each mannosyl residue; however, these intermediates have not been isolated. It is possible to accumulate truncated lipid-linked oligosaccharides and isolate these oligosaccharides from mutant cell lines which exhibit structural alterations in the carbohydrate moieties of surface glycoproteins and glycolipids. It is thought that these truncated oligosaccharides represent intermediates in the synthesis of N-linked oligosaccharides (13, 60, 61).

The final steps in the assembly of the oligosaccharide on the dolichol are the addition of glucose molecules. In vitro studies have demonstrated the Glc-P-Dol rather than UDP-Glc is responsible for the transfer of glucose to yield Glc₃Man₉GlcNAc₂-P-P-Dol (142). The Glc-P-Dol is synthesized from UDP-Glc (4, 51, 106, 123, 161). After the addition of the glucose residues the oligosaccharide is transferred to the asparagine residue. Preference in transfer is given to those oligosaccharides containing terminal glucose residues, even in mutant cell lines which produce predominantly Man₅-GlcNAc or Man₇-GlcNAc (13, 61, 77).

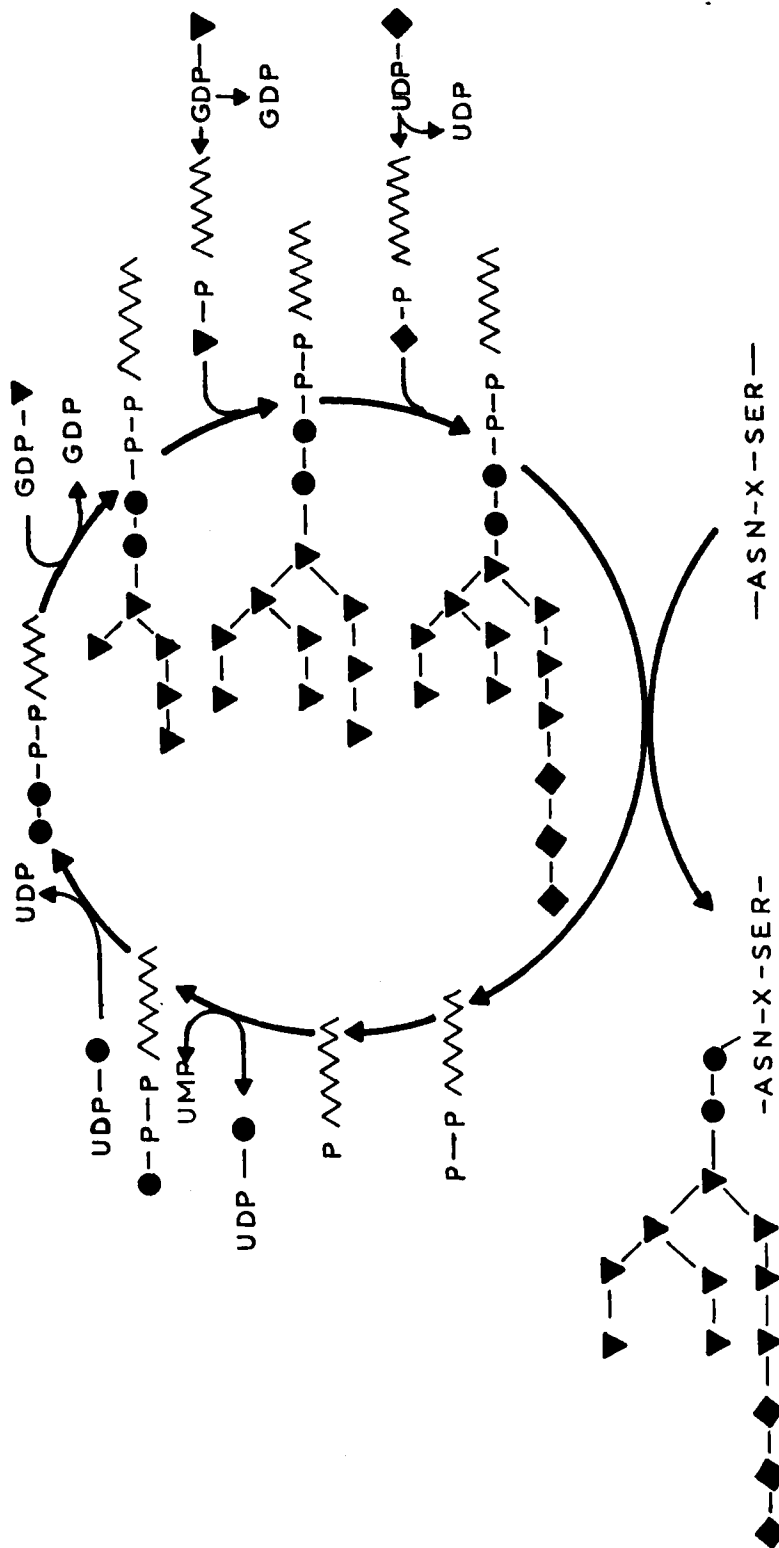


Figure 2. Dolichol phosphate cycle for synthesis of N-glycosidically linked oligosaccharides (modified from Sharon and Lis, 1981). Abbreviations: Glc, glucosamine; Man, mannose; GlcNAc, N-acetylglucosamine; ASN, asparagine; SER, serine.

The oligosaccharide is transferred en bloc to the nascent polypeptide before translation of the protein is complete (160). Since the transfer is a cotranslational event, the transfer occurs in the rough endoplasmic reticulum (5, 72, 117, 133). Once the transfer occurs the terminal glucose residues are removed immediately (59, 60, 79). After removal of the glucose residues the protein linked oligosaccharide contains only mannose and N-acetylglucosamine residues.

The high mannose glycoprotein is transported to the Golgi apparatus (90) where the protein linked oligosaccharide may retain the structure of $\text{Man}_9\text{-GlcNAc}$, or be processed further, to smaller high mannose oligosaccharides or to complex-type oligosaccharides. In order for processing to occur mannose residues must first be removed by mannosidases. Small high mannose oligosaccharides are formed by the removal of α -1,2-mannose residues. A $\text{Man}_5\text{GlcNAc}_2$ is the smallest high-mannose or simple-type oligosaccharide found in appreciable amounts in mature glycoproteins (11, 151) (Figure 3). The protein-linked $\text{Man}_5\text{-GlcNAc}_2$ also serves as an intermediate in the synthesis of complex-type oligosaccharides. The first step in the conversion of the high mannose oligosaccharide to the complex-type oligosaccharide, involves the transfer of N-acetylglucosamine by GlcNAc transferase I to the $\text{Man}_5\text{-GlcNAc}_2$ structure. As shown for the processing of the vesicular stomatitis virus G protein, the N-acetylglucosamine is added to the mannose residue linked α -1,3, to the β -1,4-linked mannose of the oligosaccharide core (150) (Figure 3). This event signals a specific α -D-mannosidase to cleave the two terminal noncore mannose residues from the $\text{GlcNAc}_1\text{Man}_5\text{GlcNAc}_2$ structure (45, 46). The core mannose exposed by

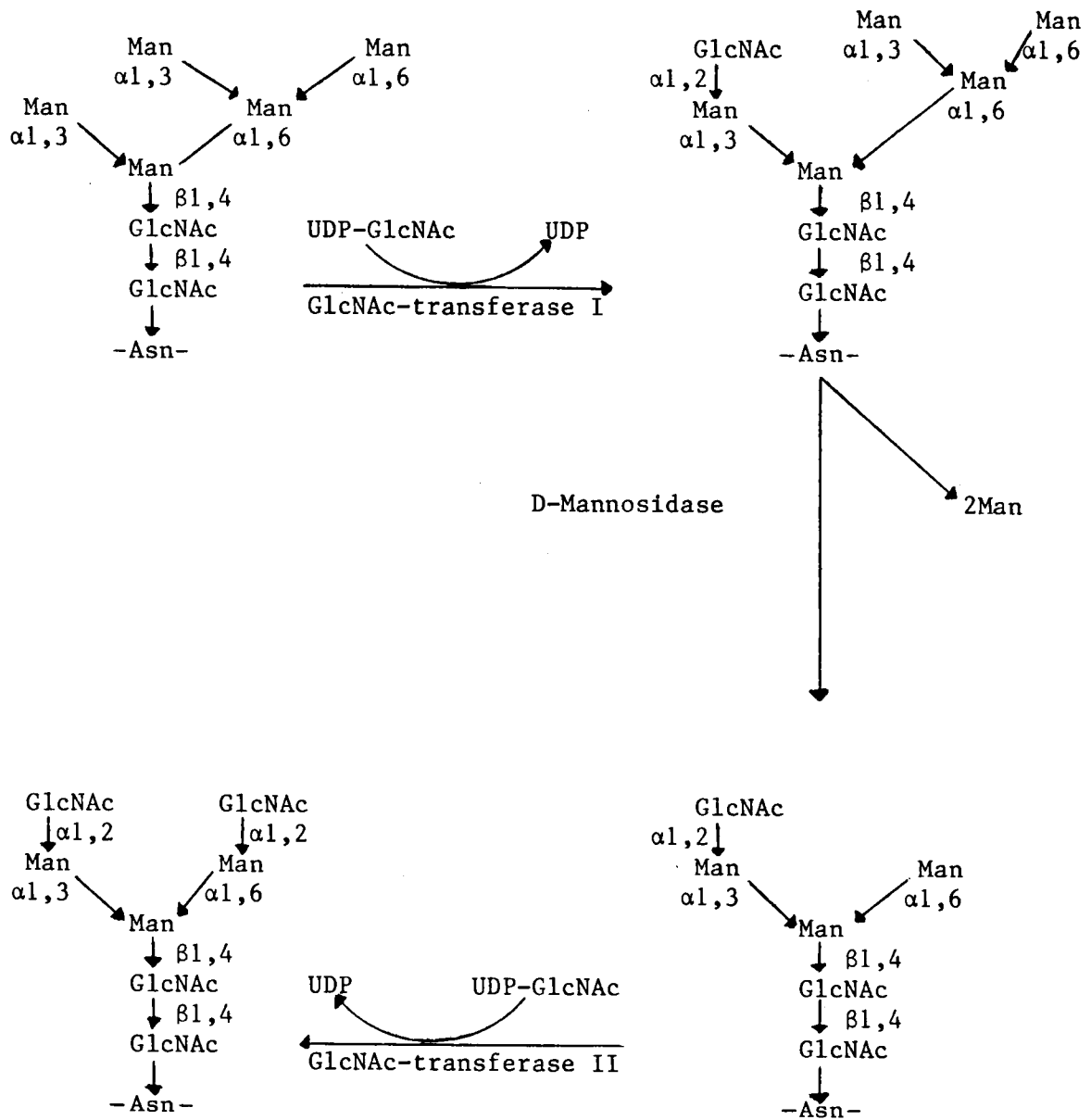


Figure 3. Proposed sequence of reactions in the late stage of processing of the complex oligosaccharide unit of the vesicular stomatitis virus (modified from Tabas and Kornfield, 1978). Abbreviations: Man, mannose; GlcNAc, N-acetylglucosamine; Asn, asparagine; UDP, uridine diphosphate.

this cleavage can now accept a second N-acetylglucosamine residue transferred by GlcNAc-transferase II (Figure 3). Two more sugar residues can be added to the oligosaccharide at this point: an α -1,6-linked fucose to the innermost GlcNAc (164), and a β -1,4-linked galactose to terminal GlcNAc residues (121, 122). The order in which these reactions occurs is not known. The addition of galactose and sialic acid to the branch structures of the oligosaccharide within the Golgi apparatus completes the synthesis of the complex type oligosaccharide (122). Among the N-linked oligosaccharides much heterogeneity can exist depending upon the extent of processing, the number of galactose, fucose, or sialic acid residues added and the presence of other modifications such as phosphorylation and/or sulfation.

O-Glycosidically Linked Glycoproteins

In many glycoproteins the oligosaccharide chains are attached to the peptide via an O-glycosidic linkage (Figure 4A) from N-acetyl-galactosaminyll to serine and/or threonine (78).

The synthesis of O-linked oligosaccharides does not involve a lipid intermediate but begins with the direct transfer of GalNAc from UDP-GalNAc to the serine or threonine in the polypeptide. The transfer is catalyzed by the enzyme N-acetylgalactosaminyll transferase (52). Strous (145) has proposed that the attachment of the initial GalNAc occurs while the nascent polypeptide is still attached to the ribosomes and that the majority of GalNAc associated with the nascent polypeptide incorporated as a monomeric saccharide. Since oligosaccharides of 20 sugar residues long were eventually found at the sites where GalNAc

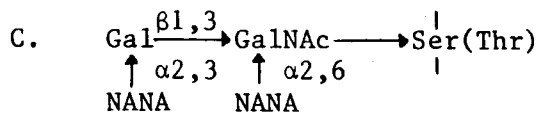
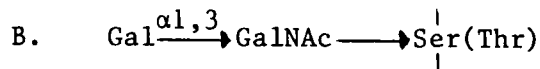
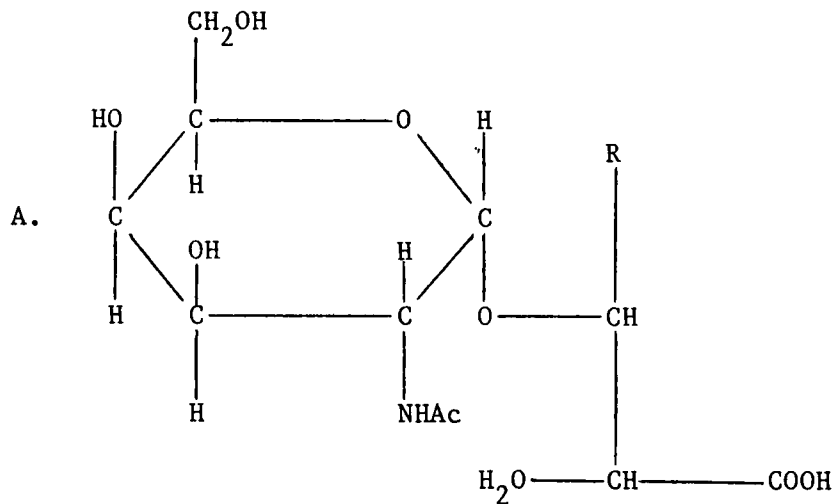


Figure 4. Glycopeptides linked through N-acetylgalactosamine to the hydroxyl group of serine and threonine. α -N-acetylgalactosaminyl-serine/threonine (A) (modified from Sharon and Lis, 1981); human IgA (B); fetuin (C) (modified from Kornfield and Kornfield, 1980). Abbreviations: Gal, galactose; GalNAc, N-acetylgalactosamine; NANA, N-acetylneuraminic acid; Ser, serine, Thr, threonine.

was initially bound it was suggested the elongation occurs posttranslationally (145). The addition of the sugar moieties by the glycosylating enzymes may be dependent on the conformation of the polypeptide. Two glycoproteins, IgA₁ (Figure 4B) and fetuin (Figure 4C), which have O-linked oligosaccharides also have a high number of proline residues (1, 140) and whether these residues create a structural signal for the addition of O-linked oligosaccharides has yet to be determined.

Further evidence that O-linked oligosaccharides are added posttranslationally was demonstrated by Gahmberg et al. (36). Their results showed that the N-linked oligosaccharides of the glycophorin A glycoprotein could be detected 5 minutes after adding label to erythrocytes in culture; however, only after 25 minutes of labeling could galactosyl residues of O-linked oligosaccharides be detected. Similar results have been shown for the synthesis of a coronavirus glycoprotein which contains only O-linked oligosaccharides (55, 94). The glycoprotein was first detected in a nonglycosylated form but was glycosylated 15-30 minutes later (55).

It is not known where the O-linked oligosaccharides are added to the protein. Holmes et al. (55) demonstrated by immunofluorescence that the coronavirus O-linked glycoprotein was restricted to tight clusters in the perinuclear region of the infected cells suggesting that this glycoprotein was associated with the rough endoplasmic reticulum or Golgi apparatus. There is also some suggestion that O-linked oligosaccharides are added at the Golgi (Johnson and Spear, in press; Nieman, personal communication) because this event appears to be inhibited by

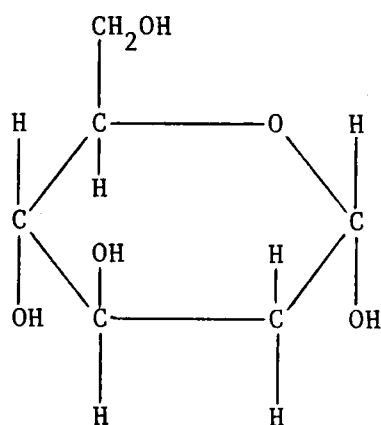
monensin, an ionophore which disturbs the Na^+/K^+ gradient of Golgi membranes (112).

III. INHIBITION OF 2-DEOXY-D-GLUCOSE

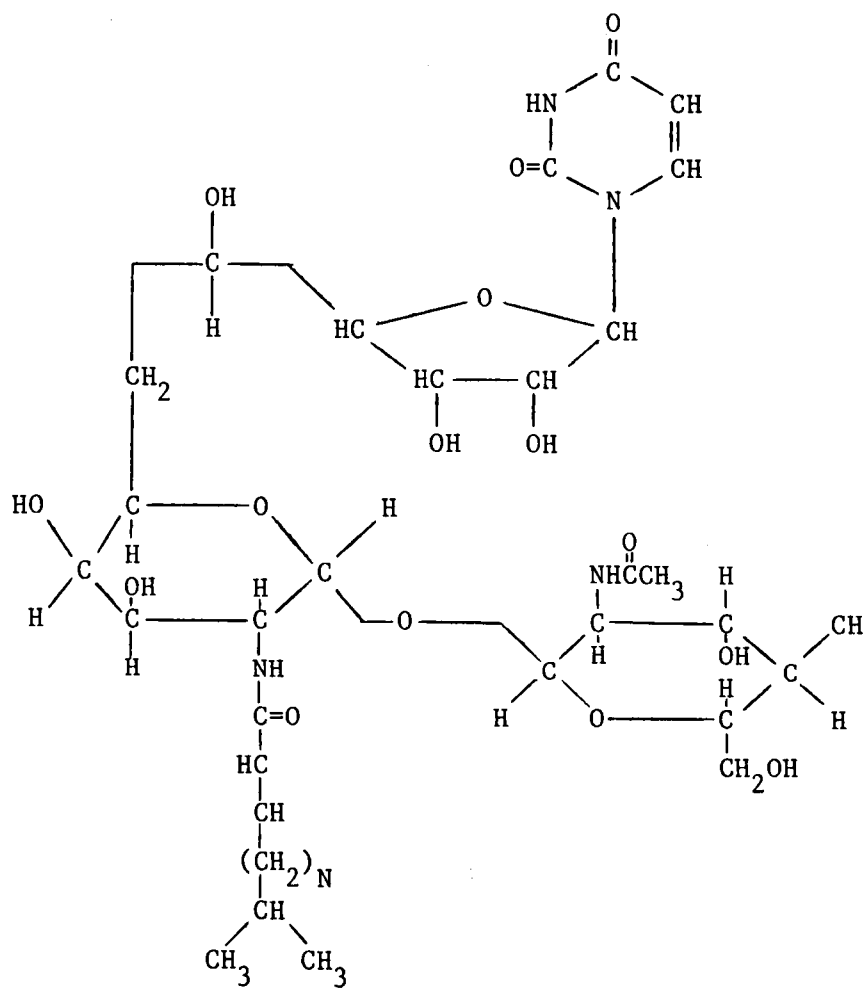
Mode of Action of 2-Deoxy-D-Glucose

The glucose and mannose analog, 2-deoxy-D-glucose (deoxyglucose) (Figure 5) was initially assumed to be metabolized in animal cells only to the 6-phosphate level (74, 138). Later it was discovered that deoxyglucose could be converted into the activated derivative UDP-2-deoxy-D-glucose (UDP-dGlc) and GDP-2-deoxy-D-glucose (GDP-dGlc) (125) and could be normally metabolized in mammalian cells (144). It is the nucleoside derivative GDP-dGlc which is primarily responsible for the effect that deoxyglucose has on protein glycosylation and N-glycosidically linked oligosaccharide synthesis.

The derivative GDP-dGlc blocks the formation of dolichol-mannosyl phosphate (Dol-P-Man) both in vivo and in vitro by replacing mannose in the dolichol phosphate derivative (126, 132). The Dol-P-Man is necessary for the addition of sugars to the lipid-linked oligosaccharide. Blocking the synthesis of Dol-P-Man thus prevents elongation of the oligosaccharide. Deoxyglucose can also be transferred from Dol-P-dGlc or GDP-dGlc to the lipid-linked oligosaccharide (132). The incorporation of deoxyglucose into the lipid-linked oligosaccharide prevents further elongation of the oligosaccharide and the transfer of the oligosaccharide to the nascent polypeptide (25). Once a complete lipid-linked oligosaccharide is synthesized its transfer to the polypeptide is insensitive to the action of deoxyglucose (24, 132). The reduced amount of carbohydrate associated with glycoproteins synthesized in the presence of deoxyglucose is probably due to fewer oligosaccharides



2-Deoxy-D-Glucose



Tunicamycin

Figure 5. Structures of 2-deoxy-D-glucose and tunicamycin.

rather than to shorter oligosaccharides being transferred from the dolichol to the nascent polypeptide (24).

The inhibition of glycosylation caused by deoxyglucose can be reversed in vivo by the addition of mannose and partially by glucose (126) and in vitro by the addition of GDP-Man (25) or dolichol-phosphate (Dol-P) (132). There is no reversal by galactose, fucose, or pyruvate (68, 119). A baby hamster kidney cell line has been isolated (88) in which incorporation of glucosamine into lipid-linked oligosaccharides or proteins is not inhibited (129) by deoxyglucose. However, treating membrane preparations derived from wild type or resistant cells in vitro with GDP-dGlc inhibited the formation of lipid-linked oligosaccharides to the same extent. It was suggested that the resistance in vivo is due to a higher GDP-Man pool in the resistant cells than in the wild-type cells causing a greater GDP-Man to GDP-dGlc ratio (129). These results explain the ability of mannose to reverse the inhibitory effects of deoxyglucose.

In contrast to GDP-dGlc, UDP-dGlc does not inhibit glycosylation in vivo or in vitro (126, 132). Deoxyglucose from UDP-dGlc is not incorporated into dolichol phosphate derivatives (132). Excess glucosamine decreases the UDP-dGlc pool of deoxyglucose-treated cells but inhibition of glycosylation is not reversed (126).

Incorporation of radiolabeled deoxyglucose into glycopeptides has been demonstrated for cellular glycoproteins (143) and viral glycoproteins (67). Datema et al. (24) have suggested that the incorporation of deoxyglucose into glycoproteins occurs as peripheral residues added

to oligosaccharide chains without participation of lipid carriers. The deoxyglucose of UDP-dGlc can be incorporated into glycoproteins in vitro (132) and in treated cells it may be incorporated at the nonreducing termini of the oligosaccharide side chains attached to the polypeptide. The transfer of deoxyglucose from UDP-dGlc directly to the glycoprotein may occur via a similar manner as the transfer of GlcNAc from UDP-GlcNAc to acceptor sites at the nonreducing termini of oligosaccharides linked to the glycoprotein (158).

Effect of 2-Deoxy-D-Glucose on Viral Replication

The antiviral activity of deoxyglucose was first described by Kilbourne (73) who reported the inhibition of influenza virus multiplication in embryonated chick eggs. Since Kilbourne's initial report a number of investigators have described similar effects of deoxyglucose on the multiplication of enveloped viruses. Virus-infected cells treated with deoxyglucose cease to produce infectious viruses and the viral glycoproteins found in cellular extracts have decreased molecular weights due to lack of carbohydrate side chains.

Studies have shown that the glycosylation of viral glycoproteins is not a prerequisite for virus particle formation. The yield of infectious influenza virus (92), respiratory syncytial virus (153), vesicular stomatitis virus and Newcastle disease virus (127) is reduced by deoxyglucose, but virus particles are still produced. In contrast, glycosylation of viral glycoproteins is necessary for assembly of vaccinia virus (107) and Semliki forest virus (65, 67, 68) particles.

The extent to which virus multiplication is inhibited in deoxyglucose-treated cells is dependent upon the virus type, the host cell and the energy source from which the virus is produced. Deoxyglucose has little inhibitory effect on the replication of vesicular stomatitis virus and Newcastle disease virus in host cells maintained with glucose-containing medium (68). In contrast, production of infectious vesicular stomatitis virus and Newcastle disease virus was significantly reduced in deoxyglucose-treated chick embryo cells when pyruvate containing culture medium was used (127). Even trace amounts of glucose in the culture medium could reduce the antimetabolic activity of deoxyglucose. Sabina and Telasco (119) also demonstrated a difference in the multiplication of vesicular stomatitis virus in deoxyglucose-treated Vero cells depending upon whether glucose or pyruvate containing medium was used. The inhibition of vesicular stomatitis virus multiplication by deoxyglucose was less apparent in glucose-containing medium. Other cell lines have been tested and the influence of the energy source on deoxyglucose inhibition of vesicular stomatitis virus multiplication was not as great (119) suggesting that the effect is also dependent upon the type of host-cell infected.

The effect of deoxyglucose on the biosynthesis of viral glycoproteins has been examined extensively. Deoxyglucose inhibits the glycosylation of viral glycoproteins and causes the accumulation of underglycosylated viral proteins in infected cells (38, 92, 126, 130, 132); however, whether these glycoproteins are inserted into virus particles is virus dependent. The underglycosylated fowl plague virus

hemagglutinin (HA_0) migrates from the rough to the smooth membranes where it is cleaved into smaller products; however, the cleavage products of HA_0 are heterogenous indicating that the carbohydrate side chains may be necessary for proper cleavage of the HA molecule into HA_1 and HA_2 (75, 130). Nakamura and Compans (92) have demonstrated that HA_0 glycoprotein of influenza virus can be isolated from plasma membranes of deoxyglucose-treated cells. They also detected the HA_0 and its cleavage products in virus particles synthesized in deoxyglucose-treated cells (92).

The polyprotein precursor of the Semliki forest virus glycoproteins is underglycosylated in the presence of deoxyglucose (65). As a result processing of the underglycosylated precursor is faulty resulting in heterogeneity of the cleavage products. Glycosylated proteins may be necessary for Semliki forest virus assembly since no virus particles are formed in the presence of deoxyglucose (67).

So far two different effects of perturbing glycoprotein synthesis of deoxyglucose has been discussed. In one case, noninfectious virus particles are produced containing aberrant glycoproteins, and in the second, aberrant glycoproteins are synthesized but without assembly of viral particles. In a third case, vesicular stomatis virus multiplication occurs in the presence of deoxyglucose and the vesicular stomatitis virus G glycoprotein is not glycosylated. A lower molecular weight G glycoprotein is synthesized lacking carbohydrate side chains (127). The underglycosylated G glycoprotein is incorporated into the virions and these virions appeared to be infectious (127).

In conclusion, the consequences of inhibiting viral glycosylation by deoxyglucose vary depending on the virus and the conditions of infection. The exact mechanism by which deoxyglucose inhibits virus multiplication is not yet fully understood.

IV. INHIBITION OF GLYCOSYLATION BY TUNICAMYCIN

Mode of Action of Tunicamycin

Tunicamycin (Figure 5, p. 18) is an N-acetylglucosamine containing antibiotic which is produced by Streptomyces lysosuperificus (152). Tunicamycin inhibits the synthesis of N-linked oligosaccharides by specifically blocking the formation of GlcNAc-pyrophosphoryldolichol, the first step in the lipid-linked oligosaccharide pathway (147, 154, 158, 159) (Figure 2, p. 10). Only the first step in the biosynthesis of the lipid-linked oligosaccharide is prevented and not the addition of the GlcNAc to GlcNAc-P-P-Dol (48) nor the formation of mannosyl-phosphoryl-polyprenol (34) or Man-P-Dol (147). The incorporation of mannose into lipid-linked oligosaccharides and the transfer of the lipid-linked oligosaccharide to a protein acceptor are not affected by the presence of tunicamycin (147).

The first step in the lipid-linked oligosaccharide pathway is catalyzed by the enzyme UDP-GlcNAc:dolichol-phosphate GlcNAc-1-phosphate transferase (47). Heifetz et al. (48) utilized a solubilized and partially purified sample of this enzyme to study the enzymatic mechanism of tunicamycin action on the in vitro incorporation of GlcNAc from UDP-GlcNAc into dolichol-pyrophosphoryl-GlcNAc. Increasing the enzyme concentrations required progressively more tunicamycin for the inhibition of GlcNAc incorporation. The inhibition of the enzyme was noncompetitive with respect to UDP-GlcNAc or Dol-P. Tunicamycin inhibited the reaction irreversibly and prevented the formation of UDP-N-acetylglucosamine from N-acetylglucosaminyldiphosphoryl dolichol.

Tunicamycin may therefore be considered a substrate-product transition analog, binding irreversibly to the UDP-GlcNAc:dolichol-phosphate GlcNAc-1-phosphate transferase (48).

Effect of Tunicamycin on Viral Replication

Tunicamycin specifically inhibits the glycosylation of proteins containing N-linked oligosaccharides and it has been used extensively in recent years to study the synthesis and biological role of virus specific glycoproteins. The extent to which tunicamycin affects these viral processes is dependent upon the individual viral system being tested. Treatment of infected cells with tunicamycin inhibits formation and release of Semliki forest virus and fowl plague virus (131), Sindbis virus (82), Sendai virus (93), and Snowshoe hare virus (9). In these cases, glycosylation of viral proteins is required for particle formation. In contrast, viral particle formation is not inhibited for influenza virus (92), measles virus (141), Newcastle disease virus (91), and coronavirus (55). These particles are noninfectious and do not contain detectable amounts of glycoproteins. In another example, vesicular stomatitis virus infectious particles are also formed in tunicamycin-treated cells, but particles containing the nonglycosylated viral glycoproteins are infectious (41). The extent of inhibition by tunicamycin on vesicular stomatitis particle formation and infectivity was dependent upon the strain of virus and the temperature at which the virus was produced (40, 41, 82). Synthesis of infectious virus was inhibited to a lesser degree at a lower temperature of replication than at a higher temperature (41).

Tunicamycin inhibits the glycosylation of virus proteins containing N-glycosidically linked oligosaccharides. In the presence of tunicamycin the following observations have been made. The accumulation of lower molecular weight viral glycoproteins synthesized in the presence of tunicamycin has been reported for Semliki forest virus (39), Sendai (93), influenza (92), Newcastle disease virus (91), Sindbis virus (81), vesicular stomatitis virus (40) and Rous sarcoma virus (26). Glycosylation of viral glycoproteins is not required for their insertion into intracellular membranes (116). However, attachment of carbohydrate side chains is required for migration to the plasma membrane for Sendai and Semliki forest virus (81, 93). In contrast, the nonglycosylated form of influenza HA glycoprotein, HA₀, does migrate to the plasma membrane but is not detected in virus particles. The nonglycosylated vesicular stomatitis virus G glycoprotein has been found both at the plasma membrane of infected cells and in the envelope of virions synthesized in tunicamycin-treated cells. The insertion of the nonglycosylated G protein appeared to be dependent on the strain of vesicular stomatitis virus and the temperature at which the virus was replicated (40, 41). Virus particles produced at 30°C in the presence of tunicamycin contained a greater amount of nonglycosylated G protein than virus particles produced at 37°C. The specific infectivity (pfu/cpm) of the virus produced at 30°C was the same whether the virus was produced in the presence or absence of tunicamycin, indicating that particles with nonglycosylated G protein were infectious (40). The nonglycosylated G protein forms aggregates and is less soluble than the glycosylated G

protein, therefore, the glycosylation may influence the folding and ultimate conformation of the glycoprotein necessary for transport (40). At the lower temperature the carbohydrate side chain may not be as critical for proper configuration since the nonglycosylated G synthesized at 30°C was more soluble than the nonglycosylated G synthesized at the higher temperature (41).

It has been suggested that in cases where the nonglycosylated forms of the viral glycoproteins are not detected in tunicamycin-treated cells, that the proteins are synthesized but are unstable and rapidly degraded (131). The fowl plague virus hemagglutinin could only be detected in tunicamycin-treated infected cells if protease inhibitors were also present (131). These results suggest that the carbohydrate side chains protect the glycoproteins from proteolytic digestion.

Glycosylation of viral proteins has been shown to be necessary for some but not all virus protein cleavage events. Semliki forest virus contains three glycoproteins, E_1 , E_2 , and E_3 , which are derived in an infected cell from a large virus specific polyprotein precursor. The cleavage of the precursor into E_1 and $p62(E_2+E_3)$ occurs in the absence of glycosylation, but cleavage of $p62$ into E_2 and E_3 is dependent upon glycosylation (39, 66). Carbohydrate side chains also may be responsible for the establishment and maintenance of specific conformations responsible for the antigenicity of the glycoprotein (66).

The molecular weight of the glycoproteins synthesized in the presence of tunicamycin may represent the approximate molecular weight

of the protein backbone structure. The molecular weights of in vitro translated vesicular stomatis virus, G, and Newcastle disease virus HN glycoproteins are similar to the molecular weights of these glycoproteins synthesized in tunicamycin-treated cells (91, 116).

Tunicamycin can be used as a tool to study those glycoproteins possessing O-linked oligosaccharides. The mouse hepatitis virus, a coronavirus, contains two viral structural glycoproteins designated E_1 and E_2 (149). The synthesis of E_2 , a glycoprotein with N-linked oligosaccharides, is not detected in the presence of tunicamycin indicating that the nonglycosylated E_2 is either not synthesized or is degraded rapidly (55). The glycoprotein E_1 is synthesized and glycosylated in the presence of tunicamycin since it contains only O-linked oligosaccharides (55). The vaccinia virus specified non-structural hemagglutinin molecule contains both tunicamycin sensitive and resistant oligosaccharides (137). The viral hemagglutinin synthesized in tunicamycin-treated cells contains O-linked oligosaccharides but not N-linked oligosaccharides (137).

V. GLYCOPROTEINS OF HERPES SIMPLEX VIRUS TYPE 1

Herpes simplex virus type 1 is a large double-stranded DNA virus consisting of an inner core of DNA, an icosahedral capsid composed of 162 capsomers, a nondescript protein structural component designated the tegument which surrounds the nucleocapsid, and a trilaminar membranous envelope containing at least four structural glycoproteins (3, 115, 139). The viral nucleocapsid is assembled in the nucleus and has the unique property among viruses of budding through the inner lamella of the nuclear membrane where it acquires its envelope and glycoproteins (23, 95, 128). Enveloped viruses accumulate in the perinuclear membrane and in the cisternae of the endoplasmic reticulum. The proposed exit of the virion from the cell is through a modified endoplasmic reticulum (23, 95, 128).

Herpes simplex virus glycoproteins are important because of their (a) involvement as carriers of antigenic determinants, (b) role in initiation of viral infection and (c) role in the social behavior of the infected cell. The antigenicity of the glycoproteins and their precursors must be taken into account when proposing subunit vaccines for controlling primary HSV infection. Recently two independent studies approached this problem by examining the ability of monoclonal antibodies directed against the individual glycoproteins to protect experimental animals against lethal HSV challenge (2, 28). The passive immunization with monoclonal antibodies established in vitro the availability of HSV glycoproteins as targets of immune surveillance. It is interesting to note that a monoclonal antibody which recognized only

the fully glycosylated HSV-2 gC did not afford passive protection, whereas a monoclonal which recognized HSV-2 gC and its precursor did give passive protection to HSV-2 challenged animals (2). It is therefore necessary to identify the glycoprotein structure which elicits the most protective immune response on the part of the organism.

Analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of HSV infected cell extracts has revealed the presence of at least 12 glycosylated viral glycoproteins of which only four have been found in association with the virion (3, 139). Some of the remainder have been identified as antigenically related precursors to the structural glycoproteins. The viral glycoproteins range in molecular weight from 50,000-130,000 (15, 30, 98, 99, 139) with the fully glycosylated glycoproteins being designated gB, gC, gD, and gE having approximate molecular weights of 120,000, 130,000, 58,000, and 80,000 respectively (3, 15, 139). The HSV glycoproteins contain type specific, unique to HSV-1 or HSV-2, and type common, shared by HSV-1 and HSV-2, properties.

The synthesis of the glycoproteins begins at the rough endoplasmic reticulum where the initial glycosylation of the nascent protein is assumed to occur (57, 128). The precursor glycoproteins obtain high mannosyl oligosaccharides (av. MW 2,000) within fifteen minutes of synthesis (57). The oligosaccharide side chains are further processed, presumably in the Golgi apparatus, in discrete steps to yield glycopeptides having an average molecular weight of 4,000-5,000 (57). The larger glycopeptides are less basic and contain fucose, galactose

and sialic acid, sugars not found in the lower molecular weight glycopeptides. The fully glycosylated proteins are present primarily in extracellular virions and the plasma membranes of infected cells, while the immature glycosylated proteins are associated with the intracellular membranes (57, 139). It has been proposed that the nucleocapsid incorporates low molecular weight precursor glycoproteins as it buds through the nuclear membrane which are then processed as the particle is transported out of the cell (Compton, personal communication).

Using the ionophore monensin, the processing of the HSV-1 glycoproteins has been further investigated (64). Monensin alters the Na^+/K^+ gradient at the smooth membranes of treated cells (112) which in turn disrupts the processing of protein-linked high-mannosyl oligosaccharides (63, 64). Treating HSV-infected cells with monensin causes an accumulation of the high mannose virus specific glycoproteins (63) which are not detected at the cell surface (63). Virions produced in the presence of monensin remain intracellular and contain the high mannose viral glycoproteins. These observations support a model in which enveloped virions move from the perinuclear space via membrane bound vesicles or altered endoplasmic reticulum to the Golgi apparatus for the processing of the glycoproteins followed by the transport of the virions to the plasma membrane and out of the cell.

HSV-1 Glycoprotein gC

The most highly glycosylated protein found in both the virions and the plasma membrane of infected cells is gC (av. MW 126,000-130,000) (29, 49, 139) which is derived from a precursor designated pgC (av.

MW 105,000-110,000) (30, 139). The antigenic determinants of gC are type specific as evidenced by the fact that hyperimmune serum to purified HSV-1 gC has the capability of neutralizing HSV-1 but not HSV-2 (20, 29, 139, 155). No monoclonal antibodies to gC HSV-1 have been isolated which react with HSV-2 gC (109, 110). Antisera to HSV-1 gC can cause cytolysis of HSV-1 infected cells in complement-dependent cytolytic tests and antibody-dependent cell mediated cytotoxicity tests (42, 97), thereby demonstrating reactivity of monospecific anti-gC with gC viral antigens at the surface of infected cells.

The function of gC still remains obscure. The glycoprotein is not necessary for replication since mutants lacking gC replicate normally and are infectious (54, 87). However, no clinical isolates have been found which do not contain gC suggesting gC may have a role in the pathogenesis of the virus.

The gC glycoprotein may have a function in suppressing viral-induced cell-fusion since the presence of gC in the plasma membrane correlates with the lack of cell fusion. Even though gC appears to have a negative influence on cell fusion its absence is not a prerequisite for cell fusion nor is cell fusion always suppressed when gC is present in the infected cell (56).

Information accumulated thus far indicates that gC is the only structural viral glycoprotein which contains both N- and O-linked oligosaccharides. The presence of O-linked oligosaccharides on an HSV-1 glycoprotein was suggested by the sensitivity of a sample containing HSV-1 glycoproteins to alkaline hydrolysis (100). The identification of

gC having O-linked oligosaccharides was based on its ability to bind to lectin-affinity columns specific for GalNAc-containing oligosaccharides (101).

The glycoprotein gC is derived from a high mannose precursor, pgC₍₁₀₅₎ which can be pulse-labeled with amino acids and the label completely converted into gC after a four hour chase period (30). Comparison of peptide digests of pgC₍₁₀₅₎, the virion associated gC, and the plasma membrane associated gC indicate all three share the same polypeptide backbone (15). The processing of pgC₍₁₀₅₎ to gC occurs in discrete steps which includes the removal of the mannosyl residues and the addition of sialic acid residues along with the attachment of the O-linked oligosaccharides (15, 43).

Based on investigations using the ionophore monensin, the majority of processing of pgC₍₁₀₅₎ to gC probably occurs within the Golgi apparatus (64). Monensin has been shown to inhibit the transport of viral membrane glycoproteins from the Golgi apparatus to the plasma membrane (63, 146). HSV-1 infected cells treated with monensin accumulated pgC₍₁₀₅₎ and its associated simple type oligosaccharides were not converted to complex-type oligosaccharides. The addition of O-linked oligosaccharides also appeared to be blocked by the presence of monensin in infected cells (Spear, personal communication). Virions isolated from monensin-treated infected cells contained pgC₍₁₀₅₎ rather than gC indicating that complete processing of the pgC₍₁₀₅₎ to gC was not necessary for its incorporation into the virion (64).

HSV-1 Glycoprotein gB

Originally the glycoprotein gB (MW 120,000) and its proposed precursor gA (MW 115,000) were considered antigenically distinct glycoproteins. They have since been recognized as sharing antigenic determinants and probably contain the same polypeptide backbone with their main difference attributed to the carbohydrate content of each (30). Monospecific antisera prepared in rabbits to either gA or gB precipitate gA and gB from infected cell extracts and no monoclonals isolated to date can antigenically differentiate the two glycoproteins (30, 109).

The glycoproteins gA and gB react predominantly as type common antigens although they have some type specific determinants as well (31, 109). In contrast to HSV-1 gC and HSV-2 gC, the map positions of HSV-1 gA and gB and HSV-2 gA and gB on the HSV-1 and HSV-2 genomes have been shown to be colinear (118). Antisera to gA and gB promote cytolysis of HSV-1 infected cells in both complement-dependent cytolytic and antibody-dependent cell mediated cytotoxicity tests (42, 97). In these tests the gB is probably the target antigen since gB and not gA, is found primarily in extracellular virions (30) and on the plasma membrane of infected cells.

Temperature sensitive mutants have been isolated which produce gA but not gB at the nonpermissive temperature (84, 120). The gA becomes incorporated into the virions produced at the nonpermissive temperature and these virions are noninfectious (84). Infectivity of the virions produced at the nonpermissive temperature could be greatly enhanced by

the addition of polyethylene glycol to infected cells following the adsorption period (84, 120). These results indicated that gB was necessary for penetration but not adsorption of the virus.

A ricin-resistant cell line, deficient in N-acetylglucosamine transferase I (89), has been isolated and when infected with HSV-1, the virus-specific glycoproteins synthesized in these cells only mature to the high mannose stage (8). In contrast to the virions produced in monensin treated cells, virions produced in the ricin-resistant cells are transported out of the cell into the extracellular medium (8). Virions produced under either condition are infectious indicating that processing of the virion associated glycoproteins is not necessary for infectivity (8). These results would tend to contradict the need for a fully processed gB for infectivity as implied by the experiments with ts mutants. It is possible that the gA of the ts mutants produced at the nonpermissive temperature has an altered conformation preventing proper interaction at the cell surface for penetration of the virions.

The precursor-product relationship of gA and gB was initially proposed by Eberle and Courtney (30). Pulse-chase studies indicated that gA and gB glycoproteins were synthesized from a common precursor polypeptide with gA labeled during the pulse and gB detected after a one hour chase period (30). The gA glycoprotein had a higher mannose content than gB indicating it may be the high mannose precursor of gB (30, 134). Haarr (43) has proposed that the processing of gB includes the sequential addition of sialic acid residues causing the glycoproteins to become more acidic.

The processing of the gB precursor (gA) probably occurs in the Golgi for the same reasons discussed for gC. Virions produce in monensin-treated cells do not contain gB and neither gA nor gB are found on the surface of monensin-treated infected cells (64). Finally, it is not known if gB also contains O-linked oligosaccharides. Conflicting reports from different laboratories describing the presence or absence of O-linked oligosaccharides on gB have yet to be resolved.

HSV-1 Glycoprotein gD

The lowest molecular weight HSV viral structural glycoprotein identified is gD (MW 59,000) which is derived in the infected cell from a high mannosyl precursor pgD (MW 52,000) (16, 33). The glycoprotein has predominantly type common antigenic determinants (15, 155) and the tryptic peptide profiles of the HSV-1 gD and the HSV-2 gD are similar (32). However, a monoclonal antibody recently prepared to HSV-1 gD neutralized HSV-1 but not HSV-2 virions (110) indicating that the two glycoproteins also contain several type specific peptides (32).

The glycoprotein gD is incorporated into virions and is expressed at the surface of infected cells where it can react with anti-gD to cause cytolysis of infected cells in antibody-dependent cell mediated cytotoxicity and complement dependent cytolytic tests (42, 97). Virions produced in the presence of monensin or in ricin-resistant cells incorporate pgD₍₅₂₎ in place of gD; however, pgD is not found in the plasma membrane of monensin-treated cells (64).

Synthesis of gD begins with the addition of a high mannose oligosaccharide (MW 1800) (33) to a core protein (MW 50,000) (111) and

this glycosylation event probably occurs cotranslationally. Data reported by Sarefini-Cessai (134) indicate that $\text{pgD}_{(52)}$ is sensitive to endo H substantiating the presence of oligomannosyl side chains attached to the polypeptide. Since conversion of $\text{pgD}_{(52)}$ to gD is blocked by monensin (64) this stage in processing probably occurs in the Golgi apparatus. The processing $\text{pgD}_{(52)}$ includes the addition of sialic acid residues causing the gD glycoprotein to be more acidic and also includes sulfation of gD (58). Based on preliminary data for the processing of gB and gC, the gD found in the virion is probably incorporated into the virion envelope originally as $\text{pgD}_{(52)}$ which is converted to gD during the egress of the virion out of the cell (Compton, personal communication).

HSV-1 Glycoprotein gE

Recently three electrophoretically distinct polypeptides ranging in molecular weight from 65,000-80,000 and having Fc-binding properties have been identified in extracts of HSV-1-infected cells (3). In pulse-chase experiments the 65,000 molecular weight polypeptide was chased into the stable 80,000 molecular weight polypeptide and all three polypeptides yielded similar protolytic digestion products suggesting that they were derived from the same gene product (designated gE) (3). HSV-1-infected cells express receptors to the Fc region of immunoglobulin IgG (162, 163) and it has been proposed that this receptor is gE since this glycoprotein has Fc-binding capabilities (103) and appears on the infected cell surface concomitant with the expression of Fc-binding activity of infected cells (104). The ability of HSV-1

infected cells to bind IgG was inhibited by first incubating the infected cells with $F(ab')_2$ fragments from anti-gE serum further demonstrating the relationship of the gE glycoprotein and the Fc binding property of infected cell surfaces (104). The gE (MW 80,000) was found in virions and the virion also had Fc binding capability (103).

The gE glycoprotein possesses type-common and type-specific antigenic determinants. Serum made against HSV-1 gE will immunoprecipitate both HSV-1 gE and HSV-2 gE from infected cell extracts. Antiserum to HSV-1 gE will neutralize HSV-1 virions in the presence of complement, but it will not neutralize HSV-2 virions (105). The gE glycoprotein has also been identified as the most sulfated HSV-1 glycoprotein and the sulfation is mainly associated with the mature gE, therefore, it must occur in the later stage of glycoprotein processing (58).

The synthesis of gE begins with the formation of an underglycosylated 65,000 molecular weight protein which is further glycosylated to an 80,000 molecular weight glycoprotein presumably by a similar fashion as discussed for other HSV-1 enveloped glycoproteins (104). In the presence of monensin the 80,000 molecular weight glycoprotein is not observed in infected cell extracts; however, the lower molecular weight gE precursors are synthesized (64).

VI. EFFECT OF TUNICAMYCIN ON HERPES VIRUS REPLICATION

Treatment of HSV-1 infected cells with tunicamycin does not significantly alter viral DNA or polypeptide synthesis (69, 102, 111). Polypeptides migrate to the nucleus of the cell and are assembled with the viral DNA into nucleocapsids, analogous to events in untreated infected cells (69, 108). These results indicate that glycosylation of viral proteins is not necessary for assembly of nucleocapsids but may be necessary for their envelopment. Katz et al. (69) reported that envelopment of nucleocapsids was not significantly affected in infected cells treated with tunicamycin. In contrast, Peake et al. (108) observed that the majority of the nucleocapsids formed in the tunicamycin-treated cells were nonenveloped. The nucleocapsids were observed in both the nucleus and the cytoplasm of tunicamycin-treated cells and only a few enveloped nucleocapsids were observed in the cytoplasm or on the cell surface (108).

The virus-like particles synthesized in tunicamycin-treated infected cells were further examined for their incorporation of HSV-1 glycoproteins. Very little if any [^3H]glucosamine label was associated with the virus particles synthesized in cells treated with tunicamycin. Neither glycoproteins nor proteins antigenically related to the HSV glycoproteins were detected in cytoplasmic or extracellular virus particles from tunicamycin-treated cells (108).

Even though the glycoproteins were not present in the virus particles synthesized in the presence of tunicamycin, they still may have accumulated in a nonglycosylated form in the infected cell.

Incorporation of glucosamine and mannose is reduced in infected cells treated with tunicamycin (69, 102, 111) and fully glycosylated viral proteins were not detected in cell extracts. Analysis of the cell extracts by SDS-PAGE showed the accumulation of nonglycosylated proteins antigenically related to the HSV-1 enveloped glycoproteins (96, 108, 111). The nonglycosylated polypeptides synthesized in the presence of tunicamycin have been examined in order to understand their relatedness with the individual HSV-1 glycoproteins (96, 108, 111).

Nonglycosylated glycoproteins synthesized in the presence of tunicamycin range in molecular weight from 44,000 to 105,000 (96, 108, 111). The lower molecular weight proteins (44,000-50,000) were reactive with antiserum specific for the HSV-1 gD glycoprotein (MW 59,000) and shared methionine tryptic peptides with the precursor of gD, pgD (MW 52,000) (111). An 85,000 molecular weight protein synthesized in tunicamycin-treated infected cells was identified as an underglycosylated analog of gC (MW 130,000) glycoprotein (108, 111) and tryptic peptide digests of both proteins showed they shared the same polypeptide backbone (111). A nonglycosylated analog of gA (MW 115,000) and gB (MW 120,000) synthesized in the presence of tunicamycin was also identified (96, 108). The molecular weight of the nonglycosylated protein was reported to have a range of 98,000-110,000 (96, 108).

The order and time of appearance of the nonglycosylated glycoprotein analogs synthesized in the presence of tunicamycin were the same as the order of appearance of the HSV-1 glycoproteins synthesized in the absence of tunicamycin (108). The glycoproteins gA, gB and gD

are synthesized soon after infection and prior to gC synthesis. In the tunicamycin-treated infected cells the 50,000 and 105,000 molecular weight proteins were detected prior to the 85,000 molecular weight protein (108).

The expression of the HSV glycoproteins at the cell surface of tunicamycin-treated infected cells was examined. No glucosamine-containing macromolecular heterosaccharides were detected on the cell surface of HSV-1 infected cells treated with tunicamycin (69). Tunicamycin also inhibited cell surface expression of reactive viral antigens as tested in antibody dependent, cell mediated cytotoxicity using specific antibodies to glycoproteins gA/gB, gC, and gD. However, a protein antigenically related to gC was detected by cell surface iodination on the plasma membrane of tunicamycin-treated infected cells (96). In contrast, iodination studies by other investigators of intact cell surfaces indicate that no viral glycoproteins or nonglycosylated analogs reach the plasma membrane of tunicamycin-treated infected cells (108). Some of the conflict in results may be due to inherent differences in the antisera preparations. Peake et al. (108) used an antisera to all the envelope glycoproteins and Norrild and Peaderson (96) used antisera made to the individual glycoproteins. Development of monoclonal antibodies will aid in deciphering the role of the carbohydrate side chains in determining the antigenicity of the glycoproteins.

VII. EFFECT OF 2-DEOXY-D-GLUCOSE ON HERPES VIRUS REPLICATION

The replication of herpes viruses has been studied in deoxyglucose-treated cells in order to determine the effect of deoxyglucose on production of both physical and infectious herpes virus particles, synthesis of viral specific glycoproteins, and functions or properties of viral glycoproteins. Recently, the efficacy of deoxyglucose as a chemotherapeutic agent against herpes virus infections has been explored.

The effect of deoxyglucose on the replication of herpes simplex virus in cell cultures was initially examined by Courtney et al. (21). In the presence of deoxyglucose there was an inhibition in the yield of infectious virus from treated infected cells while the yield of physical particles was less inhibited. The percentage of enveloped virus particles remained constant suggesting that envelopment of virus particles did occur in the presence of the inhibitor (21). Similar results have also been reported for pseudorabies- (a herpes virus) infected cells treated with deoxyglucose (85, 86).

Further insight as to the effect of deoxyglucose on the replication of herpes viruses has been provided by biochemical analysis of the viral induced glycopeptides synthesized in the presence of the inhibitor. The glycopeptides of HSV-1 synthesized in the presence of deoxyglucose exhibited a decrease in molecular weight as analyzed by SDS-PAGE (18, 76). Concomitant with the disappearance of the high molecular weight glycoproteins synthesized in the presence of deoxyglucose was the

synthesis of a new virus-induced protein(s) (18, 21, 27, 70, 76) which had an apparent molecular weight of 92,000-93,000 (18, 21). Knowles and Person (76) reported that in the presence of deoxyglucose isotopically-labeled glucosamine was incorporated primarily into a glycoprotein analogous to the 92,000 molecular weight protein reported by Courtney (18).

In view of the similarities shared between HSV-1 and HSV-2, the effect of deoxyglucose on the glycoprotein synthesis of the two viruses was compared. The replication of HSV-2 was inhibited by deoxyglucose as well as the incorporation of glucosamine into the viral glycopeptides with one notable exception. The synthesis of a high molecular weight HSV-2 glycoprotein, since designated HSV-2 gC, was not affected by deoxyglucose (19). The reason for this lack of inhibition is still being investigated.

Herpes viruses have the ability to induce syncytia or fusion of infected cells from within and it is assumed to be caused by a cell surface alteration of the infected cell, presumably the interaction of viral glycoproteins at the plasma membrane. Deoxyglucose reversibly inhibits virus-induced cell fusion of HSV-1-infected cells (37, 70, 76, 80) and pseudorabies virus-infected cells (85) supporting the assumption that glycosylated viral proteins are necessary for cell fusion.

Recently, alterations in the heterosaccharide content of the plasma membrane of infected cells treated with deoxyglucose have been studied (69). Cell surface proteins and glycoproteins of deoxyglucose-treated and untreated infected cells were analyzed for their heterosaccharide

content following digestion (69). The resulting heterosaccharides from treated-infected cells were smaller and had a molecular weight of less than 3,000. These smaller heterosaccharides were not present in digests of the proteins and glycoproteins isolated from the surface of untreated-infected cells. These results indicated that deoxyglucose blocked the appearance of high molecular weight oligosaccharides at the cell surface of infected cells (69).

The effect of deoxyglucose on viral replication, glycosylation and induced cell fusion is reversible by the addition of mannose to the culture medium. As the concentration of mannose was increased, there was a corresponding decrease in the effect of deoxyglucose on HSV-1-infected cells (18, 76). These results are consistent with the proposal that deoxyglucose acts as a mannose analog becoming incorporated into the oligosaccharide in place of mannose and thereby causing early termination of carbohydrate side chain synthesis. The incorporation of deoxyglucose into HSV viral glycoproteins has been reported (21). Deoxyglucose probably becomes associated with HSV glycoproteins by attaching to the reducing termini of oligosaccharides already attached to the protein moiety. Analysis of oligosaccharides linked to glycoproteins and synthesized in the presence of deoxyglucose should eventually reveal where on the glycoprotein the deoxyglucose is incorporated.

Studies elucidating the effect of deoxyglucose on viral macromolecular synthesis have demonstrated that the inhibitor has essentially no effect on the incorporation of isotopically labeled amino acids into

HSV specific proteins (27) and viral nonglycosylated proteins are synthesized normally (18, 76). The production of HSV and pseudorabies viral DNA also was not impaired by the action of deoxyglucose (85, 113). In contrast, deoxyglucose inhibits the synthesis of human cytomegalovirus (HCMV) DNA in infected cells (113). Amino acid incorporation of deoxyglucose treated HCMV-infected cells was also significantly decreased. The effect of deoxyglucose on HCMV DNA replication was not attributed to toxicity because host cell DNA synthesis in uninfected-treated cells was unimpaired and HSV-1 DNA replication was unaffected in the same cells treated with deoxyglucose (113). It is possible that glycosylation is necessary for HCMV protein synthesis and DNA replication in infected cells.

VIII. TREATMENT OF HERPETIC INFECTIONS WITH 2-DEOXY-D-GLUCOSE

The efficacy of deoxyglucose as a treatment of herpetic infections has been examined. Ray et al. (114) reported that deoxyglucose reduced or prevented HSV-induced ocular lesions in rabbits. The effectiveness of both topical and subconjunctival administration of deoxyglucose in treatment of herpetic keratitis was dependent on the severity of the ocular lesions. Treatment of the eyes with deoxyglucose prior to topical application with a high titer of HSV followed by postinfection treatment with the drug delayed the onset of severe lesions but did not prevent them (114).

In a clinical trial deoxyglucose was tested as a chemotherapeutic agent for treatment of human genital herpes infections (6, 7). The patients treated topically with deoxyglucose reported a faster relief of pain than those treated with a placebo. The lesions of the placebo group persisted longer than those treated with deoxyglucose, and those treated with deoxyglucose during an initial or recurrent herpes infection reportedly had a reduction in recurrent herpes infections (6).

The results of this study have been challenged by Corey and Holmes (17) who expressed concern with the difference in number of subjects included in the placebo and deoxyglucose treatment groups, the seemingly long length of virus shedding from the infected area and the duration of the lesions. They stated that the normal mean duration of lesions has been reported by others to be between nine and ten days (157) instead of the 18 days as observed by Blough and Giuntoli (6) for the placebo treatment group.

Deoxyglucose failed to prevent HSV-2 genital leison development in experimentally infected guinea pigs (135). No significant difference between the placebo and deoxyglucose-treated treated infected females was demonstrated. In other experimental trials deoxyglucose has been administered to mice infected with influenza A and hamsters infected with parainfluenza 3 or respiratory syncytial virus and the course of the viral infections was not altered (53). Aerosolyzed deoxyglucose also had no effect on parainfluenza 3 virus infections in hamsters (53).

A recent study has evaluated the effectiveness of deoxyglucose in the treatment of cutaneous infections with HSV-1 in mice, and genital infections with HSV-2 in mice and quinea pigs (71). Topical or intravaginal treatment with deoxyglucose did not significantly alter the development or severity of leisons; HSV isolation from leisons or the vaginal tract; antibody formation; mortality; or the establishment of latent infections (71). Based on these studies 2-deoxy-D-glucose is not an effective drug for the treatment of herpes virus infections.

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PART II

ENDO- β -N-ACETYLGLUCOSAMINIDASE H SENSITIVITY OF PRECURSORS
TO HERPES SIMPLEX VIRUS TYPE 1 GLYCOPROTEINS gB and gC

I. INTRODUCTION

Structural studies of viral glycoproteins containing asparagine-linked oligosaccharides reveal that the N-linked oligosaccharide side chains have a common biosynthetic origin: a high mannose precursor oligosaccharide whose synthesis begins while linked to a carrier dolichol (14, 23, 33). The completed high mannose oligosaccharide chains are then transferred en bloc from the dolichol to the nascent growing polypeptide. The newly glycosylated proteins are then transported to the Golgi where the oligosaccharide side chains may be further modified to become high mannose and complex glycans of mature glycoproteins (9, 10, 22, 28).

The biosynthesis of herpes simplex virus type 1 (HSV-1) glycoproteins includes the synthesis of five glycoproteins gA, gB, gC, gD and gE (1, 26, 27). The glycoproteins gA and gB have been demonstrated to be antigenically similar with gA being a precursor of gB (5, 8). The precursor form of gC, pgC₍₁₀₅₎, has an approximate molecular weight of 105,000 (27). The shift in molecular weight observed in the conversion of pgC₍₁₀₅₎ to gC is due in part but not completely to the addition of sialic acid to pgC₍₁₀₅₎ (3). The precursor to gD, pgD₍₅₂₎, contains an oligomannosyl core which is processed by glycosylation and sialylation to a larger, more heterogeneous oligosaccharide on the fully glycosylated glycoprotein gD (6).

Little is known about the characteristics of the intermediates involved in the biosynthesis of the high molecular weight HSV-1 glycoproteins, therefore this investigation was carried out in an attempt to

determine if the synthesis of these HSV-1 glycoproteins follows a similar pattern of synthesis as glycoproteins of other viral systems. We have investigated the biosynthesis of glycoproteins gA, gB and gC in an attempt to identify and characterize intermediates which occur during their biosynthesis.

By the use of endo- β -N-acetylglucosaminidase H (endo H) (30, 31) we have been able to further characterize the oligosaccharide structures of the glycosylated HSV-1 glycoproteins gA, gB, and gC and their precursors. Identification of high mannose oligosaccharides can be made by the susceptibility of a glycoprotein to the action of endo H (10, 22, 35). Endo H cleaves between the two proximal N-acetylglucosamine residues of the high-mannose-type oligosaccharides (30). Complex-type carbohydrate side chains are resistant to endo H, and oligosaccharides which are intermediates in processing show intermediate sensitivities (22, 29, 30, 34).

The processing of HSV-1 glycoproteins was further studied by use of the ionophore, monensin. Monensin is a linear polyether able to equilibrate Na^+/K^+ levels and shows selectivity for Na^+ (21). Monensin appears to inhibit the transport of glycoproteins from the Golgi to the plasma membrane but its effect on the actual glycoprotein processing differs among glycoproteins (11, 13, 32). It has been reported that the vesicular stomatitis virus G glycoprotein is fully processed in the presence of monensin; however, it is not transported to the plasma membrane (11). In contrast, the proteolytic cleavage of Sindbis virus glycoproteins PE2 and E2 was inhibited in ionophore-treated cells along

with the inhibition of the migration of PE2 to the plasma membrane (11). Monensin was used as a tool to investigate whether the HSV-1 glycoproteins gA, gB and gC were fully processed or blocked at an intermediate step in the presence of monensin.

II. MATERIALS AND METHODS

Cells

Human embryonic lung (MRC-5) fibroblasts and mouse L929 cells were grown in Eagle medium supplemented with 10% fetal bovine serum and 0.075% sodium bicarbonate. Virus stocks were grown in MRC-5 cells, and all virus titrations were conducted with Vero cell monolayers.

Virus

The viruses used in this study were the KOS strain of HSV-1 (25) and tsJ12, a temperature-sensitive mutant of HSV-1, KOS (15) which was kindly provided by Dr. Priscilla Schaffer (Sidney Farber Cancer Institute, Boston, Mass.).

Materials

Monensin was purchased from Calbiochem, La Jolla, California. Stock solutions of 1 mM were prepared by dissolving the ionophore in absolute ethanol and were stored at -70°C. Final dilutions were made in Eagle medium just prior to use.

The enzyme endo- β -N-acetylglucosaminidase H (endo H) was purchased from Division of Laboratories and Research, New York State Department of Health, Albany, New York. Stock solutions of 0.5 IU/ml of endo H were made in 10-20 mM sodium citrate buffer, pH 5.64, and stored at -70°C.

Isotopically labeled D-[2-³H]mannose (sp. act. 10.4 Ci/mmol), D-[1-¹⁴C]glucosamine (sp. act. 55.5 mCi/mmol) L-[³⁵S]methionine (sp. act. 1440 Ci/mmol) were purchased from Amersham, Arlington Heights, Illinois.

Infection, Labeling and Harvesting of Cells

Cells cultured in 60 to 100 mm plastic petri dishes were infected with virus at an input multiplicity of 10-20 pfu/cell. Following a 1 hr adsorption at 37°C the inoculum was removed, the monolayers were washed twice with warm phosphate buffered saline (PBS) and maintenance medium was added. Isotopically-labeled [^3H]mannose (20 $\mu\text{Ci/ml}$), [^{14}C]glucosamine (5 $\mu\text{Ci/ml}$) or [^{35}S]methionine (5 $\mu\text{Ci/ml}$) were added at 4 hr postinfection. When cells were isotopically labeled, Eagle medium supplemented with 1 $\times$ amino acids without methionine, 1 $\times$ glutamine, 2% donor calf serum, and .225% NaHCO_3 was used. At appropriate times the cells were scraped into the medium with a rubber policeman and washed twice in PBS. The pelleted cells were resuspended in water (5×10^6 to 1×10^7 cells/.2 ml), frozen, thawed and disrupted by ultrasonic vibration.

Detergent Extraction

Infected-cell extracts were prepared by extraction of infected cells with 1% sodium deoxycholate and 1% Tween-40 for 1/2 hr with constant agitation at 37°C. Insoluble material was removed by centrifugation at $100,000 \times g$ for 1 hr. The resulting soluble proteins (supernatant) were used as the antigen for the immunoprecipitation assays.

Discontinuous Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Molecular Weight Determinations

Details of the methods used for SDS-PAGE have been previously described (20). All slab gels contained 8.6% acrylamide cross-linked

with .23% N, N'-diallyl tartardiamide (DATD) as described by Gibson and Roizman (7).

Standard [^{14}C] labeled protein markers for molecular weight determinations were obtained from New England Nuclear, Boston, Mass., and contained myosin (200,000), globulins (150,000), phosphorylase B (92,500), ovalbumin (46,000), carbonic anhydrase (30,000), and lactoglobulin A (18,367). The major capsid protein of HSV-1 (154,000) was also included as a protein standard. A mixture (3,000 cpm) of these markers was coelectrophoresed with immune precipitated glycoproteins treated with and without endo H on 8.6% acrylamide gels cross-linked with DATD.

Antisera

Monospecific antisera to HSV-1 purified glycoproteins gAB and gC were used throughout this study. The purification of the glycoproteins and the characterization of the monospecific antisera prepared to the purified glycoproteins have been previously described (4, 5). Briefly, immune sera were prepared in rabbits to the gC and gAB glycoprotein components of HSV-1. Immunoprecipitation experiments demonstrated that the antisera were monospecific, i.e., anti-gC only reacting with the gC and pgC₍₁₀₅₎ glycoproteins and anti-gAB only reacting with glycoproteins gA and gB (4, 5).

Radioimmune Precipitation Assays

The details for the radioimmune precipitation assays have been previously described (5). Diluent used for radioimmune precipitation

was TNE [10 mM Tris ph 7.4), 100 mM NaCl, 1 mM EDTA], containing .1% Triton X-100. Samples of 25 μ l of solubilized cell extract were reacted with 25 μ l of rabbit antisera in a final volume of 100 μ l for 1 hr at 37°C and an additional 6 hr at 4°C. Staphylococcus aureus cells (20 μ l) (Pansorbin, La Jolla, Calif.) were then added and incubated for an additional 12 hr. Immune complexes were pelleted by centrifugation at $6,000 \times g$ for 20 min. and the precipitates were washed three times and solubilized in 35 μ l of 2% SDS, 2% mercaptoethanol (ME) and analyzed by SDS-PAGE.

Endo H Digestion of Immunoprecipitated Glycoproteins

Glycoproteins immunoprecipitated with the monospecific antisera were eluted from the Staphylococcal cells by incubating the samples in 0.8% SDS for 20 min. at 37°C followed by boiling for 30 sec. The Staphylococcal cells were removed by centrifugation. Samples to be treated with and without endo H were diluted to .2% SDS and made 50 mM with respect to sodium citrate buffer (pH 5.5). Endo H (6 μ l of .5 IU/ml) was added to samples for endo H treatment and the appropriate volume of water was added to the control samples. Following incubation at 37°C for 6 hr the samples were precipitated with 1 ml cold acetone and centrifuged at $13,000 \times g$ for 8 min. using a Fisher Model 235A microfuge. The pellet was resuspended in 35 μ l of 2% SDS and 2%-ME and stored at -20°C until analyzed by SDS-PAGE.

III. RESULTS

Endo H Digestion of HSV-1
Glycoproteins gC and pgC

To characterize the complexity of the oligosaccharide side chains of glycoproteins pgC₍₁₀₅₎ and gC, immunoprecipitates from isotopically-labeled HSV-1 infected cell extracts were analyzed. HSV-1 infected MRS-5 cells were isotopically labeled with [³⁵S]methionine from 4-24 hr postinfection and the whole cell fraction harvested at 24 hr postinfection. The isotopically-labeled cell extracts were immunoprecipitated with the monospecific anti-gC rabbit serum. The immunoprecipitates were washed, solubilized and then incubated with or without endo H. The PAGE profiles of these samples are shown in Figure 1. In the absence of endo H the fully glycosylated gC (MW 130,000) and the partially glycosylated precursor pgC₍₁₀₅₎ (MW 105,000) were clearly resolved. When endo H was present the pgC₍₁₀₅₎ glycoprotein was not detectable; however, a new protein with an apparent molecular weight of 75,000 was observed. In contrast, the migration of the fully glycosylated gC was not affected by the presence of endo H. The [³⁵S]methionine-labeled protein which migrated with a slower electrophoretic mobility than gC and which was detectable in the immune precipitates with anti-gC or normal rabbit serum probably represents the major capsid polypeptide of HSV-1. The presence of this protein which was nonspecifically precipitated served as a good control for demonstrating the lack of detectable protease activity in the endo H preparation.

Experiments were also conducted with HSV-1 infected cell extracts isotopically-labeled with [³H]mannose and immunoprecipitated with

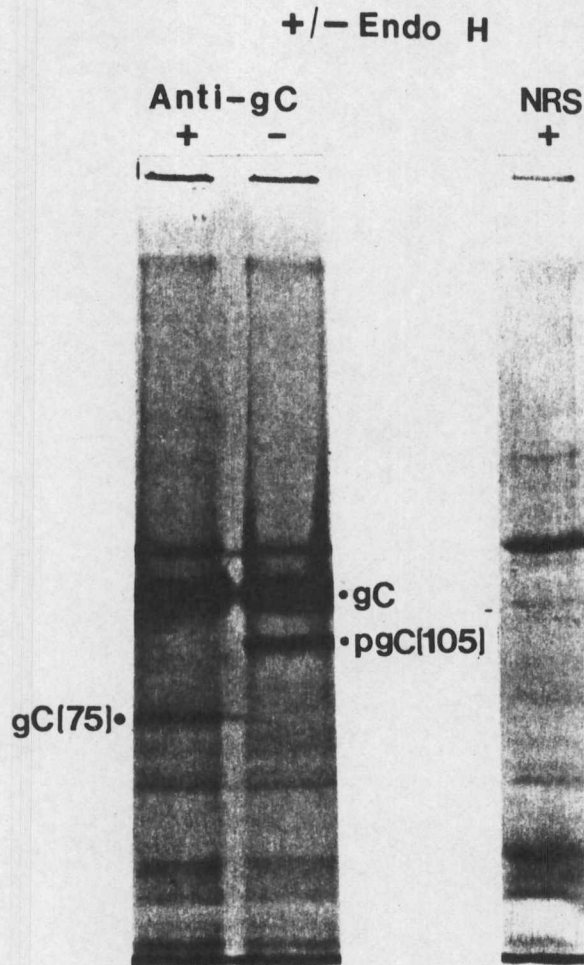


Figure 1. Endo H treatment of anti-gC immunoprecipitated [^{35} S]methionine-labeled proteins from HSV-1-infected MRC-5 cell extracts. Infected cells were labeled with [^{35} S]methionine from 4-24 hr postinfection and incubated at 37°C. Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H and prepared for SDS-PAGE analysis as described in Materials and Methods. Normal rabbit serum (NRS) was included as a control.

anti-gC (Figure 2). As observed in Figure 1, gC was not affected by the action of endo H. However, the [³H]mannose labeled pgC₍₁₀₅₎ was not detectable in the samples treated with endo H (Figure 2, lane 1). Based on endo H sensitivity and the migration of the immunoprecipitated proteins on SDS-PAGE, these results suggest that (1) gC has oligosaccharides of the complex type; (2) pgC₍₁₀₅₎ has N-linked oligosaccharides of the simple high-mannose type and (3) the nonglycosylated form of gC has an apparent molecular weight of 75,000.

To further verify that the 75,000 molecular weight protein was a digestion product of pgC₍₁₀₅₎, glycoproteins immunoprecipitated with anti-gC serum in a pulse-chase experiment were analyzed by SDS-PAGE. Infected cell cultures were pulsed at 6 hr postinfection for 5 min. with [³⁵S]methionine (50 µCi/ml) and then chased for the indicated times in Figure 3. Only pgC₍₁₀₅₎ was detected during the 5 min. pulse period; however, by 10 hr postinfection the pgC₍₁₀₅₎ had been converted to gC. The disappearance of the 75,000 molecular weight protein from endo H digested samples paralleled the disappearance of pgC₍₁₀₅₎ from the untreated immunoprecipitates (Figure 3, lanes 7 and 8). These results further suggest that the nonglycosylated form of gC has an approximate molecular weight of 75,000.

Endo H Digestion of HSV-1 Glycoproteins gA and gB

Previously we have presented data to suggest that gA represents the partially glycosylated precursor to gB (5). To characterize the complexity of the oligosaccharide side chains of glycoproteins gA and

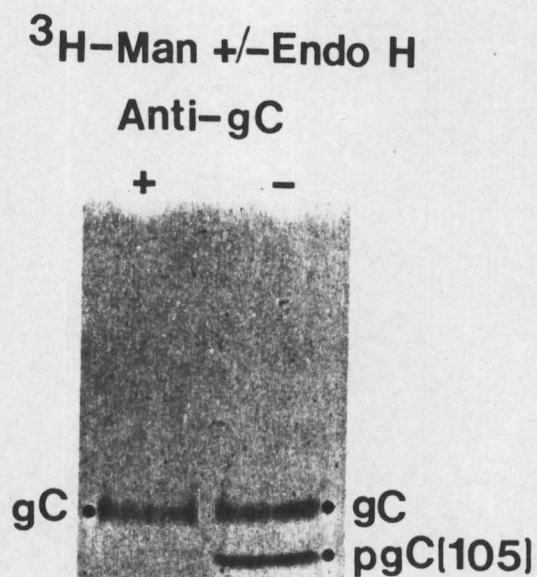


Figure 2. Endo H treatment of anti-gC immunoprecipitated [³H]mannose-labeled proteins from HSV-1-infected MRC-5 cell extracts. Infected cells were labeled with [³H]mannose from 4-24 hr postinfection and incubated at 37°C. Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H and prepared for SDS-PAGE analysis as described in Materials and Methods.

Figure 3. Endo H treatment of newly synthesized and processed forms of gC. MRC-5 cells were infected with HSV-1 and pulse labeled for 5 min. with [^{35}S]methionine (50 $\mu\text{Ci/ml}$) at 6 hr postinfection. Following the 5 min. pulse, one culture was harvested while the others were washed with PBS and chased for the indicated times in medium containing $10\times$ unlabeled methionine. HSV-1 pgC(105) and gC were immunoprecipitated from cell extracts with monospecific anti-gC serum. Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H, and prepared for SDS-PAGE analysis as described in the text.

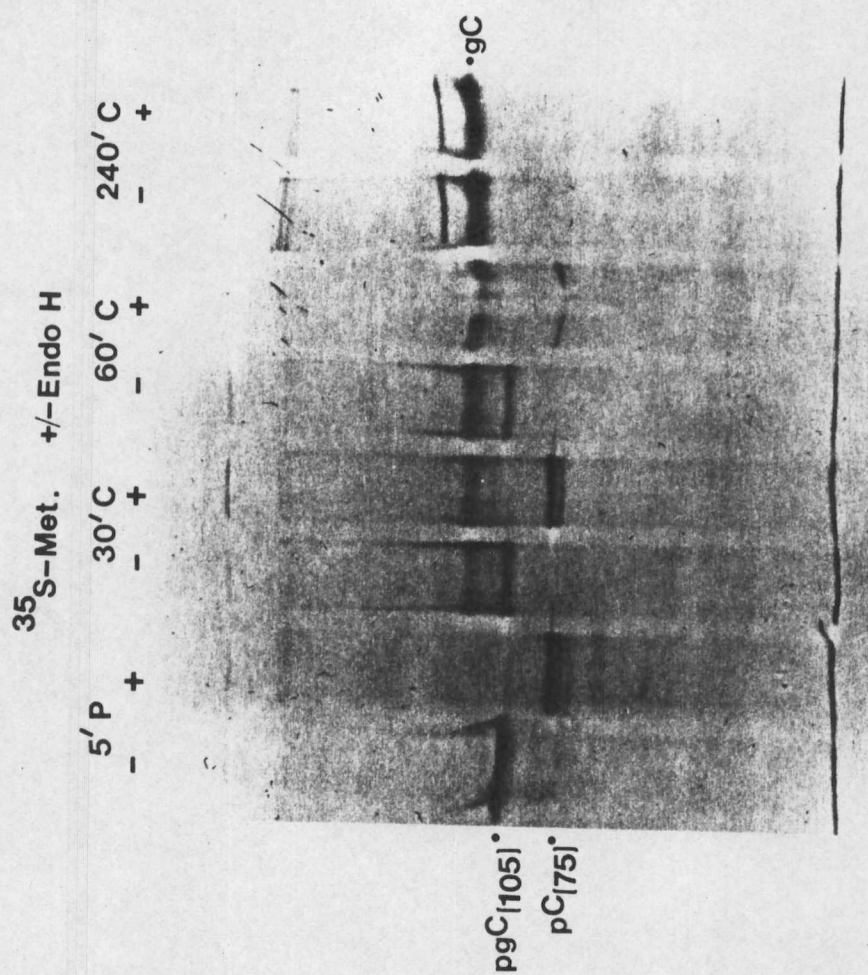


Figure 3.

gB, MRC-5 cells were infected with either HSV-1 (designated wt or wild type) or tsJl2 and isotopically-labeled with [³⁵S]methionine from 4-24 hr postinfection. The infected cells were cultured at 39°C, the nonpermissive temperature for tsJl2. The isotopically-labeled cell extracts were immunoprecipitated with monospecific anti-gAB rabbit serum. The immunoprecipitates were washed, solubilized and then incubated with or without endo H followed by analysis on SDS-PAGE (Figure 4). In the absence of endo H, gA and gB from wt infected cells migrated at a position in the gel which corresponded to apparent molecular weights of 110,000 and 120,000, respectively. However, when the immunoprecipitates of gAB were treated with endo H, there was an increase in the migration rate of both gA and gB. The apparent molecular weights of the proteins designated gB and gA were determined to be 115,000 and 97,000, respectively. To determine if these two faster migrating proteins were correctly designated as gA and gB, the experiment was repeated using the temperature sensitive mutant tsJl2. Previously it has been reported that at the nonpermissive temperature (39°C) tsJl2 infected cells synthesized gA, but not gB (15). As expected, in the absence of endo H only gA was immunoprecipitated. Following endo H digestion of the anti-gAB immunoprecipitated proteins obtained from the [³⁵S]methionine-labeled tsJl2 infected cell extract, a 97,000 molecular weight protein was detectable. However, the 115,000 molecular weight endo H digestion product detected in wt-infected cells was not detected in tsJl2-infected cells. Based on these results we suggest that the 115,000 protein is a digestion product of gB and the

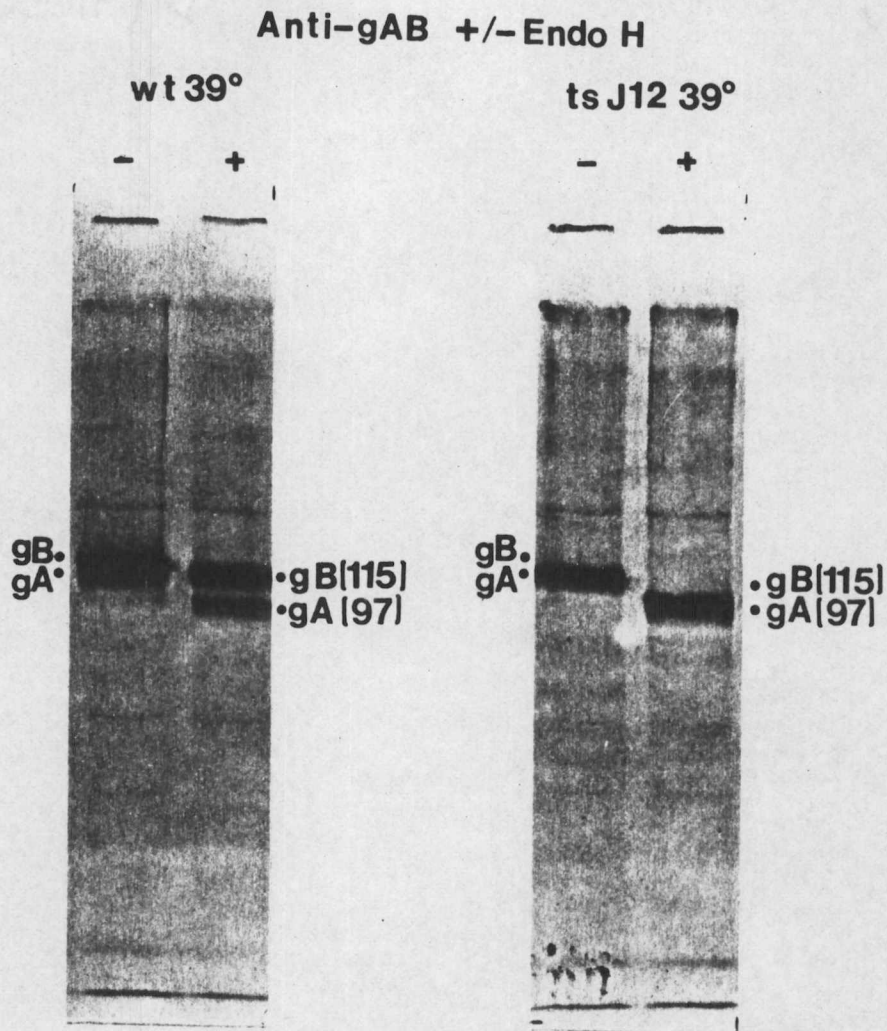


Figure 4. Endo H treatment of anti-gAB immunoprecipitated [³⁵S]methionine-labeled proteins from HSV-1 (wt) and *tsJ12* infected MRC-5 cell extracts. Infected MRC-5 cells were labeled with [³⁵S]methionine from 4-24 hr postinfection and incubated at 39°C. Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H and prepared for SDS-PAGE analysis as described in Materials and Methods.

97,000 molecular weight product is a digestion product of gA. It should be noted that the band designated gA always appeared as a doublet. The significance of this observation in relation to the synthesis and processing of gA is under investigation. Similar results were obtained with [^3H]amino acid cell extracts.

In an attempt to determine whether gA represents a high mannose type precursor to gB, immunoprecipitates of gAB from [^3H]mannose labeled infected cell extracts were also subjected to digestion with endo H. Both HSV-1 wt and tsJ12 infected cells cultured at 39°C were used in these experiments. Following endo H digestion of immunoprecipitates from wt infected cell extracts, only one [^3H]mannose labeled glycoprotein was detected (Figure 5, lane 1). To verify that this protein represented the gB glycoprotein, the gAB immunoprecipitate from the tsJ12 infected cell extracts was digested with endo H. Little if any [^3H]mannose-labeled protein was detected indicating that most of the [^3H]mannose label was cleaved from the gA synthesized in tsJ12 infected cells. Taken together, the above results suggest that the gA glycoprotein has mostly the simple or high mannose type oligosaccharides which are sensitive to endo H digestion. In contrast, gB contains both complex and simple type oligosaccharide chains.

Radioimmune Precipitation of HSV-1
Glycoproteins from Monensin-Treated
Infected Cells

The sensitivity of the HSV glycoproteins to endo H digestion described above, suggested that gA and pgC were high mannose precursors to gB and gC, respectively. Since monensin has been shown to block the

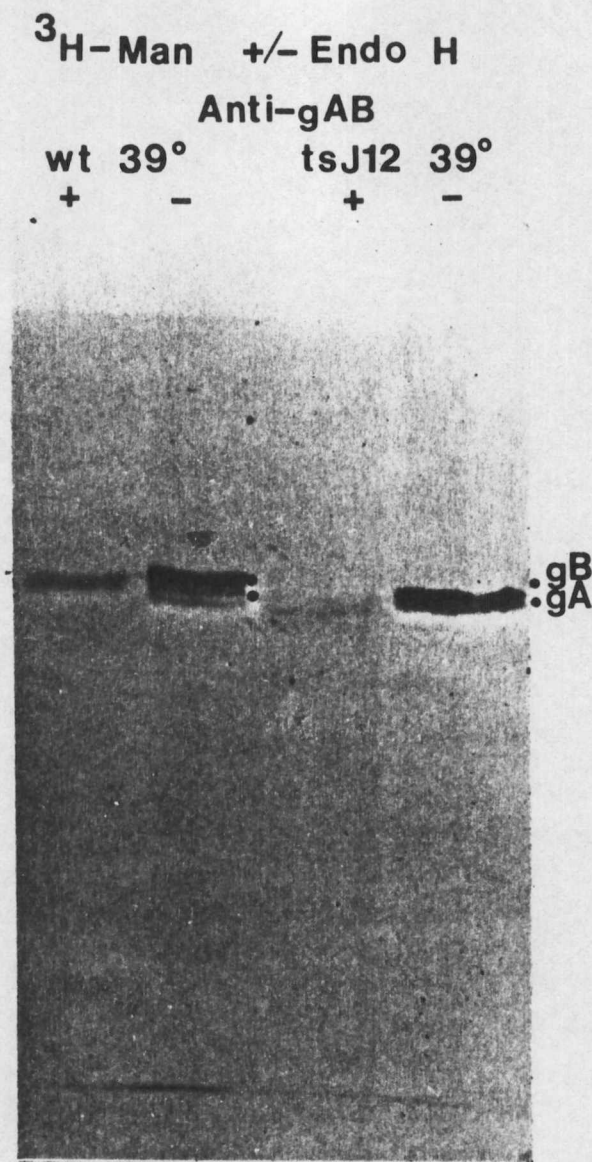


Figure 5. Endo H treatment of anti-gAB immunoprecipitated [³H]mannose-labeled proteins from HSV-1 (wt) and *tsJ12* infected MRC-5 cell extracts. Infected cells were labeled with [³H]mannose from 4-24 hr postinfection and incubated at 39°C. Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H and prepared for SDS-PAGE analysis as described in Materials and Methods.

processing of Sindbis virus glycoproteins at the high mannose stage (11), it was of interest to determine if an accumulation of pgC and gA occurred in infected cells incubated in the presence of the ionophore. Cell extracts of infected cells incubated in the presence or absence of monensin were first analyzed by SDS-PAGE. HSV-1 infected L-cells were incubated in 10^{-7} and 10^{-8} M monensin for 24 hr postinfection and labeled with [^{14} C]glucosamine 4-24 hr postinfection. At 24 hr postinfection the cells were harvested and the solubilized cell extracts were analyzed by SDS-PAGE (Figure 6). In the presence of 10^{-7} or 10^{-8} M monensin the appearance of the gB and gC glycoproteins was significantly reduced. This was especially apparent with a monensin concentration of 10^{-7} M. In contrast, it appeared that gA and pgC₍₁₀₅₎ and pgD₍₅₂₎ were synthesized and detectable in greater amounts as compared to cells cultured in the absence of the inhibitor.

To verify whether the gA and pgC₍₁₀₅₎ made in the presence of monensin do contain high mannose oligosaccharides, immune precipitates from monensin treated-infected cell extracts were incubated in the presence or absence of endo H. MRC-5 cells were infected with HSV-1 and incubated at 37°C for 24 hr postinfection with maintenance medium containing 10^{-6} M or 10^{-7} M monensin. The infected cells were labeled from 4-24 hr with [^{35} S]methionine and harvested at 24 hr postinfection. The isotopically-labeled glycoproteins were immunoprecipitated with monospecific anti-gAB and anti-gC sera and the immune precipitates were then subjected to digestion with endo H.

The effect of endo H on immunoprecipitated gAB from MRC-5 cells infected and maintained in the presence or absence of monensin is shown

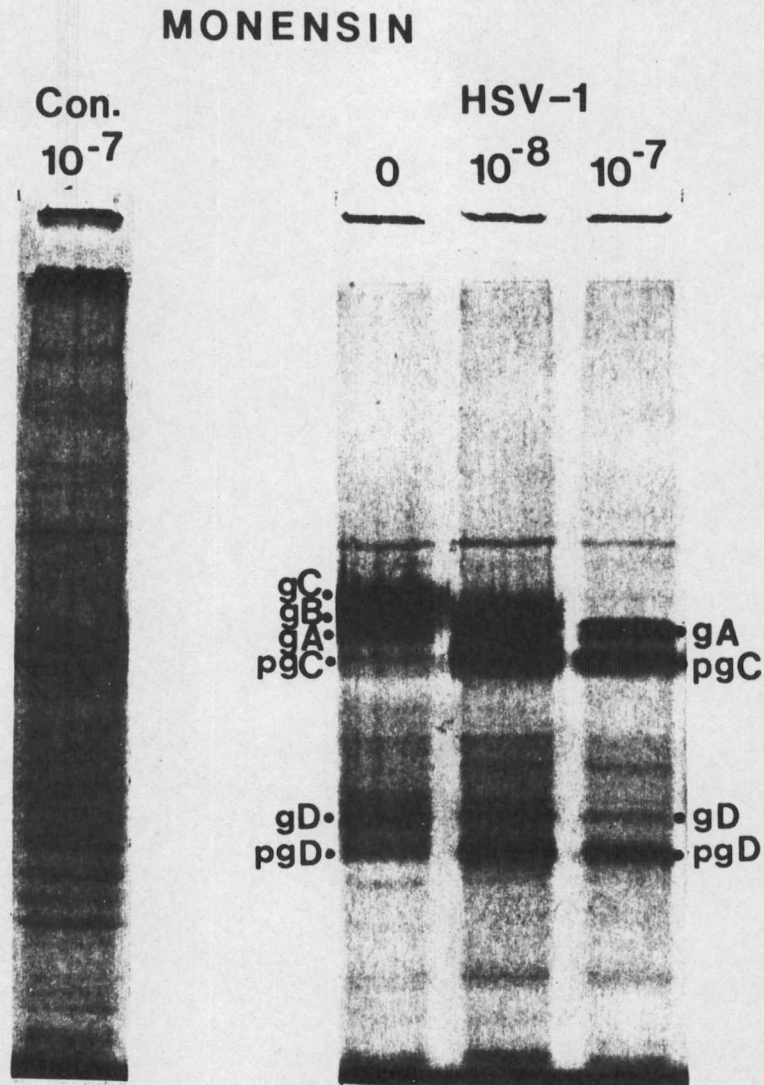


Figure 6. Effect of monensin on HSV-1 glycoprotein synthesis in L-cells. HSV-1-infected L-cells were incubated in 10^{-7} and 10^{-8} M monensin and labeled with [^{14} C]glucosamine 4-24 hr pi. Cell extracts were solubilized and analyzed by SDS-PAGE. Uninfected cells (Con, 10^{-7}) and infected cells incubated in the absence of monensin (HSV-1, 0) were included as controls.

in Figure 7. In comparison with lanes 1 and 2, only gA (110,000) was immune precipitated with anti-gAB from cells infected in the presence of either 10^{-6} M (lane 3) or 10^{-7} M (lane 5) monensin. However, this gA was sensitive to the action of endo H and was reduced in molecular weight to approximately 97,000 following endo H treatment. Results of immune precipitations using normal rabbit serum reacted with solubilized extracts of HSV-1 infected cells (lane 7), infected cells treated with 10^{-6} M (lane 8) and 10^{-7} M (lane 9) monensin were also included.

The procedure was repeated using monospecific anti-gC for immune precipitations (Figure 8). In comparison with lanes 1 and 2, there was a decrease in the amount of gC precipitated by anti-gC from cells cultured in the presence of either 10^{-6} M (lane 3) or 10^{-7} M (lane 5) monensin. In contrast, pgC₍₁₀₅₎ was immune precipitated in greater amounts than from extracts of cells incubated in monensin-free medium (lane 1). Anti-gC immune precipitates from monensin-treated cells were also incubated in the presence of endo H. In addition to the 75,000 molecular weight protein detectable after endo H treatment of pgC₍₁₀₅₎, there was a diffuse region of proteins which had a slower electrophoretic mobility.

Figure 7. Endo H treatment of anti-gAB immunoprecipitated proteins from HSV-1-infected MRC-5 cells incubated in the absence or presence of 10^{-6} M or 10^{-7} M monensin. HSV-1-infected cells were labeled with [^{35} S]methionine from 4-24 hr postinfection. Following adsorption, the cells were incubated in monensin free medium (lanes 1, 2 and 7) or medium containing 10^{-6} M monensin (lanes 3, 4 and 8) or 10^{-7} M monensin (lanes 5, 6 and 9). Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H and prepared for SDS-PAGE analysis as described in Materials and Methods. Immunoprecipitations using normal rabbit serum were also included (lanes 7-9).

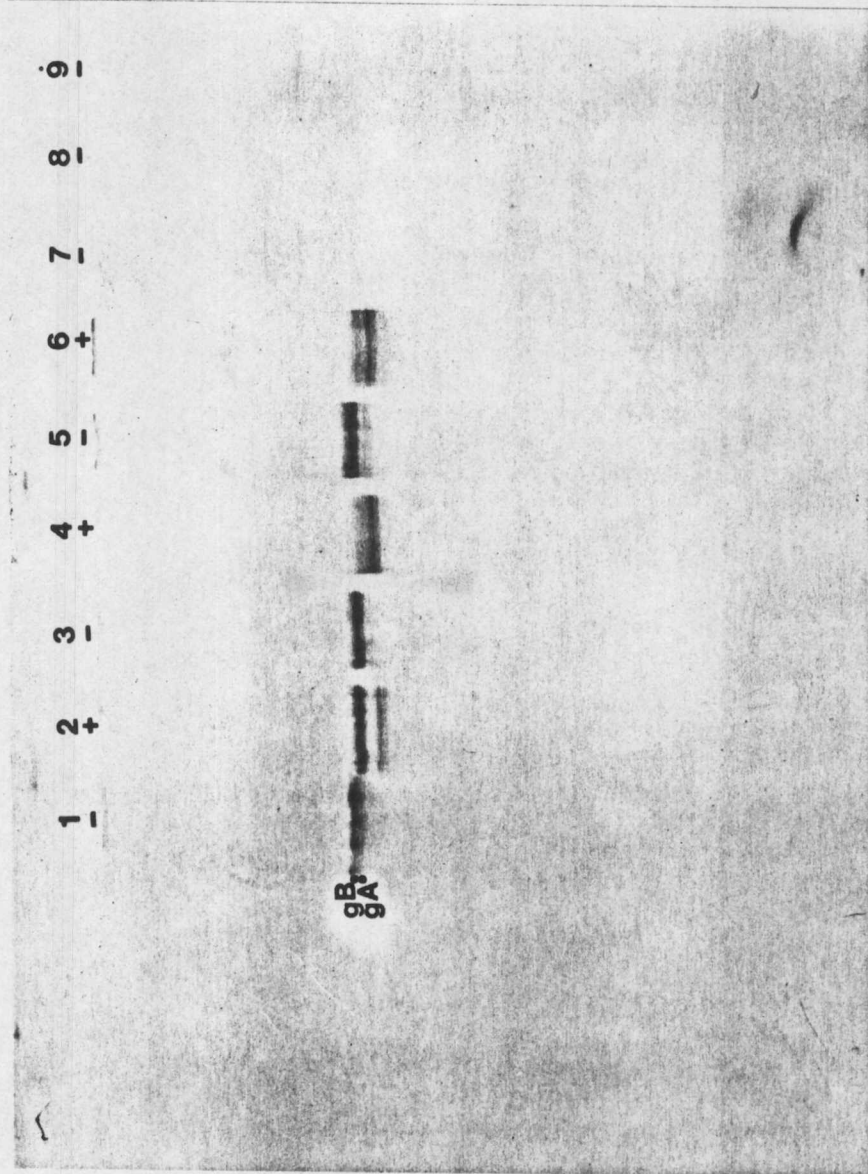


Figure 7.

Figure 8. Endo H treatment of anti-gC immune precipitated proteins from infected MRC-5 cells incubated in the absence (lanes 1 and 2) or presence of 10^{-6} M (lanes 3 and 4) or 10^{-7} M (lanes 5 and 6) monensin. The procedure was the same as described in the legend of Figure 6. Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H and prepared for SDS-PAGE analysis as described in Materials and Methods.

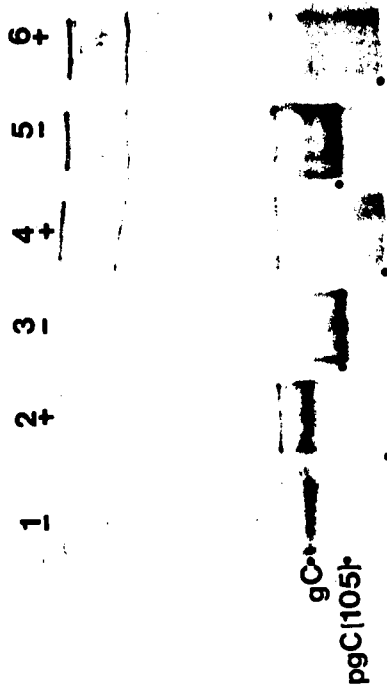


Figure 8.

IV. DISCUSSION

The purpose of this study was to further characterize the HSV-1 high molecular weight glycoproteins (gB and gC) and their precursors (gA and pgC). Based on the effect of endo H on individual glycoproteins immunoprecipitated with monospecific antisera to gC and gAB, we suggest that pgC₍₁₀₅₎ and gA represent the high-mannose precursors to gC and gB, respectively. These conclusions are in agreement with recently published data of Serafini-Cessi and Compadelli-Fiume (24). Following endo H treatment of HSV-1 infected whole cell extracts labeled with [¹⁴C]glucosamine, these authors reported the disappearance of bands corresponding to gA, pgC₍₁₀₅₎ and pgD₍₅₂₎ and the appearance of new bands. The correlation of these new bands which appeared after endo H treatment with the individual glycoproteins gB, gC or gD was not reported. Person et al. (18) have also reported that a precursor designated pgB was sensitive to endo H treatment; however, the partial sensitivity of gB was not discussed.

Based on the ability of monensin to block glycoprotein processing at the Golgi (11, 32), the accumulation of endo H sensitive gA and pgC₍₁₀₅₎ along with the inhibition of gB and gC in infected cells treated with monensin further demonstrated that gA and pgC₍₁₀₅₎ represent high mannose precursors. Since not all of the gA and pgC₍₁₀₅₎ from monensin-treated cells were equally sensitive to the action of endo H, some processing of gA and pgC₍₁₀₅₎ may have occurred in the presence of monensin. Other studies have reported that in the presence of monensin, limited removal of mannose residues does occur (11). The

removal of these carbohydrates may therefore affect the susceptibility of the oligosaccharides to endo H digestion (31).

Following endo H digestion, the [^{35}S]methionine labeled gA had an apparent molecular weight of 97,000. This molecular weight is similar to that observed for the nonglycosylated gAB synthesized in tunicamycin-treated cells (manuscript in preparation) and would suggest that gA contains only the high mannose-type oligosaccharides which are cleaved by endo H. In contrast, gB appeared to be partially sensitive to endo H and, therefore, may contain both simple and complex type oligosaccharides analogous to the mature E2 glycoprotein of Sindbis which also contains both simple and complex type oligosaccharide side chains (2, 22).

At no time did gC appear to be susceptible to the action of endo H suggesting that the N-linked oligosaccharides of gC are of the complex type. This is in agreement with Cohen et al. (3), who demonstrated the presence of sialic acid on gC but not pgC₍₁₀₅₎. Sialic acid is normally the terminal sugar of complex type oligosaccharides but is not present in simple type oligosaccharides. The disappearance of [^{35}S]methionine labeled pgC₍₁₀₅₎ after endo H digestion along with the appearance of a 75,000 molecular weight protein would suggest that the 75,000 molecular weight protein may represent the nonglycosylated gC polypeptide. This protein has a lower molecular weight than was obtained for a gC related protein isolated from tunicamycin treated-infected cells (19, Wenske and Courtney, manuscript in preparation); however, the gC protein synthesized in tunicamycin-treated cells contains O-linked oligosaccharides

(manuscript in preparation) and therefore may not represent the true nonglycosylated gC polypeptide. The presence of O-linked oligosaccharides on gC has also been suggested by Olofsson et al. (16, 17). It is conceivable that pgC₍₁₀₅₎ does not contain O-linked oligosaccharides and therefore the majority of pgC₍₁₀₅₎ is digested by endo H to a 75,000 molecular weight polypeptide. As suggested above, it also appears that pgC₍₁₀₅₎ may undergo some processing in the presence of monensin. These results could be explained in two ways, either monensin allows a partial processing of pgC₍₁₀₅₎ to gC thus producing glycoproteins with varying sensitivities to endo H or there are other oligosaccharides, other than N-linked oligosaccharides, insensitive to endo H which are present on the pgC₍₁₀₅₎ glycoproteins synthesized in the presence of monensin. Both possibilities are being considered and are under further investigation.

During the progress of our studies with monensin, we learned that D. C. Johnson and P. G. Spear (12) had obtained similar results with respect to the effects of monensin on the processing of HSV-1 glycoproteins. We recently learned that K. Kousoulas, D. Bzik and S. Person have also investigated the effect of monensin on HSV-1 glycoprotein synthesis (submitted for publication).

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PART III

GLYCOSYLATION OF HERPES SIMPLEX VIRUS TYPE 1

gC IN THE PRESENCE OF TUNICAMYCIN

I. INTRODUCTION

The carbohydrate-peptide linkages which occur between specific amino acid residues of glycoproteins and their oligosaccharide side chains may be classified into the two main types: N-glycosidic and O-glycosidic linkages. The carbohydrate-peptide linkage which occurs between N-acetylglucosamine and asparagine is of the N-glycosidic type. The anomeric carbon atom of N-acetylglucosamine is linked glycosidically via a β -linkage to the amide group of asparagine. During the synthesis of N-linked glycoproteins the high mannose oligosaccharide is first synthesized on the dolichol beginning with N-acetylglucosamine and then it is transferred en bloc to the nascent polypeptide (11, 28).

The O-glycosidic linkages include those glycoproteins with covalent linkage of N-acetylgalactosamine to serine or threonine. In this case, the anomeric carbon atom of N-acetylgalactosamine is linked glycosidically via an α -linkage to the hydroxyl group of serine or threonine. In contrast to N-glycosidic linkages, synthesis of O-glycosidically linked glycoproteins occurs by the direct sequential addition of the carbohydrates to the nascent polypeptide. The occurrence of both N-glycosidic and O-glycosidic linkages within the same glycoprotein molecule is not uncommon and has been observed in immunoglobulins (1, 2), fetuin (14, 22), erythrocyte membrane glycoproteins (6) and glomerular basement-membrane glycoproteins (26).

It is possible to use tunicamycin (24) to specifically inhibit the glycosylation of N-linked glycoproteins without inhibiting O-linked glycoproteins and thus tentatively identify glycoproteins which have

O-linked oligosaccharides (7, 10, 21). Tunicamycin is an antibiotic which inhibits the production of GlcNAc-pyrophosphoryldolichol (9), the first step in the synthesis of lipid-linked oligosaccharides (13). We have utilized tunicamycin to study its effect on the synthesis and glycosylation of the herpes simplex virus type 1 (HSV-1) glycoproteins gB and gC.

II. MATERIALS AND METHODS

Cells

Human embryonic lung (MRC-5) cells were grown in Eagle medium supplemented with 10% fetal bovine serum (FBS) and .075% sodium bicarbonate. Virus stocks were grown in MRC-5 cells and all virus titrations were conducted in Vero cell monolayers.

Virus

The KOS strain of HSV-1 and the Indiana strain of vesicular stomatitis virus (VSV) were used throughout these studies.

Radioactive Isotopes

Isotopically labeled D-[6-³H]glucosamine (sp. act. 30.3 Ci/mmole), D-[1-¹⁴C]glucosamine (sp. act. 55.5 mCi/mmol), D-[1-³H]galactose (sp. act. 8.2 Ci/mmole) and L-[³⁵S]methionine (sp. act. 1440 Ci/mmole) were purchased from Amersham, Arlington Heights, Illinois.

Inhibitors

Tunicamycin (TM) was purchased from Calbiochem, La Jolla, California. Stock solutions of 100 µg/ml were prepared by dissolving the drug in 0.01 NaOH and then diluting in Eagle medium to obtain the desired final concentration of 1.0 µg/ml for experimental purposes. Stock solutions of tunicamycin were stored at -20°C.

Infection, Labeling and Harvesting of Cells

Cells cultured in 60 or 100 mm plastic Petri dishes were infected with HSV-1 (KOS) at an input multiplicity of 10-20 pfu/cell. Following

a 1 hr adsorption at 37°C, the inoculum was removed, the monolayers were washed twice with warm phosphate buffered saline (PBS) and maintenance medium with or without 1 µg/ml of TM was added. Isotopically labeled [³H]glucosamine (20 µCi/ml), [³H]galactose (20 µCi/ml), [¹⁴C]glucosamine (5 µCi/ml) or [³⁵S]methionine (5 µCi/ml) were added at 4 hr postinfection. In experiments where cells were isotopically labeled, Eagle medium supplemented with 1x amino acids without methionine, 1x glutamine, 2% donor calf serum, and 0.225% NaHCO₃ was used. At 18 or 24 hr postinfection the cells were scraped into the medium and washed twice in PBS. The pelleted cells were resuspended in water at a concentration of 5×10^6 to 1×10^7 cells per 0.5 ml, frozen, thawed and disrupted by ultrasonication. The samples were then prepared for polyacrylamide gel electrophoresis or radioimmune precipitation as described below.

Detergent Extraction

Infected-cell extracts were prepared by treatment of infected cells with 1% sodium deoxycholate and 1% Tween-40 for 30 min. with constant agitation at 37°C. Insoluble material was removed by centrifugation at $100,000 \times g$ for 1 hr. The resulting soluble proteins (supernatant) were used as the antigen for the immunoprecipitation assays.

Discontinuous Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Details of the methods used for SDS-PAGE have been previously described (19). All slab gels presented are 8.6% acrylamide

cross-linked with 0.23% N,N'-diallyltartardimide (DATD) as described by Gibson and Roizman (8). Preparative SDS-PAGE methods were modified from those used formerly (3) in that urea was omitted from both the running buffers and gels. The appropriate fractions containing the resolved glycoproteins were pooled and concentrated using an Amicon ultrafiltration cell equipped with a PM 10 membrane.

Alkaline Hydrolysis

The purified glycoproteins obtained from preparative SDS-PAGE were subjected to alkaline hydrolysis. The samples were made 0.2 M with respect to NaOH (21) and kept at 45°C for 10 hr in the dark under nitrogen (6). Following the 10 hr incubation, samples were neutralized with 0.3 N acetic acid and 1 ml aliquots were dialyzed overnight against 10 ml of 1% SDS. Counts per minute retained in the dialysis bag or released into the dialysis buffer were determined. The percent of counts dialyzable was determined by dividing the counts dialyzed by the total number of counts remaining on the inside and outside of the dialysis tubing following dialysis.

Radioimmune Precipitation Assay

Diluent used for radioimmune precipitation (RIP) was TNE [10 mM Tris (ph 7.4), 100 mM NaCl, 1 mM EDTA], containing 0.1% Triton X-100. Samples of 25 µl solubilized cell extract, were reacted with 25 µl of rabbit antisera in a final volume of 100 µl for 1 hr at 37°C and an additional 6 hr at 4°C. Staphylococcus aureus cells (20 µl) (Pansorbin, La Jolla, Calif.) were then added and incubated for an additional 12 hr.

Immune complexes were pelleted by centrifugation at $6,000 \times g$ for 20 min. and washed three times in TNE plus 0.1% Triton X-100 and solubilized in 35 μ l of 2%-SDS, 2%-ME and analyzed by SDS-PAGE.

Antisera

Monospecific antisera to HSV-1 purified glycoproteins gAB and gC were used in this study. The purification of the glycoproteins and the characterization of the monospecific antisera prepared to the purified glycoproteins have been previously described (4, 5). Briefly, immune sera were prepared in rabbits to the gC and gAB glycoprotein components of HSV-1. Immunoprecipitation experiments demonstrated that the antisera were monospecific; anti-gC only reacted with the gC and $pgC_{(105)}$ glycoproteins and anti-gAB only reacted with glycoproteins gA and gB (4, 5).

Electrophoretic Transfer and Immunoblotting of HSV-1 Glycoproteins

HSV-1 proteins were separated by SDS-PAGE as described above. After electrophoresis the gels were prepared for transfer of the proteins to nitrocellulose (NC) paper (BA85, 0.45 m, Schleicher and Schuell, Keene, New Hampshire). Prior to the transfer, Whatman 3 mm paper and the NC paper were presoaked for 1 hr in transfer buffer consisting of 0.025 M Tris, 0.019 M glycine, and 20% methanol. The protocol followed was modified from Towbin et al. (27).

After SDS-PAGE the stacking gel was cut away and the remaining gel was pulled off the glass with a piece of dry Whatmann 3 mm paper slightly larger than the gel. The gel and paper were then set on top

(gel side up) of the immunoblotting pad which was partially submerged in transfer buffer. A rubber roller was used to remove air bubbles and to soak the underlying paper with buffer. The NC paper was then placed on top of the gel and again the air bubbles were rolled out. Two more pieces of Whatman paper were placed on top of the NC paper and all the air bubbles were removed from the "sandwich." The holder was then clamped together to securely hold the Whatmann paper, gel, NC paper and pad in place. The holder was placed into the buffer chamber so that the proteins would be electrophoresed towards the cathode unto the NC paper. The chamber was then filled with transfer buffer. Electrophoresis was performed with cooling for 2 hr at 100 V. After 2 hr the electrophoresis was stopped and the NC strips with immobilized HSV-1 proteins were removed and stored in transfer buffer for up to two days. In order to bind any remaining protein sites on the NC paper, the paper was incubated at 37°C for 1 hr in Tris-NaCl buffer composed of 0.01 M Tris, HCl (ph 7.4), 0.015 M NaCl and containing 3.0% bovine serum albumin. At the end of the hour the strips were washed with glass-distilled water and then incubated with monospecific rabbit anti-gC or anti-gAB sera. The antisera were diluted 1:50 in NETG buffer [0.05 Tris-HCl (pH 7.4), 0.15 M NaCl, 5 mM EDTA, 0.25% gelatin (wt/vol)] containing 0.05% NP40. The binding of the antiserum was carried out at 37°C for 2 hr with constant rocking. The strips were then washed with glass-distilled water and further incubated with ^{125}I -protein A. A volume of 10 ml at 10^6 cpm/ml of ^{125}I -protein A was used per NC blot. The ^{125}I -protein A was diluted into NETG buffer containing 0.05% NP40. After a 1 hr

incubation, the strips were washed 20 times (10 ml each wash) with NETG buffer containing 0.1% SDS and 0.5% Triton X-100 followed by final washes with glass-distilled water to remove the NETG buffer plus detergent. The blots were then air dried. The electrophoretic transfer of the HSV-1 proteins to the NC paper was performed using a TE Series Transphor electrophoresis unit with a Transphor power-lid, model TE 50 (Hoefer Scientific Instruments, San Francisco, Calif.).

III. RESULTS

Inhibition of gB and gC by TM

The effect of TM on the synthesis of HSV-1 glycoproteins was examined by analysis of the proteins synthesized in infected cell cultures maintained in the presence or absence of 1 µg/ml TM. HSV-1 infected MRC-5 cells were isotopically labeled with [³⁵S]methionine 4-18 hr postinfection and the whole cell fraction harvested at 18 hr postinfection. Cell extracts were then analyzed on SDS-PAGE (Figure 1, lanes 6 and 7). Little effect on viral polypeptide synthesis was observed in TM treated cells except for the appearance of a new 92,000 molecular weight polypeptide(s) and the concomittant disappearance of the high molecular weight glycoproteins.

The [³⁵S]methionine isotopically-labeled cell extracts were immunoprecipitated with monospecific anti-gAB and anti-gC sera in order to identify the polypeptides in the 92,000 molecular weight region. The immunoprecipitates were washed, solubilized and then analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included in the immunoprecipitation assays as a control. The PAGE profiles of these samples are shown in Figure 1. In the absence of TM the glycoprotein gB migrated to a position in the gel corresponding to an apparent molecular weight of 120,000 (Figure 1, lane 1) and the fully glycosylated gC migrated to a position in the gel corresponding to an apparent molecular weight of 130,000 (Figure 1, lane 3).

Immunoprecipitates from HSV-1 infected cells maintained in the presence of TM are also presented in Figure 1. Anti-gAB did not

Figure 1. Identification of HSV-1 [^{35} S]methionine-labeled 92,000 molecular weight proteins synthesized in the presence of tunicamycin. HSV-1-infected MRC-5 cells maintained in the absence (-) or presence (+) of tunicamycin (1 μ g/ml) were labeled with [^{35} S]methionine (2 μ Ci/ml) from 4-18 hr postinfection. At 18 hr postinfection the cells were harvested, solubilized and HSV-1 glycoproteins were immunoprecipitated from cell extracts with monospecific antisera, anti-gAB and anti-gC. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control.

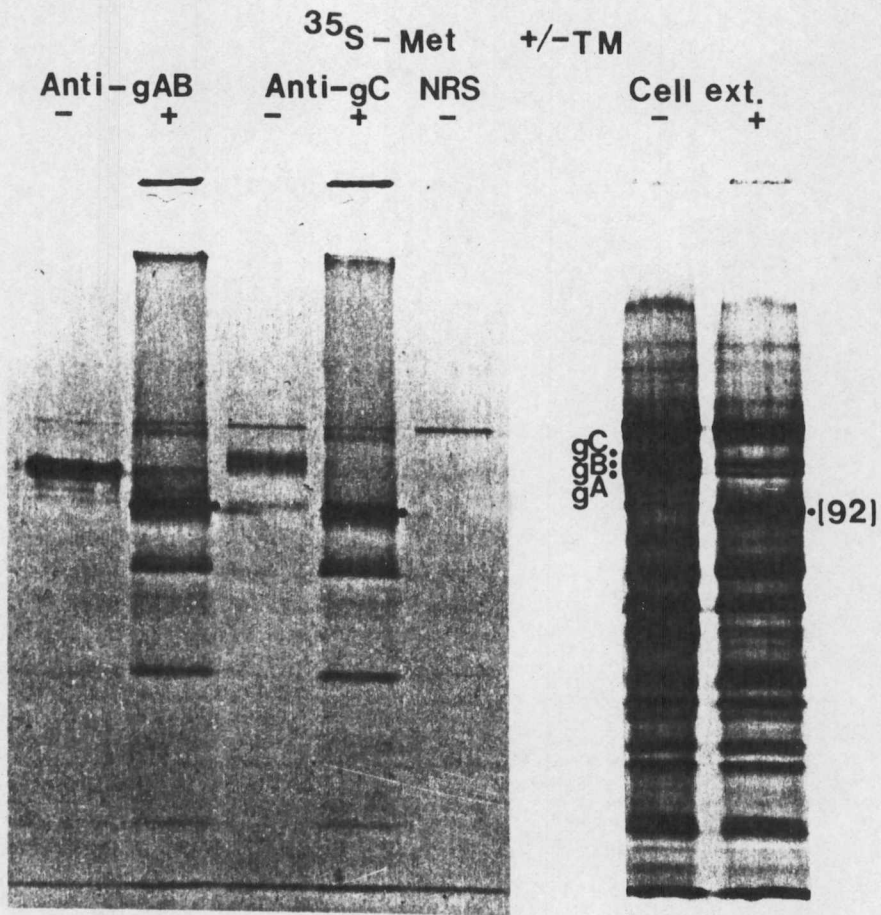


Figure 1.

immunoprecipitate significant amounts of gB from TM-treated cells; however, a protein with an apparent molecular weight of 92,000 was detected (Figure 1, lane 2). Tunicamycin also inhibited the synthesis of gC and a protein with an apparent molecular weight of 92,000 was also immunoprecipitated with anti-gC (Figure 1, lane 4). The [^{35} S]methionine-labeled proteins which migrated with a greater electrophoretic mobility than the 92,000 molecular weight proteins were detectable in the immunoprecipitates with the monospecific antisera and may be due to degradation of the 92,000 molecular weight proteins (Figure 1, lanes 2 and 4).

Effect of TM on Glycosylation of gB and gC

To determine the effect of tunicamycin on the glycosylation of gB and gC, immunoprecipitates of TM-treated infected cell extracts isotopically labeled with [^{14} C]glucosamine (5 μ Ci/ml) or [^{3} H]galactose (20 μ Ci/ml) were analyzed by SDS-PAGE. The PAGE profiles of [^{14} C]glucosamine labeled cell extracts from infected cells cultured in the presence or absence of tunicamycin are shown in Figure 2. In the absence of TM the high molecular weight glycoproteins were synthesized (Figure 2, lane 8). In contrast, HSV-1 infected cells cultured in the presence of TM exhibited a significant inhibition of isotopically-labeled proteins; however, a [^{14} C]glucosamine-labeled protein was resolved in the 92,000 molecular weight region (Figure 2, lane 7).

To determine whether the [^{14} C]glucosamine labeled protein (92,000 MW) was related to gB or gC, anti-gAB and anti-gC were used and

Figure 2. Effect of tunicamycin on the glycosylation of HSV-1 glycoproteins. HSV-1 infected MRC-5 cells maintained in the absence (-) or presence (+) of tunicamycin (1 μ g/ml) were labeled with [^{14} C]glucosamine (5 μ Ci/ml) 4-24 hr postinfection. Glucosamine-labeled proteins were immunoprecipitated from solubilized cell extracts using monospecific antisera, anti-gAB and anti-gC. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control.

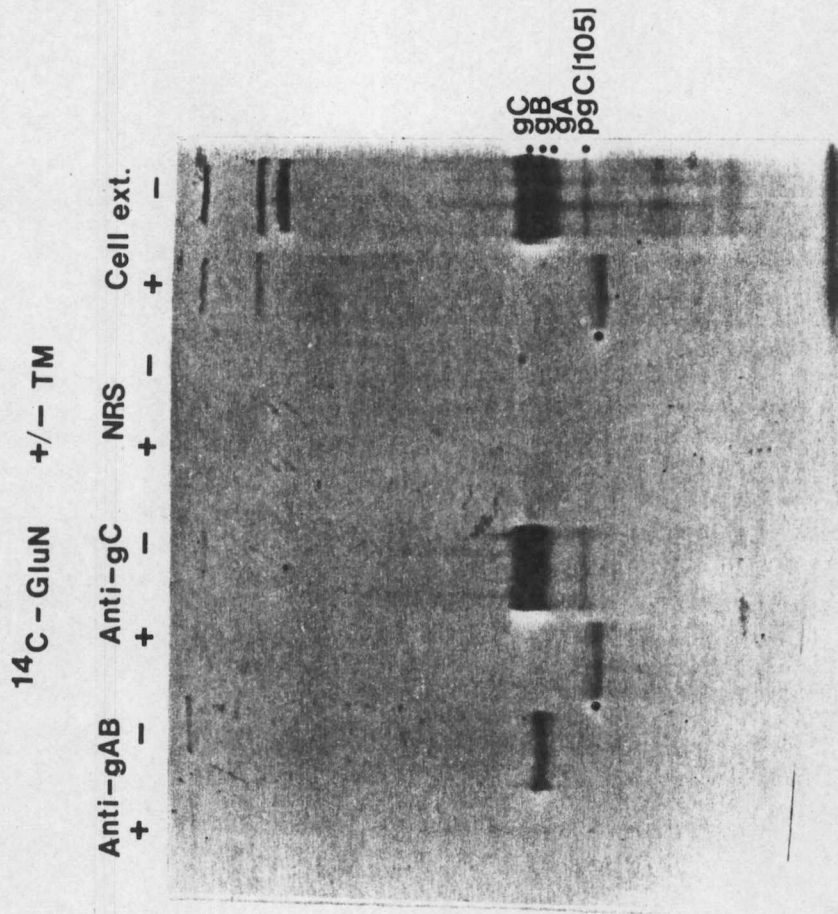


Figure 2.

the resulting immunoprecipitates analyzed (Figure 2). The monospecific anti-gC immunoprecipitated the fully glycosylated gC (MW 130,000) and the precursor pgC₍₁₀₅₎ (MW 105,000) in the absence of TM (Figure 2, lane 4). In TM-treated cell extracts the anti-gC immunoprecipitated the 92,000 molecular weight protein (Figure 2, lane 3). The glycoproteins gA and gB were immunoprecipitated by anti-gAB from cells infected in the absence of TM (Figure 2, lane 2). In contrast to anti-gC, anti-gAB did not immunoprecipitate detectable glucosamine-labeled proteins from TM-treated cell extracts (Figure 2, lane 1).

The initial carbohydrate added to threonine or serine during the synthesis of O-linked oligosaccharides is N-acetylgalactosamine (2). Since TM does not inhibit the synthesis of O-linked oligosaccharides the experiment was repeated to determine whether the 92,000 molecular weight protein could also be isotopically labeled with galactose (Figure 3). Only anti-gC immunoprecipitated a 92,000 molecular weight protein from TM-treated cells infected with HSV-1. Anti-gAB did not immunoprecipitate detectable [³H]galactose-labeled proteins from TM-treated cell extracts (data not shown). Also, cells infected with HSV-1 and treated with TM could not be isotopically labeled with [³H]mannose (data not shown).

Immune Reactivity of Immobilized HSV-1
Proteins Synthesized in the Presence
or Absence of TM

The inhibition by TM on the synthesis of HSV-1 glycoproteins was further examined by analyzing the immune reactivity of immobilized HSV-1 proteins. Unlabeled HSV-1-infected MRC-5 cells were harvested at times

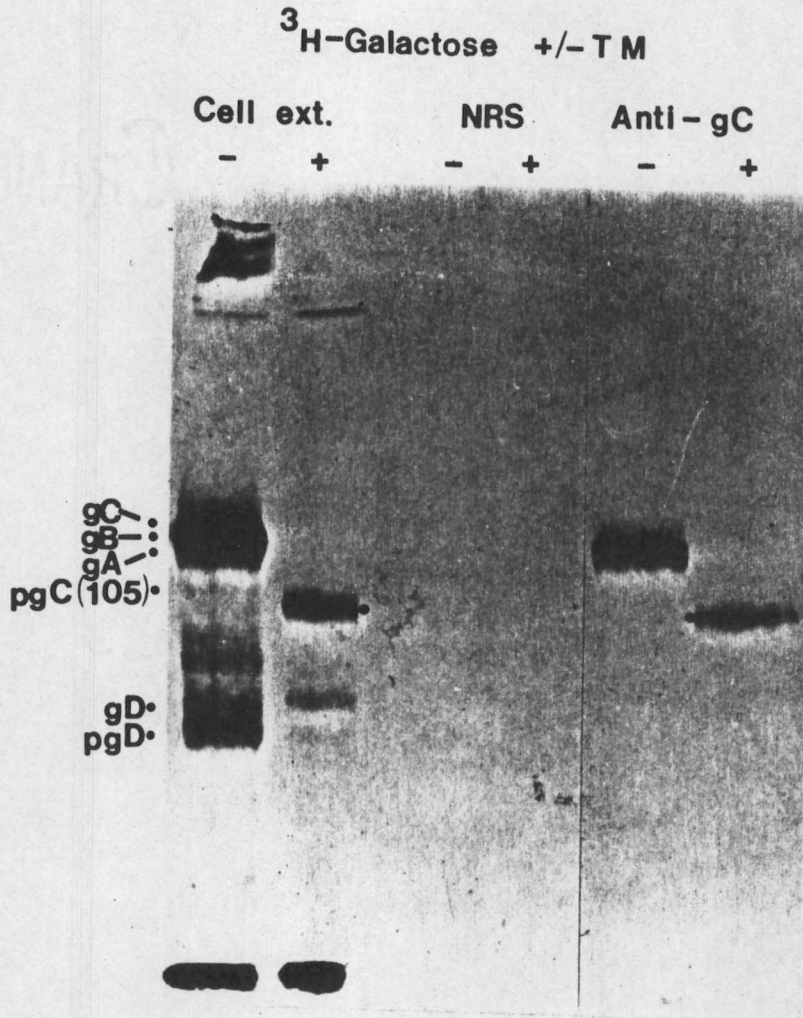


Figure 3. Immunoprecipitation of the [³H]galactose-labeled gC glycoprotein synthesized in the presence of tunicamycin. HSV-1 infected MRC-5 cells maintained in the absence (-) or presence (+) of tunicamycin (1 µg/ml) were labeled with [³H]galactose (20 µCi/ml) from 4-24 hr postinfection. Galactose-labeled proteins were immunoprecipitated from solubilized cell extracts using the monospecific antiserum, anti-gC. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control.

postinfections as indicated in Figure 4. The cells had been incubated in the presence or absence of TM (1 μ g/ml) from 1 hr postinfection to time of harvest.

Cell extracts were prepared for SDS-PAGE as described in Materials and Methods. After SDS-PAGE the proteins were transferred and immobilized on NC paper and then reacted with monospecific antisera. The immune reactivity of immobilized HSV-1 glycoproteins to anti-gAB serum is shown in Figure 4. The transferred gA and gB synthesized in the absence of TM were able to bind the anti-gAB serum. In contrast, the anti-gAB serum bound to proteins with an average molecular weight of 92,000 synthesized in TM-treated-infected cells. Cells infected in the presence of TM were then harvested at 5, 6, 7, 10, and 24 hr postinfection and tested for their ability to bind anti-gAB serum to immobilized proteins. At 6 hr postinfection only one band in the 92,000 molecular weight region was detected by anti-gAB serum. By 7 hr postinfection two bands were detected. The exact relationship of these two bands is not known. It is possible that the second band may represent a cleavage product of the higher molecular weight protein band. The band at approximately 120,000 molecular weight probably represents residual gB left-over from incoming virus particles or gB synthesized before glycosylation was completely shut off by TM.

The ability of anti-gC serum to bind to immobilized HSV-1 proteins synthesized in the presence or absence of TM is shown in Figure 5. Both pgC₍₁₀₅₎ and gC were able to bind the anti-gC serum. In contrast to the analysis of immunoprecipitates by SDS-PAGE, anti-gC bound to an immobilized 75,000 molecular weight protein synthesized in the presence

Figure 4. Binding of monospecific anti-gAB serum to immobilized proteins synthesized in the presence of tunicamycin. HSV-1-infected cells incubated in the presence (+TM) or absence (-TM) of tunicamycin (1 μ g/ml) were harvested at indicated times postinfection. Solubilized cell extracts were subjected to SDS-PAGE. The separated proteins were subsequently transferred from the gel to nitrocellulose paper by electrophoretic blotting and reacted with anti-gAB serum as described in the text.

Anti-gAB +/-TM

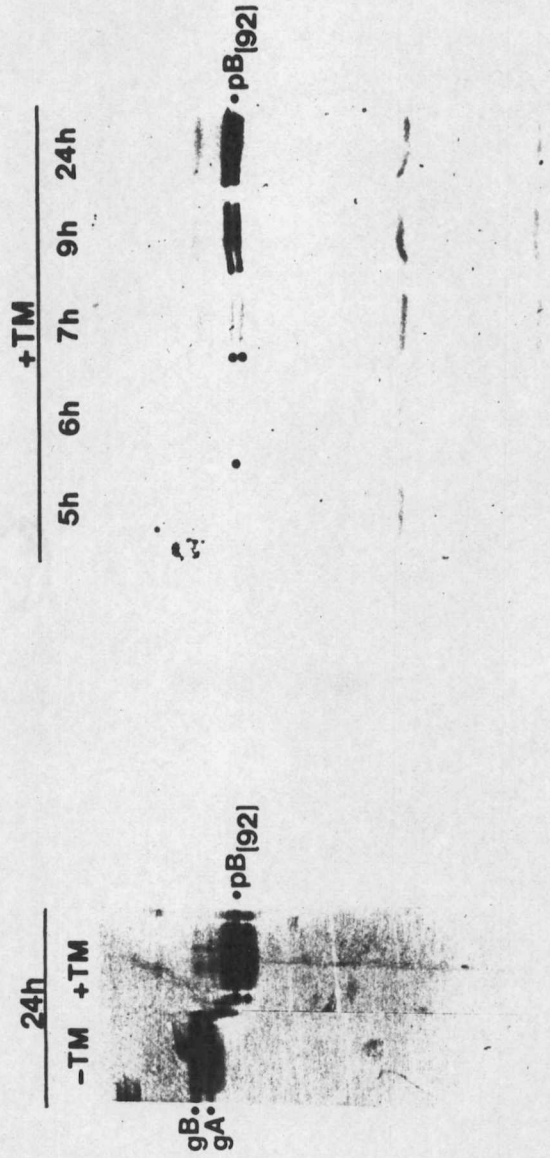


Figure 4.

Figure 5. Binding of monospecific anti-gC serum to immobilized proteins synthesized in the presence of tunicamycin. HSV-1-infected cells incubated in the presence (+TM) or absence (-TM) of tunicamycin (1 μ g/ml) were harvested at indicated times postinfection. Solubilized cell extracts were subjected to SDS-PAGE. The separated proteins were subsequently transferred from the gel to nitrocellulose paper by electrophoretic blotting and reacted with anti-gC serum as described in the text.

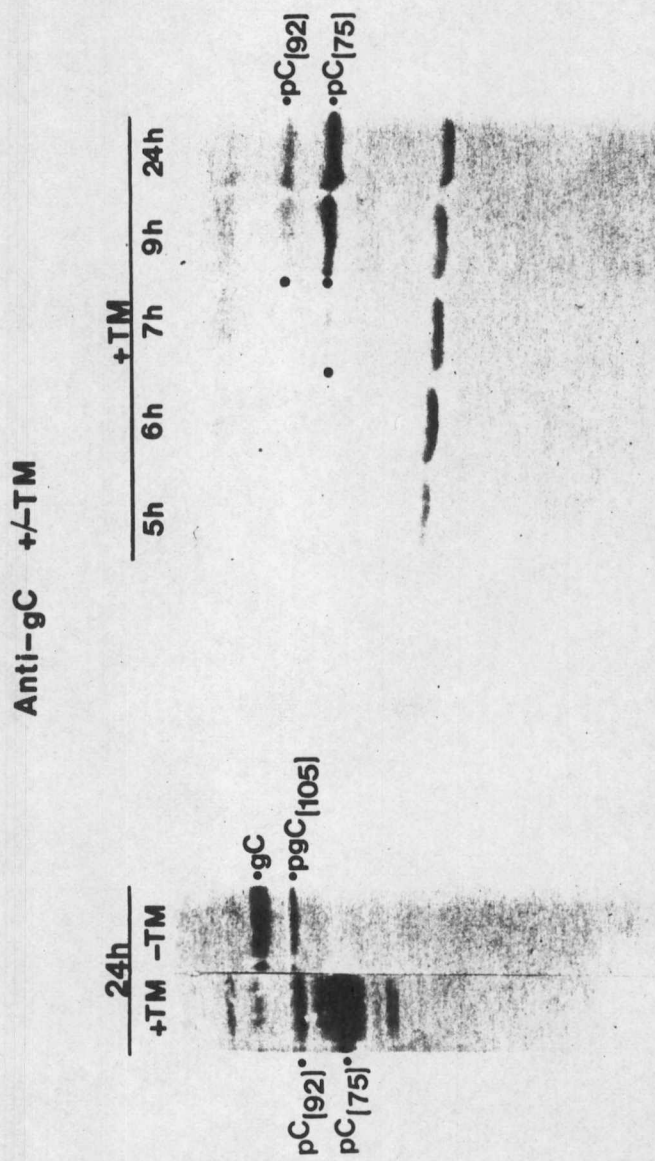


Figure 5.

of TM as well as to the protein in the 92,000 molecular region (Figure 5, lane 1). The binding of anti-gC serum to the 130,000 molecular weight region is probably due to residual gC from incoming virus particles or gC synthesized before glycosylation was completely shut off by TM.

Extracts of TM-treated-infected cells harvested at times post-infection indicated in Figure 5 (lanes 3-5) were tested for their ability to bind anti-gC serum. At 7 hr postinfection the 75,000 molecular weight protein was detected (Figure 5, lane 5). The lower molecular weight material is assumed to represent nonspecific binding since it was detected at all times postinfection in treated and untreated cultures (data not shown). At 9 hr and 24 hr postinfection both the 75,000 and 92,000 molecular weight proteins were detected. The 75,000 molecular weight protein probably represents the nonglycosylated form of gC and the 92,000 molecular weight protein represents the gC with only O-glycosidically linked oligosaccharides. The fact that the 92,000 molecular weight protein appears after the 75,000 molecular weight protein suggests that the 75,000 molecular weight protein is synthesized first and then glycosylated to a molecular weight of 92,000.

Purification of [³H]Glucosamine-Labeled 92,000 Molecular Weight Protein

In order to further characterize the gC synthesized in the presence of tunicamycin, the [³H]glucosamine labeled protein was purified by preparative PAGE. MRC-5 cells were infected with HSV-1 and labeled with

[³H]glucosamine from 4-24 hr postinfection. At 24 hr postinfection, the whole cell fraction was harvested and electrophoresed on preparative SDS-PAGE (Figure 6). The fractions (numbers 35-47) eluted from the preparative PAGE containing the glycosylated protein were pooled, concentrated, dialyzed, and further examined by SDS-PAGE (Figure 7). The pooled fraction (lane 3) migrated at the same position as the [³H]glucosamine labeled protein observed in TM-treated cell extracts and immunoprecipitated from TM-treated cell extracts by anti-gC. The purified protein was only immunoprecipitated by anti-gC and not by anti-gAB, anti-gD or anti-gF sera. The anti-gF serum was prepared to a recently identified glycoprotein which migrates at a slightly lower migration rate than gD (58,000 MW) when analyzed by SDS-PAGE. Studies are currently in progress to confirm that this glycoprotein is not related to gD or gE.

Alkaline Hydrolysis

O-glycosidically linked oligosaccharides are sensitive to mild alkaline hydrolysis whereas N-glycosidically linked oligosaccharides are resistant to alkaline hydrolysis (23). Since tunicamycin inhibits N-glycosidic linkages but not O-glycosidic linkages, glycoproteins synthesized in the presence of TM should be sensitive to mild alkaline hydrolysis. Purified HSV-1 glycoproteins synthesized in the presence or absence of tunicamycin were tested for their sensitivity to mild alkaline hydrolysis. The VSV G glycoprotein was included as a control since it is known to contain only N-linked oligosaccharides (20).

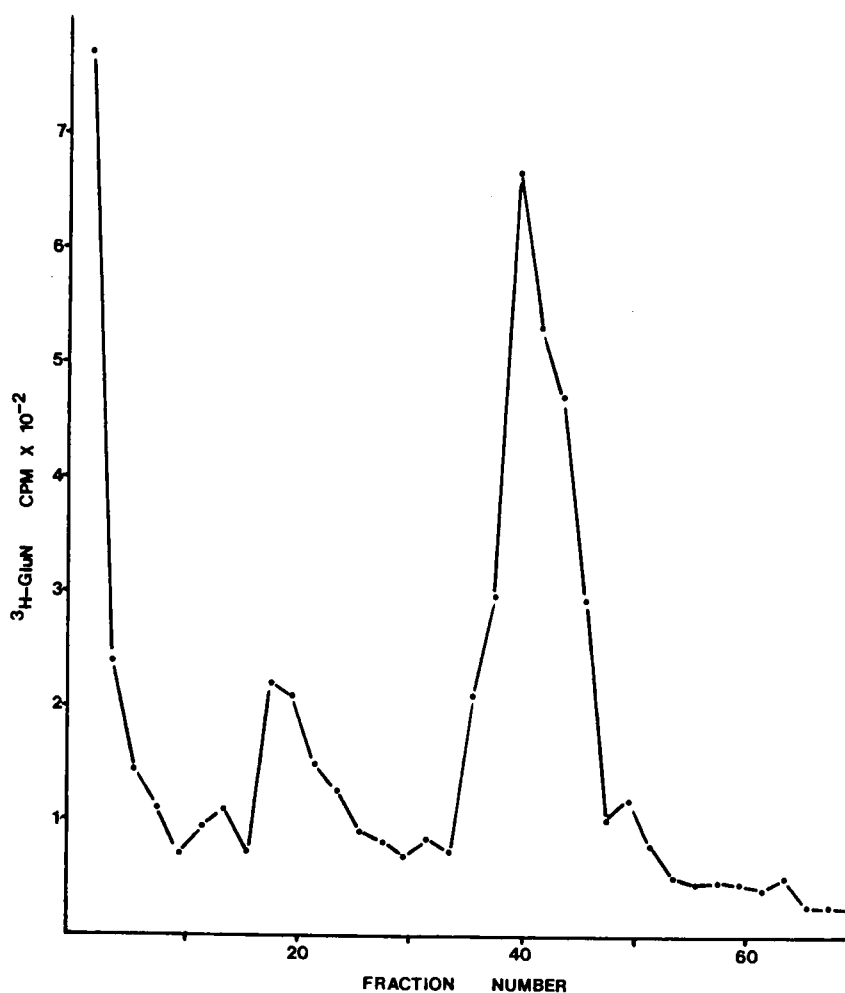


Figure 6. Preparative PAGE profile of [^3H]glucosamine-labeled HSV-1 glycoproteins synthesized in the presence of tunicamycin (1 $\mu\text{g/ml}$). Approximately 2×10^8 HSV-1 infected MRC-5 cells were labeled with [^3H]glucosamine from 4-24 hr postinfection. Labeled cell extracts were then electrophoresed on preparative SDS-PAGE and 100 μl aliquots of alternate fractions were assayed for radioactivity.

Figure 7. Immunoprecipitation of [^3H]glucosamine-labeled HSV-1 glycoprotein synthesized in the presence of tunicamycin and purified by preparative SDS-PAGE. Cell extracts of infected cells cultured in the absence (-TM) or presence (+TM) of tunicamycin and isotopically labeled with [^3H]glucosamine are included in lanes 1 and 2. The glycoprotein purified by preparative SDS-PAGE (fractions 35-47, Figure 6) was analyzed by SDS-PAGE (lane 3). Antisera specific for gC, gF, gD and gAB glycoproteins were tested for their ability to immunoprecipitate the purified ^3H -glucosamine-labeled glycoprotein (lanes 4-7). Normal rabbit serum (NRS) was included as a control.

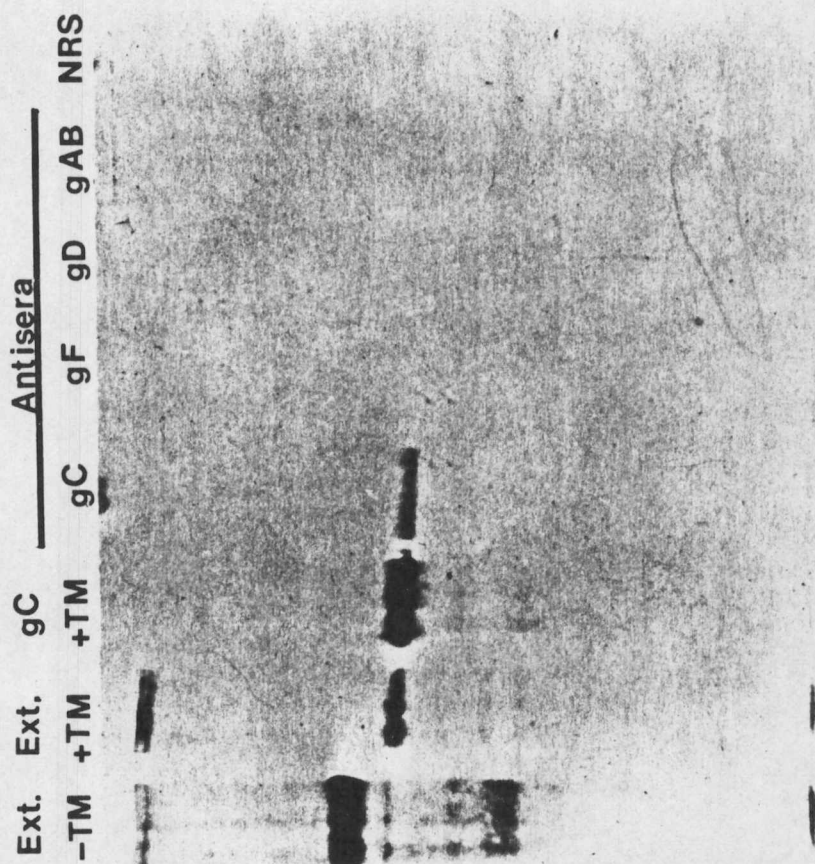


Figure 7.

Purified glycoproteins isotopically labeled with [^3H]glucosamine were subjected to mild alkaline hydrolysis (0.2 N NaOH) for 10 hr at 45°C. The samples were then neutralized and dialyzed against 1% SDS and the percent of counts dialyzable was determined. Exposure of purified gAB to mild alkaline hydrolysis resulted in 13.1% of the total [^3H]glucosamine counts being lost into the dialyzable phase. This result was similar to the 10.3% of the [^3H]glucosamine counts dialyzable following alkaline hydrolysis of the purified VSV G glycoprotein which contains only N-glycosidic linkages (20). In contrast, exposure of purified gC (130,000 MW) to mild alkaline hydrolysis resulted in 32.4% of the total [^3H]glucosamine counts being lost in the dialyzable phase, and gC (92,000 MW), synthesized in the presence of TM, lost approximately 66.2% of the [^3H]glucosamine label. These data further suggest that the HSV-1 gC contains O-glycosidically linked oligosaccharides and the HSV-1 gAB contains oligosaccharides which are only N-glycosidically linked (Table 1).

Table 1. Solubilization of Carbohydrate Moiety of Virus Glycoproteins by Alkali

Glycoprotein ^a	Treatment ^b	cpm non-dialyzable ^c	cpm dialyzable ^d	% dialyzable ^e cpm/total cpm
HSV-1 gC (synthesized in the presence of tunicamycin)	NaOH	5,401	10,500	66.2
	H ₂ O	10,575	900	7.8
HSV-1 gC (synthesized in the absence of tunicamycin)	NaOH	15,449	7,400	32.4
	H ₂ O	18,730	750	3.8
HSV-1 gAB (synthesized in the absence of tunicamycin)	NaOH	15,195	2,300	13.1
	H ₂ O	23,215	850	3.5
VSV G protein (synthesized in the absence of tunicamycin)	NaOH	19,129	2,200	10.3
	H ₂ O	20,075	550	2.7

^a[³H]glucosamine-labeled glycoproteins were purified by preparative PAGE.

^bPurified glycoproteins were treated with 0.2 M NaOH for 10 hr at 45°C.

^cCounts per minute retained in the dialysis bag.

^dCounts per minute released into the dialysis buffer.

^e
$$\frac{\text{cpm dialyzable}}{(\text{cpm nondialyzable}) + (\text{cpm dialyzable})} \times 100\%$$

IV. DISCUSSION

In this study we were able to demonstrate that HSV-1 glycoproteins normally found in infected MRC-5 cells are replaced in TM-treated cells by lower molecular weight polypeptides which are antigenically related to the glycosylated proteins and that a protein related to gC could be partially glycosylated with either [^3H]glucosamine or [^3H]galactose in TM-treated cells infected with HSV-1. Other investigators have also reported the appearance of lower molecular weight HSV-1 proteins synthesized in the presence of TM which are antigenically related to the normal HSV-1 glycoproteins (17, 18). Katz et al. reported the appearance of HSV-1 low molecular weight polypeptides synthesized in the presence of TM, however, the relationship between these low molecular weight polypeptides and the fully glycosylated HSV-1 proteins was not discussed (12).

The data presented here indicate that the HSV-1 gC contains O-linked oligosaccharides. This is based on (1) the ability for anti-gC to immune precipitate a [^3H]glucosamine or [^3H]galactose labeled protein from TM-treated infected cells, (2) the inability to label gB or gC with [^3H]mannose in the presence of TM (data not shown) and (3) the sensitivity of gC to mild alkaline hydrolysis. Olofsson et al. (15) have suggested that the HSV-1 glycoprotein gC contains O-linked oligosaccharides based on lectin binding studies. Recently they have also presented data demonstrating the sensitivity of HSV-1-specified glycoproteins to dilute alkali at low temperature (16). The partial inhibition of glycosylation by TM of gC indicates that gC also contains

N-linked oligosaccharides. Similar data has been reported with the vaccinia virus-induced hemagglutinin antigen which contains both N- and O-linked oligosaccharides (21). Holmes et al. (10) have recently shown that the E₁ glycoprotein of mouse hepatitis virus, a coronavirus, contains only O-linked oligosaccharides.

The [³⁵S]methionine labeled protein immunoprecipitated by anti-gC from TM-treated infected cells has an approximate molecular weight of 92,000. Our data suggest that this does not represent the molecular weight of the nonglycosylated gC polypeptide since the 92,000 molecular weight protein can also be isotopically labeled with [³H]glucosamine or [³H]galactose. Digestion of pgC₍₁₀₅₎ by the endoglycosidase H (endo H), resulted in a digestion product of approximately 75,000, which may represent the molecular weight of the nonglycosylated form of gC (discussed in Part II). Since endo H specifically cleaves only simple type oligosaccharides (25), it is conceivable that pgC₍₁₀₅₎ does not contain O-linked oligosaccharides and therefore the majority of pgC₍₁₀₅₎ is digested by endo H to a 75,000 molecular weight polypeptide rather than a 92,000 molecular weight polypeptide. These results suggest that the high mannose oligosaccharides are added first to the growing gC polypeptide to yield pgC₍₁₀₅₎ and then the O-linked oligosaccharides are added to pgC₍₁₀₅₎ while the N-linked high mannose oligosaccharides are being processed to complex oligosaccharides.

A viral specific 75,000 molecular weight protein synthesized in the presence of tunicamycin was detected by the immunoblotting technique. This protein was not detected in immunoprecipitates using anti-gC serum

against tunicamycin-treated-infected cell extracts. It is possible that the protein is not solubilized under the conditions used and is therefore removed with the cellular debris during preparation of cell extracts for radioimmune precipitation analysis. The detection of the 75,000 molecular protein in tunicamycin-treated infected cells agrees with the endo H experiments suggesting that the nonglycosylated form of gC has an approximate molecular weight of 75,000.

Our data also indicate that gC may not be the only HSV-1 glycoprotein containing O-linked oligosaccharides. A lower molecular weight [³H]glucosamine labeled protein has also been purified from TM-treated infected cells. This protein did not react significantly with anti-gAB, gC, or gD but it was immunoprecipitated with an antiserum made against a glycoprotein migrating at a slightly slower migration rate than gD on an SDS-polyacrylamide gel. Studies are currently in progress to determine the identity of this tunicamycin enhanced, partially glycosylated polypeptide.

In contrast to gC, our results indicate that gA and gB contain only N-linked oligosaccharides. The glycosylation of gA and gB was inhibited by tunicamycin. No proteins labeled with [³H]glucosamine, [³H]galactose or [³H]mannose could be immunoprecipitated by anti-gAB from infected cells cultured in the presence of TM. In addition, the purified gAB was resistant to alkaline hydrolysis. We therefore suggest that the 92,000 molecular weight gAB related protein may represent the nonglycosylated polypeptide form of gAB.

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PART IV

INCORPORATION OF D-MANNOSE INTO HERPES SIMPLEX VIRUS
TYPE 1 GLYCOPROTEINS gB and gC SYNTHESIZED IN THE
PRESENCE OF 2-DEOXY-D-GLUCOSE

I. INTRODUCTION

One approach to the study of viral glycoproteins is the use of glycosylation inhibitors to perturb the normal sequence of events involved in the synthesis of viral specific glycoproteins. Such an approach may result in an accumulation and therefore an identification of nonglycosylated and partially glycosylated intermediates within the virus-infected cell, and in a temporal manner allow the elucidation of the steps involved during the glycosylation of viral-induced proteins. A disruption in the addition of oligosaccharide side chains to virus-induced proteins may also reveal a necessary and specific role for the complex carbohydrate moieties of virus specific glycoproteins during viral morphogenesis. The drug, 2-deoxy-D-glucose (deoxyglucose), an analog of glucose and mannose, has been employed in the present study as a tool to induce perturbations in the normal glycosylation process, and in this fashion allow an investigation and comparison of glycoprotein synthesis in herpes simplex virus type 1 (HSV-1) infected cells. The action of this sugar analog on the inhibition of several RNA enveloped viruses has been reported (12, 13, 14, 24, 25). Kaluza et al. (12) presented evidence that deoxyglucose is incorporated into the glycoproteins as an analog of mannose, thereby terminating further elongation of the oligosaccharide chain.

Previously, it has been reported that the apparent molecular weights of HSV-1-induced glycosylated proteins are altered to lower molecular weight components when synthesized in the presence of deoxyglucose (1, 2, 3, 4, 15, 19). Other studies (1) have demonstrated

that in the presence of increasing concentrations of deoxyglucose, there was a progressive decrease in the amount of [^{14}C]amino acids incorporated into polypeptides which normally band in the gA, gB, gC region. Concomitant with this decrease was an increase in [^{14}C]amino acids incorporated into a polypeptide(s) of greater electrophoretic mobility with a lower molecular weight of approximately 92,000 (1).

The possibility that the 92,000 molecular weight proteins may represent analogs of the major HSV-1 glycoproteins is considered in this report. By the use of specific antisera to gAB and gC we have been able to demonstrate an antigenic relatedness between the 92,000 molecular weight polypeptides synthesized in the presence of deoxy-D-glucose and the fully glycosylated major glycoproteins. We also report on the increased incorporation of mannose into HSV-1 glycoproteins synthesized in deoxyglucose-treated cells. The mannose-labeled glycoproteins synthesized in the presence of deoxyglucose showed antigenic relatedness to the major glycoproteins gB and gC.

II. MATERIALS AND METHODS

Cells

Human embryonic lung (MRC-5) cells were grown in Eagle medium supplemented with 10% fetal bovine serum (FBS) and 0.075% sodium bicarbonate. Virus stocks were grown in MRC-5 cells and all virus titrations were conducted in Vero cell monolayers.

Virus

The KOS and MP (9, 10) strains of HSV-1 were used throughout these studies.

Radioactive Isotopes

Isotopically labeled D-[6-³H]glucosamine (sp. act. 30.3 Ci/mmole), D-[1-³H]mannose (sp. act. 10.4 Ci/mmole) and L-[³⁵S]methionine (sp. act. 1440 Ci/mmole) were purchased from Amersham, Arlington Heights, Illinois.

Inhibitors

2-Deoxy-D-glucose (DG) was purchased from Calbiochem, La Jolla, California. Stock solutions of 1.0% (61 mM) DG were prepared by dissolving the DG in Eagle medium to obtain the desired final concentration of 0.1% (6.1 mM) for experimental purposes. The DG was made fresh prior to use.

Infection, Labeling and Harvesting of Cells

Cells cultured in 60 or 100 mm plastic petri dishes were infected with HSV-1 (KOS) at an input multiplicity of 10-20 pfu/cell. Following

a 1 hr adsorption at 37°C, the inoculum was removed, the monolayers were washed twice with warm phosphate buffered saline (PBS) and maintenance medium with or without 0.1% DG was added. Isotopically labeled [³H]glucosamine (20 µCi/ml), [³H]mannose (20 µCi/ml) or [³⁵S]methionine (5 µCi/ml) were added at 4 hr postinfection. In experiments where cells were isotopically labeled, Eagle medium supplemented with 1x amino acids without methionine, 1x glutamine, 2% donor calf serum, and 0.075% NaHCO₃ was used. At various times postinfection the cells were scraped into the medium and washed twice in PBS. The pelleted cells were resuspended in water at a concentration of 5×10^6 to 1×10^7 cells per 0.5 ml, frozen, thawed and disrupted by ultrasonication. The samples were then prepared for polyacrylamide gel electrophoresis, endo H digestion or radioimmune precipitation as described below.

Detergent Extraction

Infected-cell extracts were prepared by treatment of infected cells with 1% sodium deoxycholate and 1% Tween-40 for 30 min. with constant agitation at 37°C. Insoluble material was removed by centrifugation at $100,000 \times g$ for 1 hr. The resulting soluble proteins (supernatant) were used as the antigen for the immunoprecipitation assays.

Discontinuous Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Details of the methods used for SDS-PAGE have been previously described (21). All slab gels presented are 8.6% acrylamide cross-linked with 0.23% N,N'-diallyltartardimide (DATD) as described by Gibson

and Roizman (7) and urea was omitted from both the running buffers and gels.

Radio Immunoprecipitation Assay

Diluent used for radioimmune precipitation (RIP) was TNE [10 mM Tris (pH 7.4), 100 mM NaCl, 1 mM EDTA], containing 0.1% Triton X-100. Samples of 25 μ l solubilized cell extract were reacted with 25 μ l of rabbit antisera in a final volume of 100 μ l for 1 hr at 37°C and an additional 6 hr at 4°C. Staphylococcus aureus cells (20 μ l) (Pansorbin, La Jolla, Calif.) were then added and incubated for an additional 12 hr. Immune complexes were pelleted by centrifugation at 6,000 $\times$ g for 20 min. and washed three times in TNE plus 0.1% Triton X-100 and solubilized in 35 μ l of 2%-SDS, 2%-mercaptoethanol (ME) and analyzed by SDS-PAGE.

Antisera

Monospecific antisera to HSV-1 purified glycoproteins gAB and gC were used in this study. The purification of the glycoproteins and the characterization of the monospecific antisera prepared to the purified glycoproteins have been previously described (5, 6). Briefly, immune sera were prepared in rabbits to the gC and gAB glycoprotein components of HSV-1. Immunoprecipitation experiments demonstrated that the antisera were monospecific, anti-gC only reacted with the gC and pGC₍₁₀₅₎ glycoproteins and anti-gAB only reacted with glycoproteins gA and gB (5, 6).

Acid Precipitations and Protein Determinations

For acid precipitations, aliquots of 0.01 ml of each sample were spotted in duplicate on GF/C Whatman filter paper, dried 15 min. at 150°C, and washed twice at 4°C with 5% trichloroacetic acid for a total of 30 min. This was followed by three 10-min. washes at 4°C with 95% ethanol. After drying at 150°C, the 2.5-cm squares of filter paper containing acid-insoluble material were placed in vials containing 5 ml of scintillation fluid and the radioactivity was counted on a Beckman LS-250 scintillation counter.

Protein determinations were carried out in triplicate using the procedure developed by Lowry et al. (18). Briefly, 15 or 25 µl of sample were diluted in 0.2 ml of water and mixed with 1 ml of a solution prepared from 2% Na₂CO₃ in 0.1 N NaOH, 2% K₂C₄H₄O₆, and 1% CuSO₄ in the ratio 10:1:1. Following a 10-min. incubation at room temperature, the diluted samples were mixed with 0.1 ml of 1 N Folin-Ciocalteu reagent (Fisher Scientific Co., Fair Lawn, New Jersey) and incubated an additional 30 min. at room temperature. The absorbance of visible light at 750 nm of each sample was then compared with an appropriate blank and established using a Gilford spectrophotometer, Model 240 (Gilford Instruments Lab., Oberlin, Ohio). The protein concentration for each sample was determined from a standard curve of known concentrations of bovine serum albumin.

Endo H Digestion of Crude Cell Lysates

Crude cell lysates (10⁶ cells/ml) to be digested with endo H were made 0.2% with respect to SDS and boiled for 5 min. After boiling,

100 μ l samples were made 50 mM with respect to sodium citrate buffer (pH 5.5). Endo H (6 μ l of .5 IU/ml) was added to samples for endo H treatment, and the appropriate volume of water was added to control samples. After incubation at 37°C for 6 hr the samples were precipitated with 1 ml of cold acetone and centrifuged at 13,000 $\times$ g for 8 min., using a Fisher model 235A microfuge. The pellet was resuspended in 35 μ l of 2.0% SDS-2.0% mercaptoethanol and stored at -20°C until analyzed by SDS-PAGE.

Endo H Digestion of Immunoprecipitated Glycoproteins

Glycoproteins immunoprecipitated with the monospecific antisera were eluted from the staphylococcal cells by incubating the samples in 0.8% SDS for 20 min. at 37°C followed by boiling for 30 sec. The staphylococcal cells were removed by centrifugation. Samples to be treated with and without endo H were diluted to 0.2% SDS and made 50 mM with respect to sodium citrate buffer (pH 5.5). Endo H (6 μ l of .5 IU/ml) was added to samples for endo H treatment, and the appropriate volume of water was added to the control samples. After incubation for 6 hr at 37°C the samples were treated as described for endo H digestion of crude cell lysates.

Immunofluorescence

1. Adsorption of fluorescein-conjugated goat anti-rabbit gamma globulin. Goat anti-rabbit gamma globulin (GAR) conjugated with fluorescein isothiocyanate (FITC) was purchased from Hyland, Division of Tarvenal Laboratories, Inc., Costa Mesa, California. Prior to usage in

the indirect immunofluorescent test, 1 ml of GAR-FITC was mixed with 0.1 gm of rabbit liver powder, incubated for 1 hr at 37°C, and centrifuged at $100,000 \times g$ for 15 min. in a type 65 rotor at 4°C. The resulting supernatant was then incubated with approximately 8×10^8 MRC-5 cells for 1 hr at 37°C, incubated overnight at 4°C, and again centrifuged at $100,000 \times g$ for 15 min. in a type 65 rotor at 4°C. The adsorbed GAR-FITC supernatant was diluted 1:2 with PBS and stored in 0.1 ml aliquots at -70°C. Immediately prior to use, an aliquot of GAR-FITC was thawed, diluted 1:8 with PBS and kept at 4°C.

2. Immunofluorescence test. The indirect immunofluorescence test described by Porter et al. (20) was employed with minor modifications. MRC-5 cells were cultured in 60 mm plastic petri dishes containing seven coverslips per dish for approximately 25 hr at subconfluency, the cells were mock infected or infected with virus. At appropriate times postinfection coverslips were subjected to three consecutive 5-min. washes in 0.85% saline, air dried for 30 min., fixed in acetone for 5 min. at room temperature, and air dried. Before staining, the cells were rehydrated for 5 min. in PBS. Cells were immediately incubated with either preimmune or immune rabbit sera directed against HSV-1-infected RK13 cells (diluted 1:8 with PBS) and the glycoproteins gA/gB and gC of the HSV-1 (5). Following a 30 min. incubation at room temperature, the coverslips were subjected to three consecutive 5 min. washes in PBS and then reacted for 30 min. at room temperature with 25 μ l of adsorbed GAR-FITC, washed three times for 5 min. each in PBS, once in distilled water, air dried, and mounted in 1:2 glycerol.

Coverslips were examined using a Zeiss microscope with a mercury light source.

III. RESULTS

Incorporation of [^3H]Monosaccharides

It has been reported previously that although deoxyglucose does not alter the incorporation of [^{14}C] amino acids into HSV-1 virus-specific proteins, the incorporation of [^3H]glucosamine is depressed by approximately 50% (5). To extend the knowledge of the effect of this sugar analog on the overall glycosylation process, the incorporation of isotopically-labeled mannose as well as isotopically-labeled glucosamine into virus-specific acid insoluble material was compared in the presence or absence of 0.1% (6.1 mM) deoxyglucose. Cultures of MRC-5 cells were infected with HSV-1 and labeled with either [^3H]mannose (10 $\mu\text{Ci/ml}$), or [^3H]glucosamine 10 $\mu\text{Ci/ml}$ from 4 to 24 hr postinfection. Results are summarized in Table 1. In close agreement with the previously reported effect of the sugar analog (3), deoxyglucose depressed the incorporation of [^3H]glucosamine by 52%. However, the incorporation of [^3H]mannose was enhanced significantly in the presence of the deoxysugar; deoxyglucose-treated HSV-1-infected cells exhibited an approximately 2.3-fold increase in [^3H]mannose incorporation when compared with untreated virus infected controls (Table 1). Since [$2\text{-}^3\text{H}$]mannose was used during this study the possibility of intracellular randomization of this isotopically labeled sugar to yield structurally related metabolites can be ruled out (25).

The incorporation of mannose into HSV-1 specific glycoproteins was examined by analyzing on SDS-PAGE infected cell extracts isotopically labeled with [^3H]mannose. Infected cells were maintained in the

Table 1. Effect of 2-Deoxy-D-Glucose on the Incorporation of [^{35}S]Methionine and [^3H]Monosaccharides into HSV-1-Infected Cells at 24 Hr Postinfection*

Isotopic Label	Control	2-Deoxy-D-Glucose	
	CPM/ μg protein	CPM/ μg protein	% Control
[^{35}S]methionine	1313.7	1357.2	103
[^3H]glucosamine	470.4	246.1	52
[^3H]mannose	232.6	542.3	233

*MRC-5 cells cultured in 60 mm plastic Petri dishes were infected with HSV-1 at an input multiplicity of 20 pfu/cell. Following adsorption for 1 hr at 37°C, the cultures were washed twice with PBS and either control medium or medium containing 0.1% deoxyglucose was added (3 ml/plate). All cultures were labeled 4-24 hr postinfection with either [^{35}S]methionine (2-3 $\mu\text{Ci/ml}$), [^3H]glucosamine (10 $\mu\text{Ci/ml}$), or [^3H]mannose (10 $\mu\text{Ci/ml}$), and harvested at 24 hr postinfection as described under Materials and Methods.

presence or absence of 0.1% DG for 1-24 hr postinfection and labeled with [^3H]mannose 4-24 hr postinfection. In the absence of DG the high molecular weight HSV-1 glycoproteins were synthesized normally and could be labeled with [^3H]mannose (Figure 1, lane 2). In contrast, DG inhibited the appearance of the high molecular weight glycoproteins; however, glycosylation still occurred in the presence of DG as evidenced by the incorporation of mannose into faster migrating glycoproteins (Figure 1, lane 1).

To determine whether the [^3H]mannose was being incorporated into gB or gC analogs synthesized in the presence of DG, immune precipitates of DG-treated-infected cell extracts isotopically labeled with [^3H]mannose (25 $\mu\text{Ci/ml}$) were analyzed by SDS-PAGE. The immune precipitations by anti-gAB of gA and gB synthesized in the absence of DG are shown in Figure 2, lane 2. Both gB and its high mannose precursor gA were immune precipitated from solubilized cell extracts of infected cells maintained in DG-free medium. In contrast, the gA and fully glycosylated gB glycoproteins were not synthesized in the presence of DG, instead, anti-gAB immune precipitated faster migrating glycoproteins (Figure 2, lane 1).

In the absence of DG the fully glycosylated gC and its precursor $\text{pgC}_{(105)}$ migrated at positions of 130,000 and 105,000 molecular weight, respectively (Figure 2, lane 4). Anti-gC did not immune precipitate gC (130,000 MW) or $\text{pgC}_{(105)}$ from DG-treated cells; however, a diffuse [^3H]mannose band (average MW, 105,000) was observed by SDS-PAGE of immune precipitations using anti-gC serum against DG-treated cell extracts (Figure 2, lane 3).

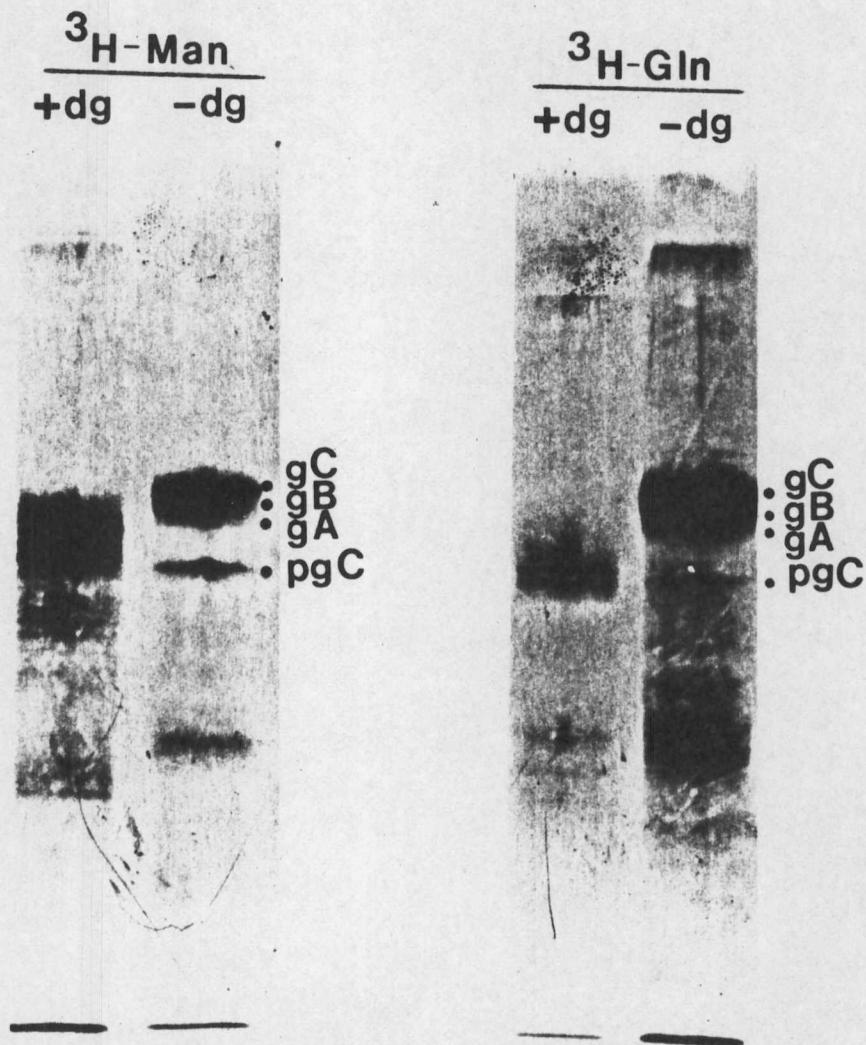


Figure 1. Effect of deoxyglucose on the synthesis of HSV-1-induced glycoproteins. HSV-1-infected MRC-5 cells maintained in the absence ($-dg$) or presence ($+dg$) of deoxyglucose (0.1%) were labeled with [^3H]mannose (20 $\mu\text{Ci/ml}$) or [^3H]glucosamine (20 $\mu\text{Ci/ml}$) from 4-24 hr postinfection. At 24 hr postinfection the cells were harvested and solubilized cell extracts were analyzed by SDS-PAGE.

Figure 2. Immune precipitation of [³H]mannose-labeled HSV-1 gAB and gC glycoproteins synthesized in the presence of 0.1% deoxyglucose. HSV-1-infected MRC-5 cells maintained in the absence (-dg) or presence (+dg) of deoxyglucose were labeled with [³H]mannose (20 µCi/ml) from 4-24 hr postinfection. Mannose-labeled proteins were immunoprecipitated from solubilized cell extracts using the monospecific anti-gAB and anti-gC sera. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control.

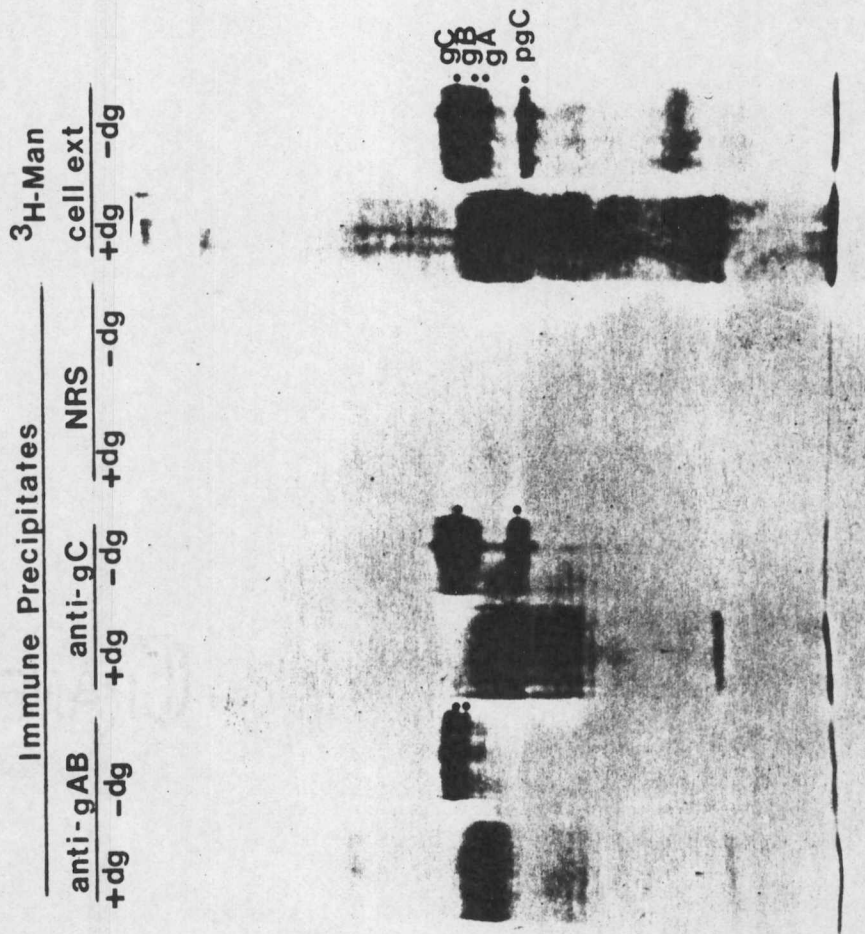


Figure 2.

These results suggest that deoxyglucose is causing an inhibition of the processing of the high mannose oligosaccharide side chains. To test whether deoxyglucose did induce the accumulation of high mannose HSV-1 glycoproteins, the effect of endo H (11, 28, 29, 30) on whole cell extracts of infected cells was determined. HSV-1-infected MRC-5 cells incubated in the presence or absence of 0.1% DG were isotopically labeled with [^3H]mannose from 4-18 hr postinfection and the whole cell fraction was harvested 18 hr postinfection. The cell fractions were solubilized in 0.2% SDS prior to endo H treatment.

After endo H treatment the samples were analyzed by SDS-PAGE (Figure 3). The glycoproteins from solubilized extracts of cells infected in the absence of deoxyglucose contained endo H sensitive and resistant oligosaccharides (11). The endo H digested sample shown in Figure 3, lane 2, was missing mannose-labeled glycoproteins gA and pgC₍₁₀₅₎ as compared to the untreated sample in lane 1. Mannose-labeled glycoproteins synthesized in the presence of deoxyglucose were also sensitive to endo H activity (Figure 3, lanes 3 and 4). In the presence of deoxyglucose the majority of the incorporated isotopically-labeled mannose was associated with HSV-1 glycoproteins having an average molecular weight of 105,000. Glycoproteins migrating in the 105,000 molecular weight region and in the lower molecular weight diffuse region contained oligosaccharides sensitive to endo H digestion (Figure 3, lane 4). Glycoproteins synthesized in the presence of deoxyglucose also contained oligosaccharides resistant to endo H digestion.

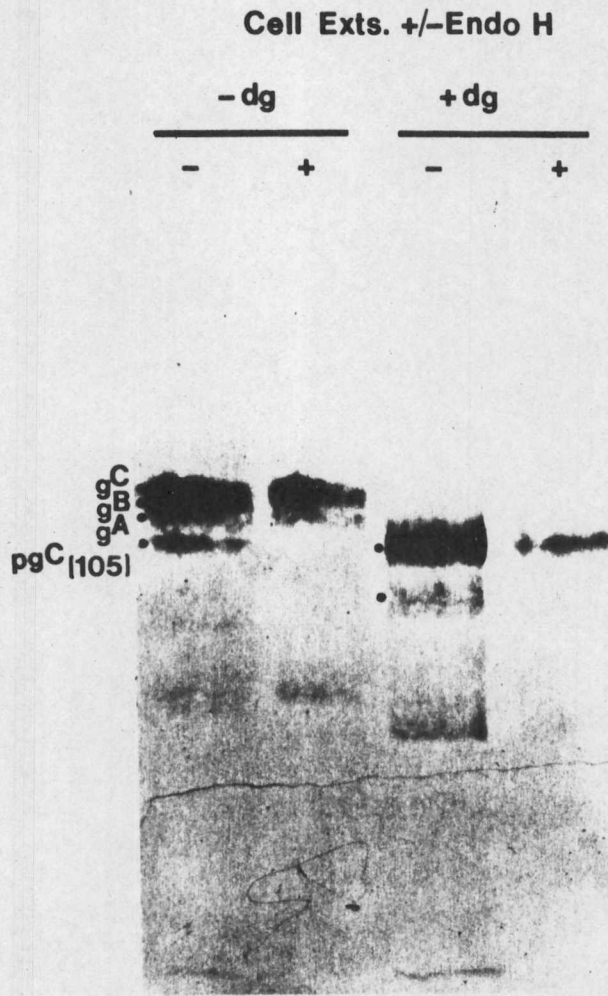


Figure 3. Endo H treatment of solubilized HSV-1-infected cell extracts. HSV-1-infected MRC-5 cells maintained in the absence (-dg) and presence (+dg) of deoxyglucose (0.1%) were labeled with [^{3} H]mannose (20 μ Ci/ml) from 4-18 hr postinfection and harvested at 18 hr post-infection. Solubilized cell extracts were incubated in the absence (-) or presence (+) of endo H and prepared for SDS-PAGE electrophoresis as described in the text.

Previously we demonstrated that gA, gB and pgC₍₁₀₅₎ but not gC contained high mannose oligosaccharide side chains. To further identify the glycoproteins synthesized in the presence of DG which were sensitive to endo H digestion, immune precipitates from DG-treated infected-cell extracts were incubated in the presence or absence of endo H. The isotopically labeled glycoproteins were immunoprecipitated with monospecific anti-gAB or anti-gC sera, and the immunoprecipitated glycoproteins were then subjected to digestion with endo H. The effect of endo H on anti-gAB immunoprecipitated glycoproteins synthesized in the presence or absence of DG is shown in Figure 4. As reported previously both gA and gB are digested by endo H, and only gB is detectable in a digested immunoprecipitate sample labeled with [³H]mannose (Figure 4, lanes 1 and 2). The results shown in Figure 4 (lane 4) also indicate that some of the mannose labeled gB (or gA) synthesized in the presence of DG do contain high mannose oligosaccharide side chains sensitive to endo H digestion. The mannose labeled glycoproteins remaining in lane 4 may represent underglycosylated gB which contains fewer complex oligosaccharide side chains than the normal 120,000 molecular weight gB.

The glycoproteins immunoprecipitated with anti-gC were also tested for their sensitivity to endo H digestion. In agreement with earlier work, the pgC₍₁₀₅₎ was sensitive to endo H digestion while the molecular weight of the fully glycosylated gC was not altered after endo H treatment (Figure 4, lanes 5 and 6). The effect of endo H digestion on glycoproteins synthesized in the presence of DG and immunoprecipitated

Figure 4. Endo H treatment of anti-gAB and anti-gC immunoprecipitated proteins from HSV-1-infected MRC-5 cells incubated in the absence (-dg) or presence (+dg) of deoxyglucose (0.1%). HSV-1-infected cells were labeled with [³H]mannose (20 μ Ci/ml) from 4-18 hr postinfection. At 18 hr postinfection the cells were harvested, solubilized and HSV-1 glycoproteins were immunoprecipitated from cell extracts with monospecific antisera, anti-gAB and anti-gC. Immunoprecipitates were solubilized, incubated in the presence (+) or absence (-) of endo H, and prepared for SDS-PAGE analysis as described in the text.

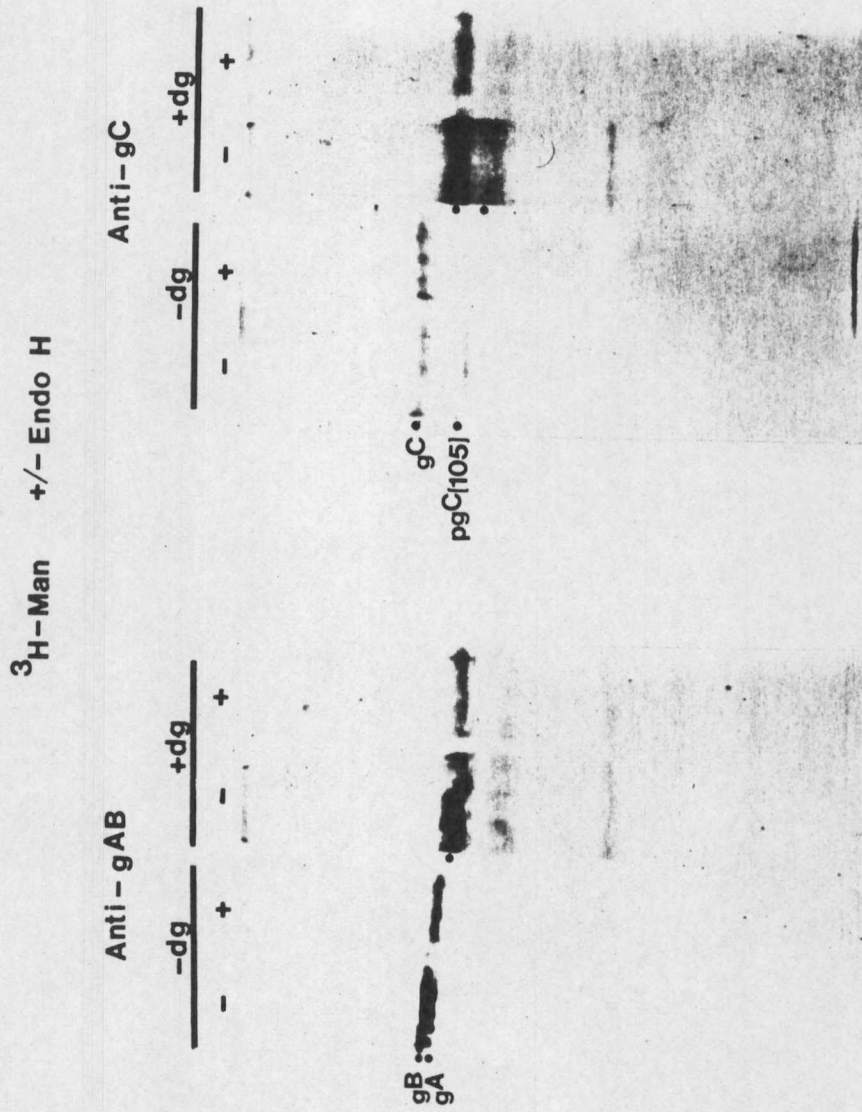


Figure 4.

by anti-gC serum is shown in Figure 4 (lanes 5-8). A mannose-labeled glycoprotein migrating with an approximately molecular weight of 105,000 appeared to be resistant to digestion with endo H while mannose-labeled glycoproteins migrating at a faster rate and comprising a diffuse region with an average molecular weight of less than 105,000 were sensitive to endo H (Figure 4, lane 8). It is possible that the diffuse region represents underglycosylated pgC₍₁₀₅₎ molecules which contain varying amounts of high mannose side chains. The 105,000 gC synthesized in the presence of DG and partially sensitive to endo H may represent an aberrant form of gC which contains complex-type oligosaccharides, only to a lesser extent than the normal 130,000 molecular weight gC.

The effect of deoxyglucose on the glycosylation of gB and gC was further examined by analyzing immunoprecipitates of deoxyglucose-treated cell extracts isotopically labeled with [³H]glucosamine (20 μ Ci/ml). The SDS-PAGE profiles of [³H]glucosamine-labeled cell extracts from infected cells cultured in the presence or absence of deoxyglucose (0.1%) are shown in Figure 1, p. 146. In the absence of deoxyglucose the high molecular weight glycoproteins were synthesized normally (Figure 1, lane 4). In contrast, HSV-1-infected cells cultured in the presence of deoxyglucose exhibited a significant inhibition of [³H]glucosamine incorporation; however, a [³H]glucosamine-labeled protein was resolved in the 92,000 molecular weight region (Figure 1, lane 3).

To determine whether the [³H]glucosamine-labeled protein (MW 92,000) was related to gB or gC, anti-gAB and anti-gC were used and

the resulting immunoprecipitates analyzed (Figure 5). The monospecific anti-gC immunoprecipitated the fully glycosylated gC (MW 130,000) and the precursor pgC₍₁₀₅₎ (MW 105,000) in the absence of deoxyglucose (Figure 5, lane 4). In deoxyglucose-treated cell extracts the anti-gC immunoprecipitated the 92,000 molecular weight proteins (Figure 5, lane 3).

The glycoproteins gA and gB were immunoprecipitated by anti-gAB from cells infected in the absence of deoxyglucose (Figure 5, lane 2). In contrast to anti-gC, anti-gAB serum did not immunoprecipitate detectable glucosamine-labeled proteins from deoxyglucose-treated cells (Figure 5, lane 1).

Radioimmune Precipitation of [³⁵S]Methionine-Labeled Glycoproteins

In previous studies (1, 4) it was shown that 0.1% DG inhibited the synthesis of the high molecular weight glycoproteins with the concomitant appearance of an amino acid labeled 92,000 molecular weight protein. We have extended these earlier results by identifying the amino acid labeled gB and gC synthesized in the presence of DG.

Cell extracts of MRC-5 cells infected with HSV-1 and maintained in the absence or presence of 0.1% DG from 1-24 hr postinfection were analyzed by SDS-PAGE. The infected cultures were isotopically labeled with [³⁵S]methionine from 4-24 hr postinfection. In agreement with earlier studies, a 92,000 molecular weight region was observed by SDS-PAGE of DG-treated-infected cells labeled with [³⁵S]methionine (Figure 6).

Figure 5. Immunoprecipitation of the [^3H]glucosamine-labeled gAB and gC glycoproteins synthesized in the presence of deoxyglucose. HSV-1-infected MRC-5 cells maintained in the absence (-dg) or presence (+dg) of deoxyglucose were labeled with [^3H]glucosamine (20 $\mu\text{Ci/ml}$) from 4-18 hr postinfection. Glucosamine-labeled proteins were immunoprecipitated from solubilized cell extracts using the monospecific anti-gAB and anti-gC sera. Immunoprecipitates and cell extracts were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control.

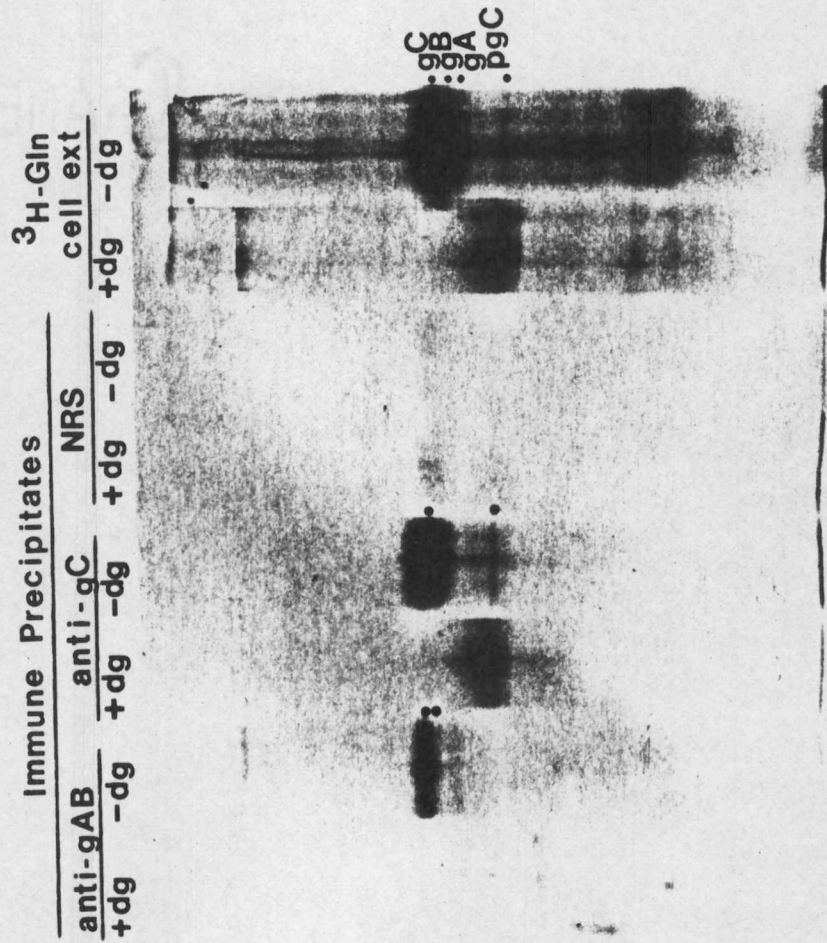


Figure 5.

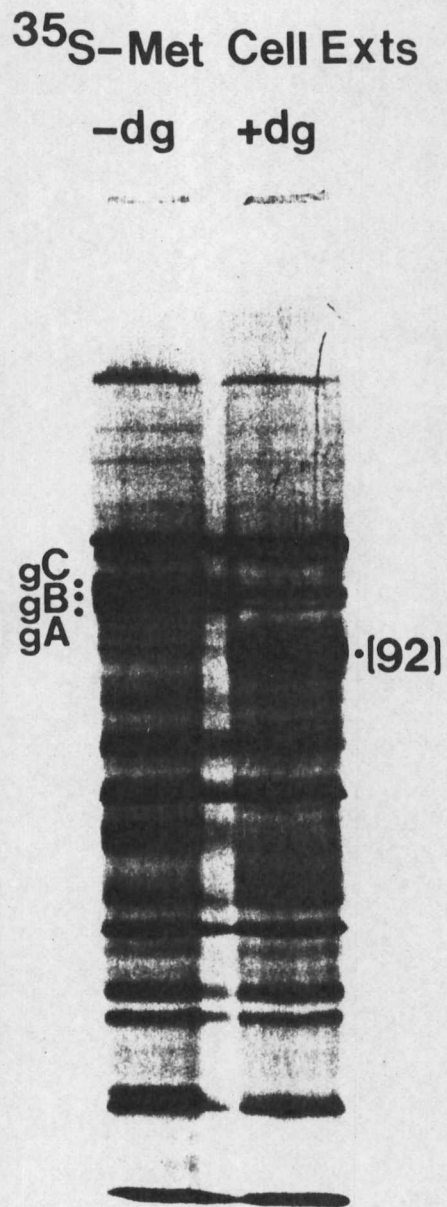


Figure 6. Effect of deoxyglucose on the synthesis of HSV-1-induced proteins. HSV-1-infected MRC-5 cells maintained in the absence (-) or presence (+) of deoxyglucose (0.1%) were labeled with [³⁵S]methionine (2 μ Ci/ml) from 4-24 hr postinfection. At 24 hr postinfection the cells were harvested and solubilized cell extracts were analyzed by SDS-PAGE.

To extend these results, the [^{35} S]methionine isotopically labeled cell extracts were immune precipitated with monospecific anti-gAB and anti-gC in order to identify the polypeptides in the 92,000 molecular weight region. The immune precipitates were washed, solubilized and then analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included in the immunoprecipitation assays as a control. The PAGE profiles of these samples are shown in Figure 7 (KOS).

In the absence of deoxyglucose the fully-glycosylated gB and its precursor gA were synthesized and migrated at positions on the gel corresponding to their apparent molecular weights of 120,000 and 110,000, respectively (Figure 7, lane 2). In the presence of DG, neither the 120,000 molecular weight gB or the 110,000 gA were synthesized (Figure 7, lane 1). The monospecific anti-gAB serum did immune precipitate a protein with a molecular weight of 92,000 from infected cells maintained in 0.1% DG (Figure 7, lane 1).

The fully-glycosylated gC and its precursor pgC₍₁₀₅₎ were immune precipitated by anti-gC serum from cells infected in the absence of 0.1% DG (Figure 7, lane 4). The glycoproteins gC and pgC₍₁₀₅₎ migrated in the gel to their apparent molecular weights of 130,000 and 105,000, respectively. When cells were infected in the presence of 0.1% DG the [^{35} S]methionine-labeled gC and the 105,000 pgC were not detected. However, the anti-gC serum did immune precipitate a protein with an approximate molecular weight of 92,000 from cells infected in the presence of 0.1% DG (Figure 7, lane 3).

Figure 7. Identification of HSV-1 [³⁵S]methionine-labeled 92,000 molecular weight proteins synthesized in the presence of 0.1% deoxyglucose. HSV-1 (KOS and MP) infected MRC-5 cells maintained in the absence (-) or presence (+) of deoxyglucose from 4-24 hr postinfection. At 24 hr postinfection the cells were harvested, solubilized and the HSV-1 glycoproteins were immunoprecipitated from cell extracts with monospecific antisera, anti-gAB and anti-gC. Immunoprecipitates were analyzed by SDS-PAGE. Normal rabbit serum (NRS) was included as a control.

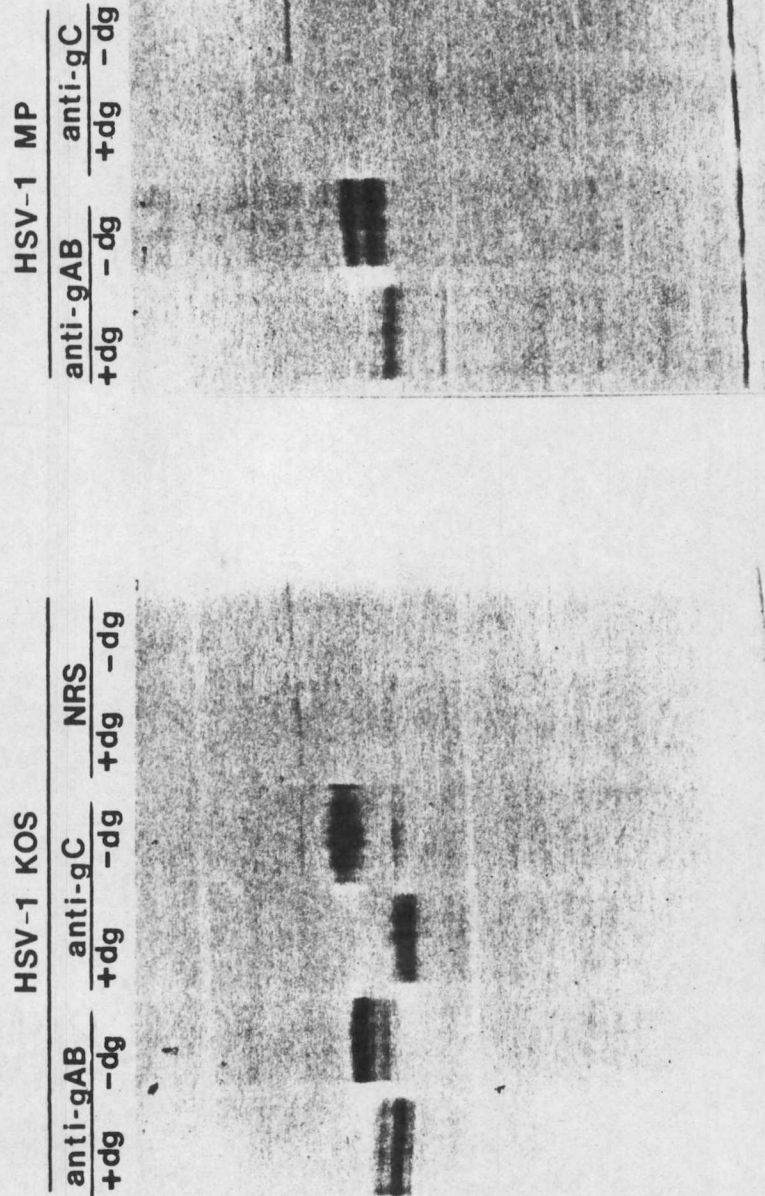


Figure 7.

In order to further characterize the 92,000 molecular weight proteins synthesized in the presence of DG the experiment was repeated using the HSV-1 MP mutant which does not synthesize gC (9, 10). Cells infected with MP were isotopically labeled with [^{35} S]methionine 4-24 hr postinfection. At 24 hr postinfection the cells were harvested and immunoprecipitations were carried out on solubilized cell extracts using monospecific sera anti-gAB and anti-gC.

As in the KOS infection, the gA and gB were synthesized in MP-infected cells and were immunoprecipitated by anti-gAB (Figure 7, lane 7). Anti-gAB also immunoprecipitated a 92,000 molecular weight protein synthesized in the presence of 0.1% DG (Figure 7, lane 6). Consistent with the MP strain not synthesizing gC, anti-gC did not immunoprecipitate gC or pgC₍₁₀₅₎ from normally infected cells (Figure 7, lane 10). The anti-gC also failed to immunoprecipitate a 92,000 molecular weight protein from MP infected cells maintained in 0.1% DG (Figure 7, lane 9). These results indicate that in the presence of DG a gC analog and a gB analog both having an approximate molecular weight of 92,000 are synthesized in wild-type infected cells.

Indirect Immunofluorescence of HSV-1 Infected Cells

The effect of inhibitors on the distribution of internal virus specific antigens within acetone-fixed herpesvirus-infected MRC-5 cells was examined. Typical patterns of fluorescence staining observed in HSV-1-infected cells treated with antisera to the high molecular weight glycoproteins and the individual glycoprotein gAB and gC at 12 hr

postinfection are shown in Figure 8. In the absence of deoxyglucose HSV-1-infected cells exhibited diffuse cytoplasmic fluorescence (Figure 8A). Infected cells grown in the presence of deoxyglucose gave a distinct perinuclear fluorescence indicative of an accumulation of altered glycoproteins within deoxyglucose HSV-1-infected cells (Figure 8B). This perinuclear fluorescence was observed whether the cells were treated with the antiserum to the high molecular weight glycoproteins or monospecific antiserum to gAB or gC (Figure 8B and 8C). Preimmune serum yielded only background fluorescence when reacted with HSV-1-infected cells (Figure 8D).

Figure 8. Patterns of internal immunofluorescence observed in HSV-1-infected cells cultured in the presence or absence of 0.1% deoxyglucose at 12 hr postinfection. (A) Untreated HSV-1-infected cells stained with antiserum made against the high molecular weight HSV-1 glycoproteins (courtesy of R. Dix); (B) deoxyglucose-treated HSV-1-infected cells stained with anti-gAB serum; (C) deoxyglucose-treated HSV-1-infected cells stained with anti-gC serum; and (D) deoxyglucose-treated HSV-1-infected cells stained with normal rabbit serum.

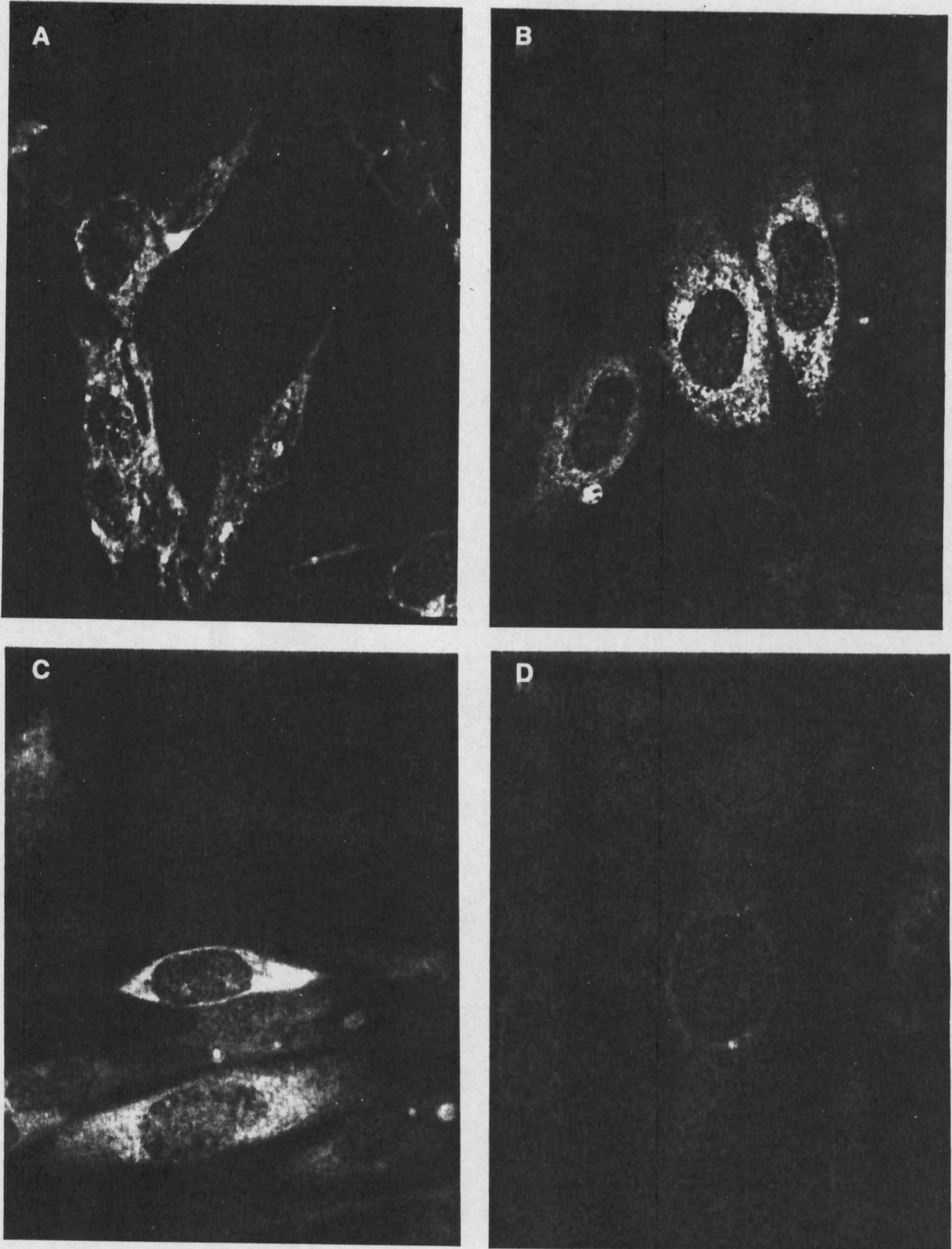


Figure 8.

IV. DISCUSSION

Deoxyglucose treatment of cells infected with RNA containing viruses has been reported to significantly inhibit the incorporation of mannose into viral specific glycoproteins (12, 25). In contrast, we observed a dramatic increase of mannose into acid precipitable material from HSV-1-infected cells treated with deoxyglucose. This study attempted to identify and characterize the HSV-1 glycoproteins synthesized in the presence of deoxyglucose which incorporated the majority of isotopically-labeled mannose.

Prior to this study we had reported the synthesis of a viral-induced 92,000 molecular weight protein(s) in the presence of deoxyglucose (1, 4). This protein was not synthesized in untreated-infected cells. Concomitant with the appearance of the 92,000 molecular protein was the disappearance of the high molecular weight glycoproteins from DG-treated-infected cells (1, 4). It was assumed that the 92,000 molecular weight protein(s) represented nonglycosylated forms of the high molecular weight glycoproteins. This study identified underglycosylated gB and gC proteins in the 92,000 molecular weight region.

In support of the fact that deoxyglucose is a glycosylation antagonist we observed a depression of [^3H]glucosamine incorporation into virus-specific, acid-insoluble material from HSV-1-infected cells maintained in the presence of 0.1% DG. In contrast, we consistently observed a significant increase of [^3H]mannose incorporation in the presence of deoxyglucose. The mannose-labeled gB and gC synthesized in the presence of deoxyglucose incorporated significant amounts of

[³H]mannose and migrated faster than the fully-glycosylated gB and gC (on SDS-PAGE). The high mannose glycoproteins synthesized in presence of DG may represent a population of virus-induced glycoprotein intermediates which are precursors during the synthesis of the HSV-1 major envelope glycoproteins. The data on endo H sensitivity indicate that glycoproteins synthesized in the presence of deoxyglucose contain high mannose simple-type oligosaccharides. The gB and gC related glycoproteins synthesized in DG-treated cells also contained oligosaccharides resistant to endo H activity. Presumably these represented complex-type oligosaccharides (27); however, they could also be aberrant oligosaccharides which are not recognized by endo H.

Since DG has been shown to become incorporated into viral-specific glycoproteins (3, 12) we postulate that DG may be incorporated in place of proximal residues and that the enzymatic activity responsible for the removal of excess mannose residues may not recognize the incorporated deoxysugar. The fact that there was an increase in mannose incorporation in the presence of DG with a parallel decrease in other monosaccharides indicates that DG is inhibiting the processing of HSV-1 glycoproteins in the high-mannose intermediate step. The incorporation of deoxyglucose in place of mannose residues on the lipid-linked oligosaccharide and preventing its transfer to the nascent polypeptide could also cause the synthesis of nonglycosylated glycoproteins in the presence of deoxyglucose (1, 12). The deoxysugar may also incorporate in place of N-acetylglucosamine residues known to be added after the en bloc transfer of the high-mannose oligosaccharide side chains to the

intermediate glycoprotein structures (17) and cause the processing to terminate prematurely yielding an accumulation of major envelope glycoprotein intermediates possessing excess mannose residues.

The antigenic expression of altered glycoprotein components within DG-treated infected cells was examined by immunofluorescence. The positive fluorescence observed in the perinuclear space of deoxyglucose-treated HSV-1-infected cells when reacted against monospecific antisera suggest that nonglycosylated glycoproteins need the carbohydrate side chains for proper transport. In studies with RNA viruses it has been demonstrated that partially glycosylated and nonglycosylated viral proteins are not transported to the plasma membrane (8, 16, 23) indicating that glycosylation may play a role in the transport of glycoproteins.

Results of earlier investigations have been extended (1, 2, 3, 4) to demonstrate a 92,000 molecular weight gC and a 92,000 molecular weight gB are synthesized in the presence of 0.1% DG. Based on the ability of the 92,000 molecular weight gC, but not gB, synthesized in the presence of tunicamycin (discussed in Part III) or deoxyglucose to incorporate glucosamine, it now appears that the 92,000 molecular weight gB represents the nonglycosylated gB and the 92,000 molecular weight gC represents the gC containing only O-linked oligosaccharides.

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PART V

OVERVIEW

I. INTRODUCTION

A major focus is being placed on the study of HSV-1 viral glycoproteins because of the integral role they play in viral infection, maturation and pathogenesis. The glycoproteins have been shown to be intimately associated with the attachment and penetration of virus particles into infected cells, the assembly of the virus particles, and the eventual expression and release of the particles at cellular membrane organelles. The HSV-1 glycoproteins are the primary antigens for induction of the humoral and cellular immune responses. Because of the role these glycoproteins play in eliciting an immune response they are one of the prime candidates for use in subunit vaccines. It therefore becomes important to characterize the glycoproteins as they pertain to the immune response in order to prevent the possibility of presenting an ineffectual vaccine to the general public.

The involvement of viral glycoproteins in viral replication is dependent upon the system under study and, therefore, it is necessary to consider each virus separately. It must be pointed out, however, that since the general mode for glycosylation of proteins has been conserved during evolution, the results obtained from investigating the glycosylation of proteins from one virus have applicability to other viral systems and glycosylation schemes in general.

The studies described above provide a better understanding of HSV-1 viral glycoprotein synthesis and indicate areas for future research into interactions between the virus and the infected host cell. At the onset of this research the principle objective was to determine whether the

biosynthesis of the HSV-1 glycoproteins, particularly HSV-1 gB and gC glycoproteins, followed the same biosynthetic pathway shown for the prototype G glycoprotein of the vesicular stomatitis virus. This involved the identification of the intermediate precursors to glycoproteins gB and gC and the characterization of the oligosaccharide side chains attached to the precursors and the fully glycosylated proteins in order to establish the processing events which occurred between the intermediates and the mature glycoproteins.

A number of approaches were used to elucidate the biosynthetic pathway of gB and gC. Because the initial glycosylation of HSV-1 glycoproteins occurs cotranslationally and then proceeds in a dynamic fashion without the intermediates accumulating into intracellular pools, it was necessary to manipulate the infected cell in order to accumulate the intermediates of the HSV-1 glycoproteins. The glycosylation inhibitors tunicamycin and 2-deoxy-D-glucose were employed for two primary reasons: (1) to identify the nonglycosylated polypeptide backbone, and (2) to perturb the glycosylation in order to amass intermediates or precursors involved in glycoprotein synthesis. Since no N-linked oligosaccharides are added to the glycoproteins in the presence of tunicamycin, it was used to identify the nonglycosylated forms of the glycoproteins. In addition, it was used to identify potential glycoproteins containing O-linked oligosaccharides.

The use of deoxyglucose was advantageous because it permitted the synthesis and accumulation of putative precursor HSV proteins which were partially glycosylated. A limitation to using deoxyglucose is that it

can incorporate as an analog of mannose or glucose and therefore the specific mechanism by which deoxyglucose inhibits glycosylation may vary with the virus under study (4, 7, 19, 22). The ionophore monensin was also used to cause the accumulation of partially glycosylated precursors to the HSV-1 glycoproteins. Specifically, it blocked the processing of the glycoproteins at the high mannose stage (12).

The use of glycosylation inhibitors may cause the synthesis of aberrant glycoproteins which are not representative of the normal precursor glycoproteins. One way to circumvent this problem was to make use of ts mutants which processed glycoproteins only to a certain point at the nonpermissive temperature (14, 20). The employment of ts mutants eliminates the risk that the results will be due to a chemical agent causing a generalized toxic effect rather than specifically perturbing the system of interest. For example, from the description of the monensin experiments in the literature one could falsely conclude that HSV-1 glycoprotein processing of the high mannose precursor is a prerequisite for the virion's egress out of the cell (12) when in fact virions containing high mannose oligosaccharides can be eliminated into the extracellular medium (1, 14). In the case of the monensin-treated cells, the block in virion release was probably caused by monensin inhibiting intracellular membrane flow (25).

The identification of the HSV-1 glycoproteins containing high-mannose oligosaccharides was accomplished by treating glycoproteins accumulated under various conditions with the specific glycosidase, endo- β -N-acetylglucosaminidase H (endo H) (28). Endo H cleaves between

the two proximal N-acetylglucosamines of high-mannose oligosaccharides (12, 17). When using endo H it was necessary to control for the presence of contaminating proteases which could have cleaved the glycoproteins thus giving false results. To reduce this possibility, parallel endo H digestion samples were incubated with or without the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The digestion products observed in the presence or absence of PMSF were consistently the same indicating that they were caused by endo H activity.

Based on the results obtained in this study and the data reported in the literature, it is now possible to propose a biosynthetic pathway for the HSV-1 glycoproteins gB and gC. The scheme depicted in Figure 1 illustrates the following discussion.

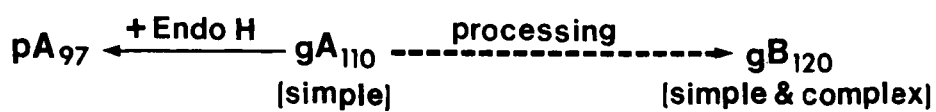
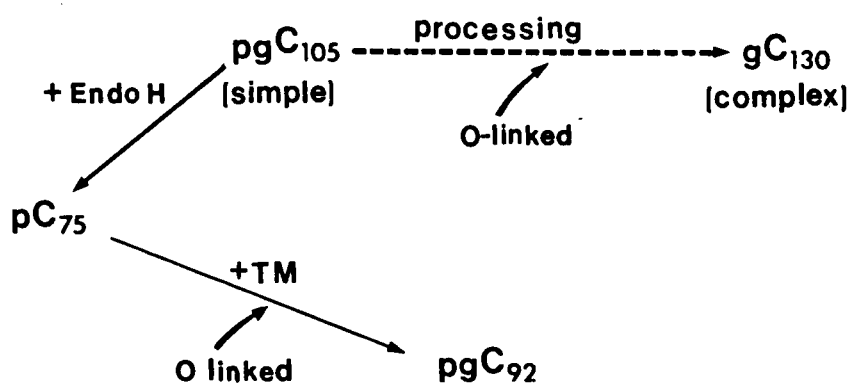


Figure 1. Proposed biosynthetic pathway for HSV-1 glycoproteins gC and gB.

II. BIOSYNTHESIS OF HSV-1 GLYCOPROTEIN gB

The oligosaccharides of gB were known prior to these studies to be N-glycosidically linked, but the nature, simple vs. complex, of the oligosaccharides had not been determined. The endo H digestion results of gB obtained in this study indicated that gB contains both simple- and complex-type oligosaccharides. It was demonstrated that the mobility of [³⁵S]methionine-labeled gB glycoprotein on SDS-gels increased after endo H digestion, indicating the presence of simple-type oligosaccharides on gB. Following endo H digestion of [³H]mannose-labeled gB some of the [³H]mannose label remained associated with the digested gB indicating that many but not all of the carbohydrate side chains of gB are of the complex type. The assumption that the [³H]mannose-labeled glycoprotein remaining in anti-gAB immune precipitates after endo H treatment was indeed gB was verified by the results of a parallel experiment using the tsJ12 mutant which does not synthesize gB at the nonpermissive temperature (14). When tsJ12-infected cells were grown at the nonpermissive temperature only gA was immunoprecipitated from solubilized cell extracts with anti-gAB serum. When the tsJ12 gA, isotopically labeled with [³H]mannose, was treated with endo H no [³H]mannose-labeled glycoprotein was detected by SDS-PAGE analysis confirming that the endo H resistant oligosaccharides observed in anti-gAB immune precipitates were associated with the gB glycoprotein. The HSV-1 gB glycoprotein is therefore analogous to the Sindbis virus glycoprotein in that both contain simple- and complex-type oligosaccharide side chains (18).

The possibility that gB contains a small amount of O-linked oligosaccharides must be considered. Other investigators have suggested that O-linked oligosaccharides are found on gB based on their observations that oligosaccharides on gB were released by alkaline borohydride or the enzyme N-acetyl galactosamine oligosaccharidase which preferentially removes O-linked oligosaccharides (Johnson and Spear, personal communication).

The data obtained in this research do not support the presence of O-linked oligosaccharides on gB. The gB could not be metabolically labeled with glucosamine or galactose when synthesized in the presence of tunicamycin or deoxyglucose. The synthesis in the presence of tunicamycin of a nonglycosylated gB which increased in molecular weight due to the addition of O-linked oligosaccharides was not detected. Alternatively, it is possible that the varied results may be due to the use of different cell lines. The detection of O-linked oligosaccharides was made on a gB synthesized in epithelial cells (Johnson and Spear, personal communication), whereas the absence of O-linked oligosaccharides in this study is based on the synthesis of gB in fibroblast cells. If some glycoproteins (i.e., gB) require N-linked oligosaccharides for transport to the Golgi where presumably O-linked oligosaccharides are added, then this would explain why gB is not isotopically labeled with galactose or glucosamine in the presence of tunicamycin.

Understanding the biosynthesis of glycoprotein gB was of interest because the identification of a mannose-rich oligosaccharide precursor structure was lacking. The prime candidate for the high-mannose

precursor to gB was gA primarily because gA had already been proposed as a precursor to gB (5). As discussed above, the oligosaccharides of gA are primarily of the high-mannose nature since no [^3H]mannose-labeled gA was detected by SDS-PAGE analysis after endo H digestion of gA, and this observation was the same whether the gA was synthesized in wt or tsJ12 infected cells.

Eberle and Courtney (5) suggested that during the synthesis of gB, gA is processed to a lower molecular weight precursor from which gB is derived. The lower molecular weight glycoprotein may represent gA which has had the outer mannose residues removed from the oligosaccharides destined to be complex type. The synthesis of three gB related glycoproteins, a glycoprotein with a migration rate between gA and gB, the gA glycoprotein, and the lower molecular weight gB precursor glycoprotein have been observed in ricin-resistant cells (1). More research is necessary to properly define the relationship of these antigenically similar glycoproteins to gB. Interestingly, analysis of tunicamycin-treated-infected cell extracts by the immunoblotting technique revealed only one 92,000 molecular weight band reacting with anti-gAB serum at 6 hr postinfection, whereas at 7 hr postinfection a slightly lower molecular weight protein was also detected. Whether this second band represents a cleavage product, perhaps the protein minus its signal sequence, needs to be investigated.

The processing of gA to gB includes the removal of mannosyl residues along with the addition of GlcNAc and sialic residues (9). Based on the inhibition of this processing in monensin-treated-infected

cells, glycoprotein maturation probably occurs in the smooth membranes of the Golgi apparatus. The maturation of the virion-associated glycoproteins also must occur within the Golgi since virions containing only the immature-high mannose oligosaccharides are isolated from infected cells treated with monensin (12).

Since endo H cleaves between the two N-acetylglucosamine residues of the high mannose oligosaccharide, the 97,000 molecular weight gA remaining after endo H digestion would still contain one GlcNAc per oligosaccharide side chain removed. This may account in part for the discrepancy in the molecular weight of the gA after endo H digestion and the 92,000 molecular weight gA (gB) synthesized in the presence of tunicamycin or deoxyglucose. The 92,000 molecular weight gA probably represents the nonglycosylated polypeptide backbone of gA (gB). At no time could this 92,000 molecular weight protein be isotopically labeled with mannose, glucosamine, galactose, or fucose. Recently, a nonglycosylated 85,000 molecular weight protein antigenically similar to gA has been translated in vitro from HSV-1 mRNA (11). To verify that the 92,000 and 85,000 molecular weight glycoproteins represent the same structure it will be necessary to compare tryptic peptide digests of the two proteins.

III. BIOSYNTHESIS OF HSV-1 GLYCOPROTEIN gC

The demonstration of both N- and O-linked oligosaccharides on the gC glycoprotein immunoprecipitated from HSV-1-infected cell extracts is the first suggestion that a viral structural glycoprotein may contain both types of oligosaccharide side chains. The actual demonstration of both N- and O-linked oligosaccharides on the virion associated gC still needs to be shown. The N-linked oligosaccharides of the gC glycoprotein are primarily of the complex-type as observed by the lack of change in the mobility of gC on SDS-gels following endo H digestion. The presence of O-linked oligosaccharides on gC has been suggested in the literature based on alkaline borohydride hydrolysis of HSV-1 glycoprotein associated oligosaccharides (15) and the ability of gC to bind to lectin affinity columns specific for GalNAc residues (16). The data presented in this study, demonstrating the incorporation of glucosamine or galactose, but not mannose, into the gC glycoprotein synthesized in the presence of tunicamycin substantiate the presence of O-linked oligosaccharides on the gC glycoprotein. In addition, this study also provided evidence that the [^3H]glucosamine-labeled 92,000 molecular weight gC synthesized in tunicamycin-treated cells and the 130,000 molecular weight mature gC glycoprotein were both sensitive to alkaline hydrolysis.

The gC glycoprotein is derived from its high-mannose precursor designated pgC₍₁₀₅₎ (5, 26). The high-mannose nature of the pgC₍₁₀₅₎ was verified by the inability to detect [^3H]mannose-labeled pgC₍₁₀₅₎ on SDS-gels following endo H digestion. Once the pgC₍₁₀₅₎ was converted to

gC, a 75,000 molecular weight protein was no longer detected in samples exposed to the action of endo H. Since only a 75,000 molecular weight nonglycosylated protein could be detected after endo H digestion of pgC₍₁₀₅₎, the O-linked oligosaccharides are probably added after the processing of the high-mannose oligosaccharides has commenced.

The HSV-1 glycoproteins synthesized in ricin-resistant cells are only processed to the high-mannose state (1), and preliminary data obtained from other laboratories (Johnson and Spear, personal communication) indicate that O-linked oligosaccharides are not added to the glycoproteins synthesized in these cells. Based on these results it might be assumed that the processing of N-linked oligosaccharides is a signal for the addition of O-linked oligosaccharides. Alternatively, the ricin-resistant cells may also be deficient in transferases responsible for the addition of O-linked oligosaccharides as the glycosyl transferases of these cells have not been fully characterized. The presence and processing of N-linked oligosaccharides on the gC glycoprotein synthesized in MRC-5 cells are not essential for the addition of O-linked oligosaccharides. The gC synthesized in tunicamycin-treated MRC-5 cells incorporated both glucosamine and galactose. The presence of N-linked oligosaccharides is not critical for the addition of O-linked oligosaccharide side chains to the glycophorin A protein (6) or the vaccinia hemagglutinin protein (24).

The enzymes necessary for the addition of O-linked oligosaccharides to gC may be located within the same membranes as the GlcNAc transferase I, mannosidase enzymes, etc., necessary for the processing of

simple-type chains to complex-type side chains. The two processing events may therefore occur simultaneously rather than the former being dependent on the latter. There is some indication that the gC synthesized in monensin-treated-infected MRC-5 cells does incorporate a small amount of O-linked oligosaccharides. It is evident that reconciling these differences will necessitate characterizing the glycosyl transferases involved and determining the influence of the host cell and the virus itself on the glycosylation of viral glycoproteins.

The determination of the molecular weight of the nonglycosylated gC glycoprotein was shown by two means. The treatment of pgC₍₁₀₅₎ with endo H yielded a 75,000 molecular weight protein, and a 75,000 molecular weight gC synthesized in tunicamycin-treated-infected cells was detected by the immunoblotting technique. Since the 92,000 molecular weight gC synthesized in the presence of tunicamycin or deoxyglucose could be isotopically labeled with glucosamine or galactose in the presence of tunicamycin, it represents the gC glycoprotein containing only O-linked oligosaccharides. Preliminary studies indicate that the 75,000 molecular weight protein synthesized in the presence of tunicamycin can be chased into the 92,000 molecular weight gC in agreement with O-linked oligosaccharides being added posttranslationally (6, 10). The molecular weight of the nonglycosylated gC is in agreement with the molecular weight of in vitro translated gC from HSV-1 mRNA (Spear, personal communication, Wagner, personal communication).

IV. EFFECT OF 2-DEOXY-D-GLUCOSE ON HSV-1

GLYCOPROTEIN SYNTHESIS

The precise mechanism by which deoxyglucose influences the glycosylation of HSV-1 glycoproteins is not known. The presence of deoxyglucose in the culture medium of HSV-infected cells caused an increase in the incorporation of [^3H]mannose into HSV-1 glycoproteins in direct contrast to the decrease of mannose incorporation into the glycoproteins of several RNA-containing viruses produced in deoxyglucose-treated cells (13, 21). This same phenomenon was observed in an in vitro study which reported that UDP-deoxyglucose stimulated the incorporation of [^{14}C]mannose into endogenous glycoproteins while GDP-deoxyglucose suppressed the incorporation of [^{14}C]hexoses into glycoproteins (23). The deoxyglucose from UDP-deoxyglucose was probably incorporated in place of GlcNAc, from UDP-GlcNAc, near the nonreducing termini of the protein-linked oligosaccharides. Since it was reported earlier that HSV-1 glycoproteins incorporate isotopically-labeled deoxyglucose (2), it is entirely plausible that the deoxyglucose replaces the first GlcNAc at the nonreducing terminal end (Figure 3, Part I, p. 12), thereby preventing proper recognition by the mannosidase, and causing an accumulation of glycoproteins with aberrant simple-type carbohydrate side chains. The incorporation of glucosamine into HSV-1 proteins was decreased by approximately 50% in the presence of deoxyglucose in agreement with the proposed block in further processing of the protein-linked oligosaccharides. The O-linked oligosaccharides are probably responsible for much of the incorporated

[³H]glucosamine in the presence of deoxyglucose. Experiments designed to study the effect of UDP-deoxyglucose vs. GDP-deoxyglucose on the incorporation of mannose into HSV-1 glycoproteins are necessary to elucidate the mechanism by which deoxyglucose effects the glycosylation of HSV-1 proteins.

The HSV-1 genome is large enough to conceivably code for proteins capable of modifying the cellular glycosylation enzymes. Alteration of the cellular glycosyl transferases could account in part for the unexpected incorporation of mannose into HSV-1 glycoproteins synthesized in the presence of deoxyglucose. Altered kinetic properties and acceptor specificities of galactosyl and sialyl transferases have been noted between uninfected and HSV-1-infected cells (17). However, further experimentation such as physiochemical characterization of the enzymes from different infected cell lines are required to understand the nature of the changes in glycosyl transferase activities of cells infected with HSV-1.

V. DISCUSSION

The determination of the steps and intermediates involved in HSV-1 glycoprotein synthesis must now be followed by studies to define where the processing events occur within the HSV-infected cell. It is proposed (Compton, personal communication) that the HSV-1 glycoproteins are initially glycosylated at the rough endoplasmic reticulum and then migrate in the high mannose form to two intracellular positions, namely the Golgi apparatus and the nuclear membrane. The glycoproteins which migrate to the Golgi apparatus are further processed and are then transported to the plasma membrane (12). A second processing pathway is proposed for those glycoproteins which enter the nuclear membrane. These immature glycoproteins become assembled into the virion at the nuclear membrane and are processed on the virion probably after the transport of the virus particles to the Golgi apparatus. The virions may then exit the cell via modified vesicles. Johnson and Spear (12) reported the accumulation of virions in dilated vacuoles of HSV-1-infected cells treated with monensin. The smooth vacuoles appeared to be discrete entities and were not connected to the nuclear or rough endoplasmic membranes. The authors state that their observation argued for a model in which enveloped-herpes particles move from the perinuclear space via membrane vesicles to the Golgi apparatus and then onto the cell surface and extracellular space. If the virions migrate from the nuclear membrane to the Golgi apparatus and from there to the plasma membrane via vesicles, structures should exist containing virions with either immature or mature glycoproteins depending upon whether they

represent pre- or post-Golgi processing. Alternatively, the virions may migrate to the Golgi apparatus by a modified cytoplasmic membrane system and be packaged at the Golgi into vesicles for transport out of the cell. At this time the mechanism for the transport of the virions out of the cell is not fully understood.

Recent studies have demonstrated that different intracellular pathways exist for the transport of glycoproteins from the Golgi apparatus to the cell surface depending upon whether the glycoproteins are membrane or secretory proteins. Smilowtz (25) demonstrated that the two glycoproteins acetylcholine receptor (ACHR), a membrane glycoprotein, and acetylcholine esterase (ACHE), a secretory glycoprotein, were effected differently in monensin-treated embryonic chick skeletal muscles. The ACHR glycoprotein continued to appear and accumulate at the plasma membrane while the ACHE glycoprotein accumulated within intracellular-perinuclear membranous structures which did not communicate with the extracellular medium.

Two distinct pathways for transport of secretory and membrane glycoproteins to the surface of pituitary tumor cells have also been described (8). The adrenocorticotrophic hormone glycoprotein accumulates in secretory granules and its release can be stimulated four- to five-fold with 5 mM 8-bromoadenosine 3':5'-cyclic monophosphate, whereas the release of the endogenous MuLV glycoprotein, gp70, is stimulated less than two fold under the same conditions (8). Gumbiner and Kelly (8) proposed that the gp70 viral glycoprotein was transported by a constitutive pathway, while mature ACTH is stored in secretory granules

where its release is enhanced by stimulation. This latter pathway is thought to occur primarily in specialized secretory cells such as endocrine and exocrine cells or neurons, where it can be stimulated or induced by appropriate regulators. It is interesting to speculate that HSV-1 or HSV-2 may use this mechanism for maturation in latently infected neurons. It is conceivable that the induction of a latently infected neuron to release HSV virions is due to the stimulation of a secretory glycoprotein pathway.

Not only does the study of HSV-1 glycoprotein synthesis enhance our knowledge of HSV-1 replication, but it also has direct application to expanding our knowledge of cellular glycosylation mechanisms. The opportunity exists through the use of viruses to directly compare the glycosylation pathways between different cell types by keeping constant the genetic information of the protein moiety. The unique aspects of HSV-1 glycoprotein synthesis have provided investigators with a fascinating system to probe.

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CONCLUSIONS

The results from this study support the following conclusions which will add to our understanding of HSV-1 glycoprotein synthesis:

1. The glycoprotein gB contains N-glycosidically linked oligosaccharides which are representative of both simple- and complex-type oligosaccharides. The gC glycoprotein contains both N- and O-glycosidically linked oligosaccharides and the N-linked oligosaccharides are complex-type carbohydrate side chains.
2. The glycoproteins gB and gC are both synthesized from precursors which contain primarily high-mannose simple-type oligosaccharides. With the use of endo H, gA was identified as the high-mannose precursor of gB, and pgC₍₁₀₅₎ was verified as the high-mannose precursor of gC.
3. The nonglycosylated polypeptide backbone of gA has an approximate molecular weight of 92,000. In the presence of tunicamycin the O-linked oligosaccharides are added to the 75,000 molecular weight gC protein posttranslationally and the resulting gC protein has a molecular weight of 92,000.
4. Mannose-labeled gB and gC glycoproteins are synthesized in deoxyglucose-treated-infected cells, and their associated carbohydrate side chains are representative of both endo H resistant and sensitive oligosaccharides.

VITA

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