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I am submitting herewith a dissertation written by Henry K. Su entitled "Characterization of the Synthesis and Processing of the Herpes Simplex Virus Type 2 Glycoprotein, gG." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.

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CHARACTERIZATION OF THE SYNTHESIS AND PROCESSING OF THE HERPES SIMPLEX VIRUS TYPE 2 GLYCOPROTEIN, gG

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Henry K. Su

December 1985

DEDICATION

This dissertation is dedicated to my family,

especially to my parents

for their constant love and support;

And to Susie;

for her companionship and love.

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Above all, I would like to express my utmost gratitude to my mentor Dr. Richard Courtney for everything--thank you, sir.

ABSTRACT

The synthesis and processing of herpes simplex virus type 2 glycoprotein gG was studied with the aid of a variety of techniques including the use of monospecific, monoclonal and polyclonal antibodies in immunoprecipitation, immunoblotting and immunofluorescence studies. The intermediates of gG processing were characterized by *in vitro* translation studies as well as the use of glycosidic enzymes and inhibitors of glycoprotein processing. The results of this study showed a number of components were involved in the synthesis of gG. These included a 100,000 molecular weight unglycosylated polypeptide (designated 100K), three high-mannose containing components designated 104K, 72K and 31K (104,000, 72,000, and 31,0000 molecular weight, respectively) and the fully glycosylated mature gG of 108,000 molecular weight (108K). Based on these results as well as kinetic studies and pulse-chase experiments, the following scheme of events in the synthesis and processing of gG is suggested. The gG glycoprotein is synthesized as a cotranslationally glycosylated 104K high-mannose intermediate that is cleaved into the 72K and 31K high-mannose containing glycoproteins. The high-mannose intermediate is then further glycosylated to generate the mature 108K gG.

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REVIEW OF THE LITERATURE

CHAPTER I

GLYCOPROTEIN BIOSYNTHESIS

Introduction

Glycoproteins belong to a large family of proteins that have covalently attached carbohydrates. This diverse group of macromolecules has been shown to play crucial roles in many biological reactions in cell growth, differentiation and communication. Glycoproteins can be found as enzymes, hormones, mediators of immune responses, and structural components or receptors on cellular as well as viral membranes (Sharon and Lis, 1982).

The contribution of the carbohydrate moieties to the functions of glycoproteins is not fully understood; however, recent studies have indicated that they function as carriers of biological information in a variety of ways. In particular, carbohydrate moieties have been shown to act as specific markers recognized in various processes such as the sorting of lysosomal enzymes and virus-cell interactions. For example, specific recognition of the mannose-6-phosphate sugar on lysosomal enzymes has been implicated in the facilitated uptake of these proteins into the lysosomes (Neufeld et al., 1975). Several investigations have also demonstrated that the sialic acid residues on erythrocyte membrane glycoproteins can act as the specific-receptors for influenza virus glycoprotein in viral attachment and agglutination of the erythrocytes (Gottschalk, 1960; Paulson et al., 1979; Markwell and Paulson, 1980).

Additionally, carbohydrate moieties can also influence the folding and final conformation of glycoproteins resulting in changes in their solubility and biological activity. One example of this is the role of the disaccharide on the antifreeze glycoprotein of Antarctic fish. Removal or modification of the disaccharide units resulted in loss of the glycoprotein's ability to depress the freezing point of water (Vandenheede et al., 1972; Shier et al., 1972). In vesicular stomatitis viral glycoprotein synthesis, inhibition of the addition of oligosaccharides to the G protein by tunicamycin resulted in altered solubility of the protein and prevented the transport of the protein to the plasma membrane for virus assembly (Leavitt et al., 1977; Gibson et al., 1981). Further, growth of VSV in a glycosylation defective mutant cell line results in the transfer of a truncated oligosaccharide chain to the G protein during synthesis. Such an event results in the production of the temperature-sensitive virus in the mutant cells, suggesting that proper oligosaccharide structure is important in the folding of the glycoprotein (Kornfeld et al., 1979; Gibson et al., 1980).

The biosynthesis of viral glycoproteins has been intensively studied. Studies of viral glycoproteins have contributed significantly to the understanding of the mechanisms involved in the biosynthesis of glycoproteins since viral glycoproteins utilize the same glycosylation machinery as cellular membrane glycoproteins. Additionally, viral glycoproteins have proved to be extremely useful models for elucidating the biosynthesis and processing mechanisms because they can be easily monitored in infected cells.

Membrane Glycoprotein Synthesis

A model membrane glycoprotein is exemplified by the structure of the widely studied G protein of VSV. Amino acid sequence derived from the cDNA sequence for the VSV G protein (Rose and Gallione, 1981; Gallione and Rose, 1983) has revealed four structural domains of the molecule. The first 16 amino acids form the signal sequence, which is important in the translocation of the polypeptide into the endoplasmic reticulum (ER), followed by the main portion of the protein containing 446 amino acid residues with asparagine residues 178 and 335 being glycosylated (Rothman and Lodish, 1977; Toneguzzo and Ghosh, 1978; Rose and Gallione, 1981). The molecule is anchored in the membrane by a stretch of 20 hydrophobic amino acids also known as anchor or membrane sequences followed by a 29 amino acid carboxyl end in the cytoplasmic domain (Chatis and Morrison, 1979). Removal of the region spanning the membrane to the carboxyl-terminus has been shown to result in a truncated product that is secreted (Rose and Bergman, 1982, 1983). Additionally, hybrid proteins constructed with only this portion of the G protein fused to a secretory protein (rat growth hormone), generated a protein that is synthesized as a transmembrane protein anchored by the G protein portion in the membrane (Quan and Rose, 1984). The membrane-spanning and cytoplasmic domains of G protein can therefore function to anchor G protein as well as other hybrid proteins containing this region in the membrane.

The synthesis of membrane glycoproteins requires the translocation of the newly synthesized polypeptides on the polyribosomes to the lumen of the ER where further synthesis and processing can occur (Palade,

1975). An important insight into this process is provided by studies that led to the signal hypothesis proposed by Blobel and Dobberstein (1975). These investigators first noted in an in vitro system that a stretch of 15 to 30 hydrophobic amino acids in secretory proteins was cleaved after they were translocated into the ER. These sequences have since been demonstrated in a variety of secretory membrane-associated proteins and they are commonly referred to as "signal sequences."

The recognition of the signal sequence for translocation of membrane proteins has been shown to require two main components, the signal recognition particle (SRP) (Walter and Blobel, 1980) and the SRP receptor or "docking" protein (Gilmore et al., 1982a; Meyer et al., 1982). Studies by Walter and Blobel (1980) have shown that the SRP is an 11S nucleoprotein composed of six polypeptides of apparent molecular weights of 72, 68, 54, 19, 14 and 9 kilodaltons (K). In addition, this 11S cytoplasmic nucleoprotein also contains a 300 nucleotide RNA (Walter and Blobel, 1982). The activity of this SRP requires both the protein and the RNA components and such activity is abolished when the particle is separated. However, this activity can be restored when the particle is reconstructed (Walter and Blobel, 1983). The SRP receptor has been purified from the ER as a disulfide-linked protein of 72K bearing a 60K cytoplasmic domain (Meyer and Dobberstein, 1980). Removal of this 60K domain from the ER by proteolytic cleavage abolishes the translocation function but this function can be restored by adding the 60K fragment back to the cleaved ER membranes (Meyer and Dobberstein, 1980).

The interactions of these different components in the translocation process is depicted in Figure 1 (Walter et al., 1984). Following the translocation of the signal peptide sequence on the ribosome, further polypeptide elongation is arrested by the binding of the SRP to the ribosome (Walter and Blobel, 1981b; Meyer et al., 1982). The signal sequence of nascent membrane proteins has been shown to be involved in this recognition (Anderson et al., 1982) since incorporation of certain amino acid analogs can render this signal sequence unrecognizable (Hortin and Boime, 1980; Walter and Blobel, 1981a). The elongation inhibition by the SRP is released once the SRP has delivered the nascent polypeptide located on the ribosomes to the ER membrane. This release of elongation arrest has been shown to occur through the binding of the SRP to the SRP receptors on the ER (Gilmore et al., 1982b). This binding releases the SRP from its bound ribosome allowing both the SRP and its receptor to be recycled (Gilmore et al., 1982a and b). With the resumption of elongation the polypeptide is vectorially translocated into the lumen of the ER where the signal sequence can be removed by the ER signal peptidase (Walter and Blobel, 1981a and b). The process following this initial targeting to the membrane is poorly understood. Formation of a "pore-like" structure has been proposed for facilitating the entry of the remainder of the polypeptide into the ER (Walter et al., 1984) but this has yet to be confirmed. The translocation is completed once the hydrophobic membrane anchor sequences are inserted into the ER membrane.

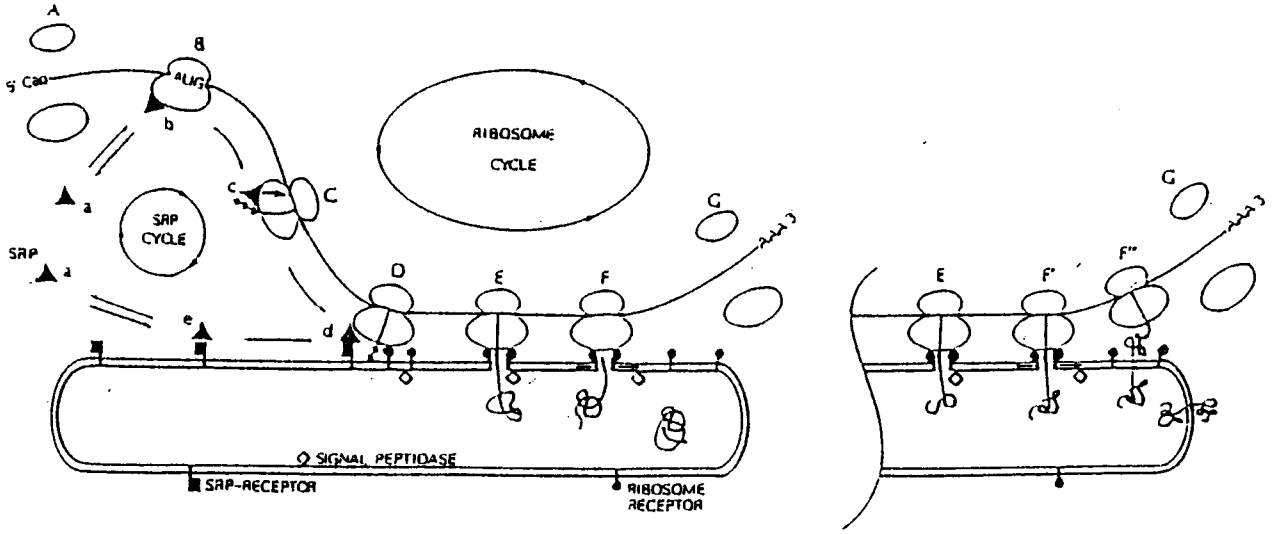


Figure 1. Model for the interaction of the SRP and the SRP-receptor in the translocation process (taken from Walter, P., R. Gilmore, and G. Blobel. 1984. Protein translocation across the endoplasmic reticulum. Cell 38:5-8).

Types of Oligosaccharide Chains on Membrane Glycoproteins

The nascent membrane glycoproteins inserted into the ER are further processed by the addition of carbohydrate chains as they pass through the ER and the Golgi. The oligosaccharide chains present on membrane glycoproteins can be classified into two main groups of structures based on the kinds of linkage involved between the carbohydrate chain and the polypeptide backbone. The first group is the O-glycosidically-linked or O-linked oligosaccharides and they are attached from an N-acetylgalactosamine (GalNAc) residue on the sugar chain to a serine or a threonine on the polypeptide backbone. O-linked structures are also known as "mucin-types" because of their abundance on mucin proteins. O-linked sugar chains have been described for viral glycoproteins associated with coronaviruses (Holmes et al., 1981) and herpes simplex viruses (HSV) (Olofsson et al., 1981a, 1981b; Johnson and Spear, 1983; Wenske and Courtney, 1983). The other group of oligosaccharide chains are the N-acetylglucosamine (GlcNAc) residues coupled to the amide nitrogen of asparagine or N-linked structures (Berger et al., 1982). The most commonly studied example of an N-linked glycoprotein is the G protein of VSV. It should be pointed out that membrane glycoproteins can contain all N-linked or all O-linked or both types of carbohydrate chains.

O-glycosidically Linked Carbohydrate Structures

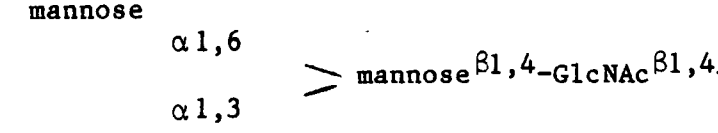
O-linked carbohydrates can be studied after release from their polypeptide backbone by β-elimination in the presence of alkaline borohydrate (Spiro, 1972). Studies of O-linked oligosaccharide chains

have revealed that they are characterized by the presence of GalNAc residue(s) with or without galactose (Gal), fucose (Fuc) and sialic acid (SA). Additionally some structures have been shown to contain GlcNAc residues as well (Kornfeld and Kornfeld, 1980). The simplest O-linked structures are disaccharides such as N-acetylneuraminic acid (NeuNac) linked α2,6 to GalNAc found in most submaxillary mucins and Gal β1,3 to GalNAc found in certain Antarctic fish antifreeze proteins (Shier et al., 1972; Sharon and Lis, 1982). The Gal β1,3 GalNAc disaccharide structure appears to be commonly found in O-linked structures usually in a substituted form. In fetuin, human chorionic gonadotropin and glycophorin, two SA residues are linked α2,6 and α2,3 to the GalNAc and Gal residues respectively (Sharon and Lis, 1982). A more complex structure is present on the disaccharide in porcine submaxillary mucin with GalNAc β1,3 and Fuc α1,2 linked to the Gal residue and SA 2,6 attached to the GalNAc sugar (Williams et al., 1980). The disaccharide structure can also be elongated by Gal β1,3 or β1,4 GlcNAc repeating units plus other substitutions to achieve an even more complex branched structure with up to as many as 20 residues as in blood group glycoproteins. Structures of the O-linked oligosaccharide chains on viral glycoproteins are still poorly understood.

Asparagine-linked Oligosaccharide Structures

Analyses of the asparagine-linked carbohydrates from viral and cellular glycoproteins have revealed three general types of structures termed high-mannose or simple, complex, and hybrid types. Typical structures of each of these types are shown in Figure 2 (Kornfeld and

Kornfeld, 1985). The striking feature of all of these oligosaccharide types is that they share a common pentasaccharide core.



The high-mannose oligosaccharide chains have an additional two to six mannose (Man) residues attached to this core. In the complex type structures, branches of sialyl lactosamine sequence (SA, Gal, GlcNAc) are frequently attached to the terminal Man residues of the pentasaccharide core (as shown in Figure 2). Fucose in an α1,6 linkage to the asparagine-linked GlcNAc and a GlcNAc β1,4 linked to the β Man residue are frequently observed. The lactosamine sequence Gal α1,4 GlcNAc may contain an α1,3 linked Fuc to the Gal residue instead of SA but addition of both is not detected (Paulson et al., 1978; Beyer et al., 1979). The Gal may also be substituted with α1,2 or α1,6 linked fucose residues (Kobata, 1984) or with another α1,3 linked Gal residue (Eckhardt and Goldstein, 1983; Dorland et al., 1984; Spiro and Bhoyroo, 1984). The SA residues can be varied to include more than one per outer lactosamine branch structure. Multiple branches or branch structures with repeating lactosamine disaccharides have also been described (Kornfeld and Kornfeld, 1985). Finally, the hybrid-type oligosaccharides have features of both the high-mannose type and the complex-type chains. A GlcNAc residue and/or Gal β1,4-GlcNAc group can be linked on one α-mannosyl residue, as in the complex-type chains with one or two α-mannoses on the other mannosyl residue like the high-mannose type chains (see Figure 2).

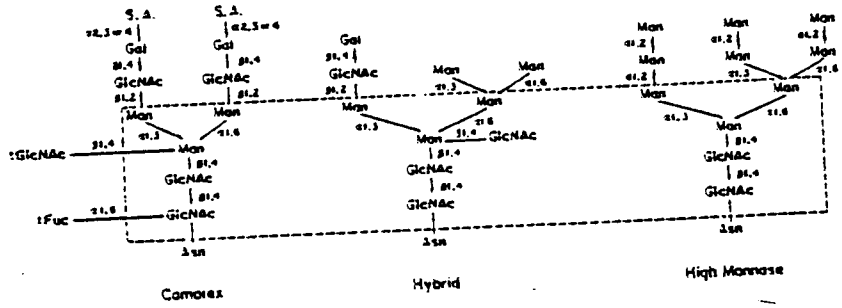


Figure 2. Structures of the major types of asparagine-linked oligosaccharides. The common pentasaccharide core is indicated in the boxed area. (Figure taken from Kornfeld, R., and S. Kornfeld. 1985. Assembly of asparagine-linked oligosaccharides. Ann. Rev. Biochem. 54:631-664.)

Assembly of O-linked Sugar Chains

The addition of O-glycosidically linked sugars on serine or threonine appears to occur in a sequential manner with the product of one glycosyltransferase being utilized as the substrate for the next (Schachter and Roseman, 1980; Beyer et al., 1981). The first residue synthesized results from the transfer of GalNAc to the hydroxyl group of threonine or serine by the enzyme UDP-GalNAc:polypeptide transferase in the absence of any intermediates (Hanover et al., 1980). Examination of sequences around this addition site has not revealed any specific peptide sequence for this reaction. In general O-glycosyl residues are frequently found in regions rich in proline (Hanover and Lennarz, 1981). Secondary and tertiary structures of polypeptides may thus be important for the accessibility of the transferase (Hill et al., 1977). The site of this addition appears to occur predominantly in the cis-Golgi (Roth, 1984; Dunphy and Rothman, 1985) though some GlcNAc transferase activity has been detected in the ER (Hanover et al., 1980; Kim et al., 1971; Ko et al., 1972; Strous, 1979).

The sequential pathway for the synthesis of O-linked oligosaccharides of the well-studied porcine submaxillary mucin is shown in Figure 3. After the formation of GalNAc-thr/ser, the Gal residue is added next. The addition of SA could also be added onto the GalNAc before Gal but this would result in the termination of the oligosaccharide since Gal transferase would not transfer Gal to SA-GalNAc-thr/ser (Schachter et al., 1971). Similarly, Beyer et al. (1979) have shown that the preferred order of addition of the next

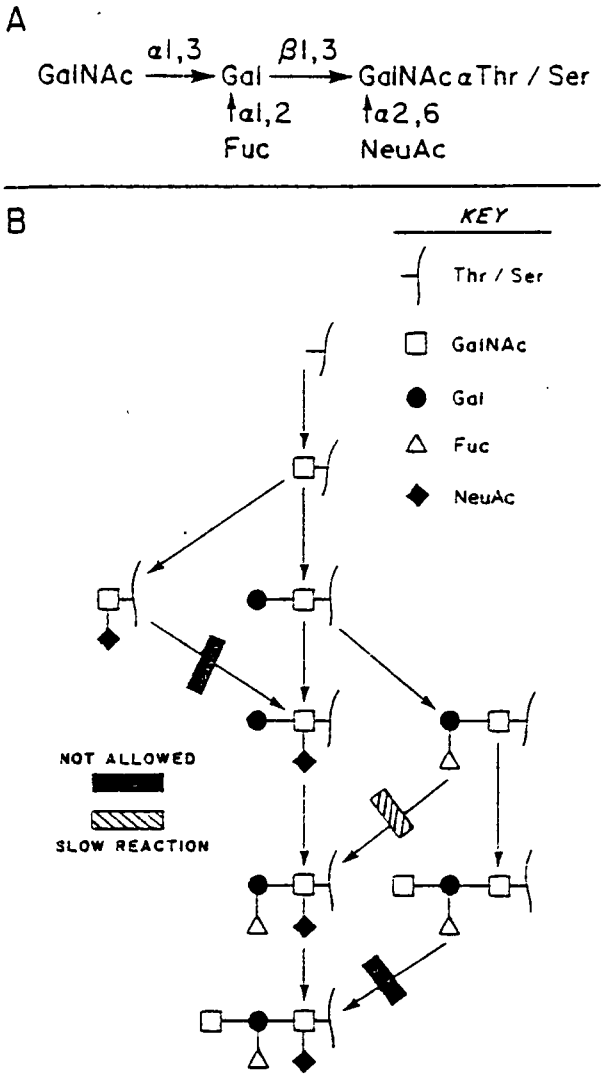
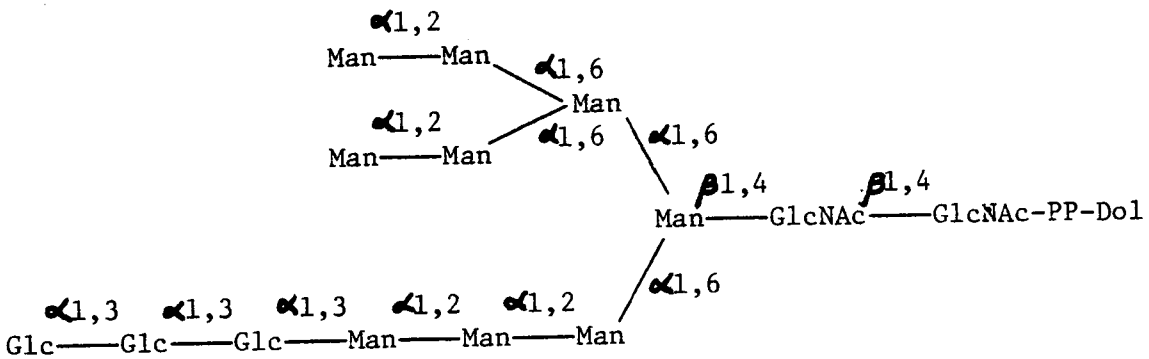


Figure 3. Biosynthesis of the O-linked oligosaccharides of porcine submaxillary mucin. A. The most complete oligosaccharide structure of the mucin. B. Alternate and preferred pathways of synthesis of this structure. (Taken from Berger, E. G., E. Buddecke, J. P. Kamerling, A. Kobata, J. C. Paulson and J. F. G. Vliegenthart. 1982. Structure, biosynthesis and functions of glycoprotein glycans. Experientia 38:1129-1258.)

residues to be SA, Fuc followed by GalNAc. Such an order is again dictated by the specific glycosyltransferase requirement of a specific acceptor-structure for the addition of the appropriate sugars. The addition of the sugars Gal, SA and Fuc to the GalNAc has been suggested to occur in the medial region of the Golgi whereas a terminal GalNAc may be added in the transportion of the Golgi (Dunphy and Rothman, 1985).

Assembly of N-linked Oligosaccharides

Different asparagine-linked oligosaccharides share a pentasaccharide core because a common pathway is involved in their biosynthesis. This occurs as a result of the common oligosaccharide-lipid dolichol phosphate (Dol-P) carrier:



The generation of this lipid-linked oligosaccharide intermediate is depicted in Figure 4. The first step starts with the reversible transfer of GlcNAc-1-phosphate from UDP-GlcNAc to Dol-P to yield GlcNAc-PP-Dol. This reaction is inhibited specifically by the antibiotic tunicamycin (Takatsuki et al., 1975; Tkacz and Lampin, 1975) which is thus a specific inhibitor of N-linked glycosylation. The next GlcNAc is added irreversibly to the GlcNAc-P-P-Dol also from UDP-GlcNAc

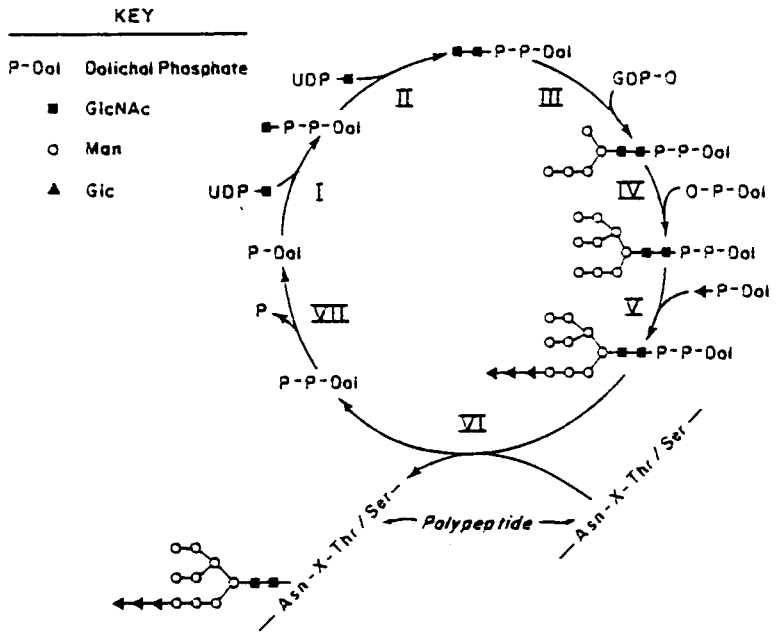


Figure 4. Synthesis of the lipid-linked oligosaccharide precursor on dolichol phosphate. (Taken from Berger et al., 1982.)

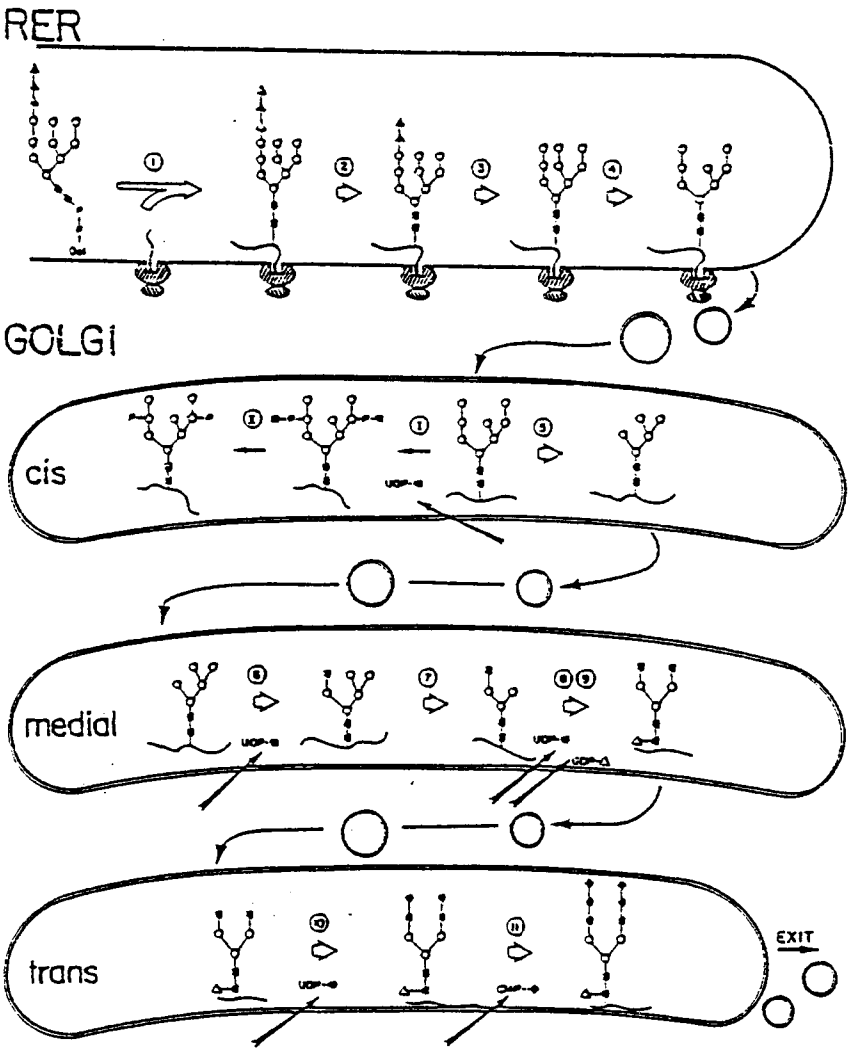
to form $(GlcNAc)_2$-P-P-Dol. This is followed by the stepwise transfer of 5 Man residues from the donor UDP-Man. Four more mannoses are next added onto $(Man)_5(GlcNAc)_2$-P-P-Dol in a similar stepwise manner but from Dol-P-P-Man donors. Finally, this precursor lipid-oligosaccharide structure is completed with the addition of 3 Glc residues from Dol-P-P-Glc. The site of transfer of the sugar residues to the lipid-dolichol carrier is not fully resolved but it has been proposed that some of these structures may be assembled in the cytoplasm and then translocated into the ER possibly by some protein mediated mechanism (Kornfeld and Kornfeld, 1985).

After the assembly of the oligosaccharide-dolichol intermediate, the oligosaccharide chain has been shown to be transferred en bloc to an acceptor polypeptide in the lumen of the ER (Hubbard and Robbins, 1979). This reaction is carried out by a portein-oligosacharidyl transferase which recognizes the asparagine (ASN) residue in the sequence ASN-X-Ser/Thr. Where X can be any amino acid except proline (see review by Struck and Lennarz, 1980). This reaction appears to require the presence of glucose in the lipid-linked oligosaccharide for efficient transfer suggesting that the presence of glucose may serve as a sort of signal for this process (Spiro et al., 1979; Hubbard and Robbins, 1979). The presence of this tripeptide signal, however, does not always result in glycosylation, as only 1/3 of the potential ASN-X-Ser/Thr sites on proteins are actually glycosylated (Kronquist and Lennarz, 1978). These same authors also suggest that the conformations of the rest of the polypeptide molecule around the acceptor site might be important in determining whether glycosylation would occur.

Processing of N-linked Oligosaccharides

Following the transfer of the oligosaccharide chains to the acceptor proteins, the precursor oligosaccharides undergo a series of modifications to produce the various N-linked structures. These processing events can occur cotranslationally as in VSV G protein in HeLa cells (Atkinson and Lee, 1984). Under special conditions of reduced temperature in HEp-2 cells, processing of HSV glycoproteins has been shown to occur post-translationally as well (Compton and Courtney, 1984). The pathway and subcellular localization of the various enzymatic reactions is depicted in Figure 5. The first reaction in this process is the removal of the terminal glucose residue by α1,2 glucosidase I soon after the transfer of the oligosaccharide to protein. α1,2 glucosidase II then removes the two inner glucose residues and this is followed by the removal of one or more mannose residues by ER-specific α-mannosidase (Kornfeld and Kornfeld, 1985). The removal of the glucose residues in the ER appears to be important in facilitating the vesicular transport of the oligosaccharide to the cis-cisternae of the Golgi apparatus (Lodish and Kong, 1984; Schlesinger et al., 1984).

Further processing of the oligosaccharide occurs as the glycoprotein passes through the different cisterna of the Golgi. In the cis cisternae of the Golgi the high-mannose oligosaccharide can be further trimmed by the Golgi α1,2 mannosidase I (Rothman et al., 1984 a and b). Most high-mannose carbohydrates on mature membrane glycoproteins are believed to result from the removal of a variable number of the α1,2 mannose residues by this enzyme. The



KEY:

The reactions are catalyzed by the following enzymes: (1) oligosaccharyltransferase, (2) α-glucosidase I, (3) α-glucosidase II, (4) ER α1,2-mannosidase, (I) *N*-acetylglucosaminylphosphotransferase, (II) *N*-acetylglucosamine-1-phosphodiester α-*N*-acetylglucosaminidase, (5) Golgi α-mannosidase I, (6) *N*-acetylglucosaminyltransferase I, (7) Golgi α-mannosidase II, (8) *N*-acetylglucosaminyltransferase II, (9) fucosyltransferase, (10) galactosyltransferase, (11) sialyltransferase. The symbols represent: ■, *N*-acetylglucosamine; ○, mannose; ▲, glucose; △, fucose; ●, galactose; ◆, sialic acid.

Figure 5. Schematic pathway of oligosaccharide processing on newly synthesized glycoproteins. (Taken from Kornfeld and Kornfeld, 1985.)

$(Man)_5(GlcNAc)_2$ structures that are destined to become complex structures are then first conjugated with a GlcNAc residue from UDP-GlcNAc in a reaction catalyzed by N-acetylglucosaminyl transferase I in the medial cisternae of the Golgi (Dunphy et al., 1985). The addition of the GlcNAc appears to provide the signal for the removal of the next two mannoses leaving behind the three Man residues found on the common pentasaccharide core. Subsequent addition of GlcNAc by N-acetylglucosaminyl transferase II on the Man, and Fuc residue by fucosyl transferase (to the innermost GlcNAc) continues in the medial cisternae of the Golgi (Dunphy and Rothman, 1985). The final additions of the terminal residues of Gal and SA by galactosyl and sialyl transferases respectively, have been shown to occur in the trans cisternae of the Golgi (Roth and Berger, 1982; Dunphy and Rothman, 1985). The processed membrane glycoproteins can then be transported to the plasma membrane where they can be incorporated into viral envelopes.

CHAPTER II

HERPES SIMPLEX VIRUS TYPE 2 (HSV-2) GLYCOPROTEINS

Introduction

The glycoproteins detected in HSV-2 infected cells can be differentiated into at least five antigenically distinct viral gene-products known as gB-2, gC-2, gD-2, gE-2 and gG-2. These proteins can be identified on sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis (PAGE) as molecules with average apparent molecular weights of 120, 75, 59, 90 and 108 kilodaltons (K) respectively. Of these glycoproteins, gB-2, gC-2, gD-2 and gE-2 have been shown to share type-common antigenic determinants, DNA sequence homology and colinear gene-arrangements with herpes simplex virus type 1 (HSV-1) glycoproteins gB-1, gC-1, gD-1 and gE-1, respectively. Because of the similarities between the glycoproteins of HSV-1 and HSV-2, many of the characteristics of the better studied HSV-1 glycoproteins have been applied to the HSV-2 glycoprotein as well.

Biosynthesis of HSV-glycoproteins

The general pathway for the synthesis of HSV glycoproteins is assumed to follow that described for other viral and cellular membrane glycoproteins (as described in the preceeding section). Briefly, after the synthesis of the polypeptide on polyribosomes associated with the ER, the nascent chains are believed to be vectorially translocated into the lumen of the ER where the N-linked high-mannose oligosaccharides

are added. Further processing of the glycoproteins then occurs as the glycoproteins are transported through the Golgi cisternae. Here high-mannose structures are believed to be processed to the complex types. O-linked carbohydrates and other modifications are added to the glycoproteins before their export to the cell surface membranes.

Recent findings lend support for these assumptions. Studies by Matthews et al. (1983) using an in vitro translation system have shown that HSV-1 gD synthesized in the absence of microsomal membranes contained presumably, a 3K signal peptide. They further showed that addition of dog pancreatic microsomes to this in vitro translation system resulted in the detection of gD products in the microsomes with the loss of this 3K signal peptide. Addition of three N-linked high-mannose oligosaccharide chains on gD also occurred when the microsomes were present. Synthesis of the high-mannose intermediates in the ER in vivo has also been suggested by the association of only high-mannose containing HSV glycoprotein precursors with the nuclear fraction of infected cells (Compton and Courtney, 1984).

Further processing of the HSV glycoproteins is assumed to occur in the Golgi. In the presence of monensin, an inhibitor of normal Golgi processing and transport functions, the processing of high-mannose HSV glycoprotein intermediates to complex forms was shown to be inhibited (Johnson and Spear,1982; Wenske et al., 1983) as were other modifications of the glycoproteins such as acylation and O-linked glycosylation (Johnson and Spear, 1983). In addition, in the presence of monensin, HSV glycoproteins were not transported to the cell surface suggesting the involvement of the Golgi in this process. Taken

together, these observations are consistent with the utilization of a similar processing and transport mechanism by HSV glycoproteins as has been described for other membrane glycoproteins (see also review by Kornfeld and Kornfeld, 1985).

Glycoprotein B-2

The mature gB glycoprotein of HSV-2 can be detected on PAGE as a component with an apparent molecular weight of 120K. Under non-reducing conditions oligomers of gB-2 of 250K molecular weight have also been detected in HSV-2 infected cells (Eberle and Courtney, 1982). Mapping studies using intertypic recombinants have located the gB-2 gene to a region between 0.30 to 0.42 map units on the HSV-2 genome, colinear with that for the gB of HSV-1 (Ruyechan et al., 1979). The region specifying gB-1 has now been narrowed to 0.348 to 0.366 map units on the HSV-1 genome (Bzik et al., 1984). Although finer mapping of gB-2 has not been published it is assumed that the open reading frame for gB-2 also resides in a similar region on the HSV-2 genome.

In addition to their colinear gene-location on their respective genomes, gB-2 and gB-1 also share many immunological and biochemical properties. Reactivity of both polyclonal and monoclonal antibodies have indicated gB-2 and gB-1 to possess common as well as unique antigenic determinants (Eberle and Courtney, 1980; Pereira et al., 1981; Vestergaard and Norrild, 1979). The presence of common antigenic sites have been confirmed by partial gene-sequence comparisons of the two glycoproteins (Bzik et al., 1984). Biochemically, gB-2 has been observed to have a similar apparent molecular weight to that of gB-1

and the synthesis of both glycoproteins involved the conversion of a gA (now designated at pgB 110) to the mature gB (Eberle and Courtney, 1980; Balachandran et al., 1982). Further studies of the processing of gB-1 have shown that it is synthesized from a non-glycosylated precursor (pgB 97), detected in the presence of tunicamycin, to the high-mannose intermediate pgB 110 which was subsequently processed to yield the mature gB-1 (Wenske and Courtney, 1983). Similar processing may presumably be involved in the synthesis of gB-2.

Functionally, gB-1 appears to be required for HSV-1 virion infectivity and fusion activity, which is putatively associated with the infectious process (Little et al., 1981; Sarmiento et al., 1979). Such a role for gB-2 has not been demonstrated. In HSV-2 infected cells, gB-2 was detected as early as 1-3 hours (h) post-infection and increasing amounts were made at 11 h (Balachandran et al., 1982).

Glycoprotein C-2

The gC-2 (formerly gF-2) can be detected in HSV-2 infected cells as 67, 69 and 75K components. Pulse-chase studies suggested that the 75K mature gC-2 is derived from the 67K and 69K components (Zezulak and Spear, 1983). In studies with the high-mannose specific endoglycosidase, endo H and α-O-N-acetylgalactosaminyl oligosaccharidase, which cleaves O-linked structures, Zezulak and Spear (1983) showed that the 67K and 69K components to represent different glycosylated high-mannose intermediates of the 75K. The mature 75K gC-2 was shown to contain both N-linked complex oligosaccharides as well as O-linked structures since it was endo H resistant but sensitive

to α-O-N-acetylgalactosaminyl oligosaccharides. The native polypeptide synthesized in an in vitro translation experiment has been shown to have a molecular weight of 60K (Swain et al., 1985).

gC-2 has recently been shown to map between 0.62 and 0.64 map units on the HSV-2 genome which is colinear with that of gC-1 [0.62 to 0.64 map units on the HSV-1 genome (Frink et al., 1983; Lee et al., 1982a; Para et al., 1983; Zezulak and Spear, 1984)] suggesting gC-2 to be the HSV-2 counterpart of gC-1. DNA sequencing data have also noted regions of homology between the coding sequence of gC-2 and gC-1 (Swain et al., 1985) and the presence of similar sequence homology has also been demonstrated by the observation that antibodies to a synthetic peptide corresponding to amino acids 128 to 139 of gC-1 also reacted with gC-2 (Zweig et al., 1984). Several other investigations have also noted cross-reactivity between the antisera against gC-1 and gC-2 (Zweig et al., 1983; Zezulak and Spear, 1983). However, similarities of gC-2 to gC-1 appear to be limited as Swain et al. (1985) have noted only limited homology between the coding sequence of gC-2 and gC-1 and the presence of a deletion near the region coding for the amino terminus that resulted in 31 fewer amino acids in gC-2 as compared with gC-1.

The role of both gC-1 and gC-2 appeared to be non-essential for viral replication though their lack of expression appeared to be associated with the syncytial marker (Zezulak and Spear, 1984). The precise role of these "true late" gene products (Balachandran et al., 1982; Compton and Courtney, 1985) is unknown at present.

Glycoprotein D-2

As compared with gC-2, gD-2 of HSV-2 appears to show marked similarities to its counterpart gD-1 of HSV-1. Common antigenic determinants between gD-2 and gD-1 have been demonstrated in cross-reactivity studies with both polyclonal and monoclonal antibodies (Eisenberg et al., 1982; Pereira et al., 1982) and Eisenberg and coworkers have further mapped the common antigenic determinants to specific epitopes on the two molecules (Eisenberg et al., 1985). Tryptic peptide analysis (Eisenberg et al., 1980; Balachandran et al., 1982) and partial amino acid sequence comparisons (Eisenberg et al., 1984) have shown only minor differences between gD-2 and gD-1. Colinear gene location within 0.90 to 0.945 map units on their respective genomic maps (Ruyechan et al., 1979; Watson et al., 1982) and extensive amino acid sequence homology (85% as deduced from their DNA sequences) (Lee et al., 1982b; Watson, 1983; Lasky et al., 1984), lend support for the common origin of gD-1 and gD-2.

The synthesis and processing of gD-2 appears to be also similar to that of gD-1. As mentioned previously, Matthews et al. (1983) have identified the presence of 3K signal sequence on gD-2 that was shown to be cleaved during the co-translational addition of three N-linked high-mannose chains to form pgD-2 of 52K molecular weight. The mature gD-2 of 59K is presumed to be further processed from pgD-2 by the addition of O-linked carbohydrates and processing to the N-linked complex structures.

Although gD-1 has been shown to be involved in cell fusion (Noble et al., 1983) no direct evidence of such a role for gD-2 has been

obtained. The feasibility of gD as a potential vaccine against both HSV-1 and HSV-2 is currently under investigation by several laboratories.

Glycoprotein gE-2

The gE glycoprotein has been shown to possess the receptor activity for the Fc-portion of immunoglobulin G detected on HSV-infected cells and HSV virions (Bauke and Spear, 1979; Para et al., 1982a). Both glycoproteins of HSV-1 and HSV-2 appear to be antigenically related (Para et al., 1982b) and studies with intertypic recombinants have also determined gE-1 and gE-2 to map colinearly to the unique short region within 0.924 to 0.951 of HSV-1 (Lee et al., 1982b) and 0.886-0.935 of HSV-2 (Hope and Marsden, 1983), respectively.

The synthesis of gE-2 is still poorly understood. Synthesis of gE-2 has been detected at 1-3 h post-infection but maximum synthesis was seen only at 3-5 h with decline of synthesis observed by 5-7 h (Balachandran et al., 1982). Pulse-chase experiments have indicated gE-2 to be synthesized from a 75K intermediate (Para et al., 1982b; Hope and Marsden, 1983). The mature gE of HSV-1 has been shown to contain N-linked oligosaccharides (Hope and Marsden, 1983) and the glycoprotein also appears to be sulphated (Hope et al., 1983; Hope and Marsden, 1983) and acylated in the Golgi (Johnson and Spear, 1983). Similar modifications may also be present on gE-2.

Glycoprotein G-2

The HSV-2 gG (formerly known as gC-2) is probably the least studied of the HSV glycoproteins. No cross-reactivity has been shown

with specific monoclonal and polyclonal anti-gG antibodies to any of the glycoproteins of HSV-1 (Eberle and Courtney, 1981; Balachandran et al., 1982; Marsden et al., 1978; Roizman et al., 1984). This glycoprotein has been shown to map within the unique short region of the HSV-2 genome in an area which does not code for any known HSV-1 or HSV-2 glycoprotein [Marsden et al. (1978) (who described at 92K component now shown to represent gG-2) and Roizman et al. (1984)]. Additionally, studies by Marsden et al. (1984) have further mapped the location of gG-2 to within 0.846 to 0.924 map units of the HSV-2 unique short region. Recent studies by Balachandran and Hutt-Fletcher (1985) have also reported on the identification of several potential gG-2 precursors synthesized in HSV-2 infected cells. The gG-2 glycoprotein is the subject of further investigation in this dissertation.

CHAPTER III

POSTTRANSLATIONAL CLEAVAGE OF VIRAL MEMBRANE GLYCOPROTEINS

Introduction

The processing of several viral membrane glycoproteins involve proteolytic cleavage of precursor molecules in addition to glycosylation to generate the final mature glycoproteins. Unlike the cotranslational removal of the signal peptides, this cleavage modification occurs after complete protein synthesis and translocation has been established. This form of posttranslational modification can be also distinguished from proteolytic breakdown events in that the cleavage involves specific breakage of peptide bonds of the precursor polypeptide that results in stable biologically active product(s). Such modification has been observed in the processing of the fusion and hemagglutination-neuraminidase glycoproteins, F and HN, respectively, of paramyxovirus, hemagglutinin, HA of orthomyxovirus, E_2 glycoprotein of togavirus and the envelope (env) glycoprotein of retrovirus. Results presented in this dissertation also suggest the possible involvement of posttranslational cleavage in the synthesis of gG-2 of HSV-2. Hence, the review will summarize some of the information on the cleavages which occur during processing of glycoproteins HA, F, HN, E_2 and env.

Synthesis of the Viral Glycoproteins HA, F, E_2, env and HN

The synthesis of these glycoproteins appears to follow the processing pathway as discussed previously for the viral and cellular

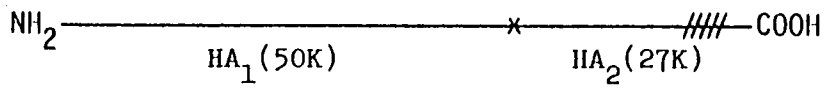
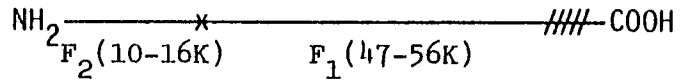
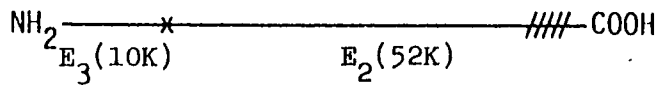
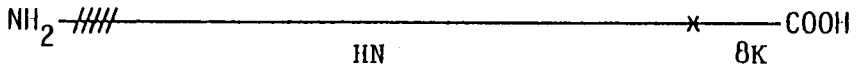
membrane proteins. Glycoprotein precursors for HA, F, E_2 and env have been shown to be synthesized on the rough ER where they are cotranslationally translocated into the ER and glycosylated (Porter et al., 1979; Blumberg et al., 1985; Bonatti et al., 1979; Hunter et al., 1983). In the case of the precursor to E_2 (pE_2), which is synthesized from a polycistronic 26S messenger RNA, insertion of the nascent polypeptide into the lumen of the ER requires the removal of the completed amino-terminal capsid protein from the growing polyprotein to reveal the signal sequence (Bonatti et al., 1979). Following translocation, the signal peptides are removed, except for pE_2 (Bonatti and Blobel, 1979) and the high-mannose sugars attached. The high-mannose containing precursors are then further processed to the complex glycoproteins in the Golgi. Additionally, the E_2 glycoprotein is also acylated (Schmidt and Schlesinger, 1980) and the cleavage event to be discussed below, then generates the mature glycoproteins.

The synthesis of HN appears to differ from that of the other glycoproteins HA, F, E_2 and env in that the protein is anchored to the membrane by the amino terminal portion of the polypeptide. In amino acid sequencing experiments the carboxyl end of the HN is shown to lack hydrophobic amino acids. Instead the amino-terminus is blocked from Edman degradation suggesting this end to be associated with the membrane (Schuy et al., 1984). Recent nucleic acid-sequencing of HN has revealed a single hydrophobic stretch of amino acids sufficient in length for a membrane anchor sequence at the amino terminal (Heibert et al., 1985). The mechanism of translocation of this protein is still

unclear, but it is assumed that the addition of three N-linked oligosaccharide chains occur by a similar glycosylation pathway as with other glycoproteins (Prehm et al., 1979).

Site of Cleavage

The posttranslational modification by cleavage of the glycoprotein precursors of HA, F, E_2 and env appear to involve very similar cleavage patterns. The cleavage sites and the arrangement of the products on the precursor molecules are depicted in Figure 6. In each instance, the precursor molecule is anchored in the membrane by a stretch of hydrophobic amino acids termed the "anchor sequence" at the carboxyl-end of the molecule such that cleavage of the precursor generates two fragments on the same side of the membrane, with the carboxy-fragment remaining inserted into the membrane by the carboxy-terminus. The resulting amino-fragment can be either attached to the carboxy-fragment by disulfide-bonds as with HA, F and env (Wilson et al., 1981; Harwick and Bussell, 1979; Pauli et al., 1978) or noncovalently associated as in E_2 and E_2 (Garoff et al., 1982). A similar tetrapeptide sequence is present at the cleavage site of the precursors of HA, F, E_2 and env (see Figure 7). This basic amino acid region may constitute a specific cleavage site recognized by the trypsin-like host protease shown to cleave the precursors of F (Choppin and Scheid, 1980), suggesting that a similar post-translational cleavage mechanism may be involved in the processing of these glycoproteins.

Precursor	Structural arrangement	Reference
HA_0		Porter et al., 1979.
F_0		Matsumoto et al., 1982.
pE_2		Garoff et al., 1980.
$pr95K_{env}$		Hunter et al., 1983.
HN_0		Hiebert et al., 1985.

KEY:

⋇ site of cleavage

//// anchor sequence

Figure 6. Structural arrangement of precursors HA_0, F_0, pE_2, $pr95K_{env}$, and HN_0.

Sequence	Virus	Reference
...-arg ser-lys-arg↑ser-... E_3 ↑ E_2	Sindbis	Rice et al., 1981.
...-arg-ser-arg-arg↑ala-... gp60 ↑ p21	HTLV	Seiki et al., 1983.
...-arg-val-arg-arg↑ser-... gp60 ↑ p30	BLV	Rice et al., 1984.
...-arg-ala-lys-arg↑phe-... gp52 ↑ gp36	MMTV	Dickson and Peters, 1983.
...-arg-arg-lys-arg↑ser-... gp85 ↑ gp37	RSV	Hunter et al., 1983.
...-arg-his-arg-arg↑ser-... E_3 ↑ E_2	Semliki forest virus	Garoff et al., 1980.
...-arg-glu-lys-arg↑gly-... HA_1 ↑ HA_2	Influenza	Porter et al., 1979.

Figure 7. Basic tetrapeptide associated with the cleavage sites. The cleavage site is indicated by the arrow (↑).

The cleavage of HN appears to be different from that of the precursors of HA, F, E_2 and env due to the anchorage of HN at the amino-terminal of the molecule. In virulent strains of Newcastle disease virus (NDV), the glycosylated precursor HN_o is cleaved to generate a 74K active HN and a glycopeptide from the carboxy-terminal of 8K (Nagai et al., 1976a), which is absent from the virion (Garten et al., 1980). Examination of the nucleic acid sequence of the HN gene, (Heibert et al., 1985) has shown no basic tetrapeptide that would indicate a cleavage site for trypsin-like enzymes. Not surprisingly, the cleavage generation of the 8K and active HN can be achieved by a variety of enzymes including trypsin, elastase and thermolysin (Nagai and Klenk, 1977). Though such cleavage has not been observed with the HN of many other paramyxovirus, in NDV, the cleavage event appears to be important in the activation of the biological function of HN (Garten et al., 1980).

Location of Cleavage Event

The cellular location of the cleavage event by the common trypsin-like host protease for precursors of HA, F, E_2 and env is unresolved. With the env glycoprotein of Rous sarcoma virus, cleavage of the 95K env precursor has been shown to occur after the processing of high-mannose to the complex glycoprotein in the Golgi since only complex structures were detected on the products gp85 and gp37 (Wills et al., 1984). However, pulse-chase studies by Klemenz and Diggelman (1977) have shown no detectable intracellular gp85 and gp37 suggesting that the precursor is cleaved extracellularly. With Sendai virus,

cleavage of F_0 to generate active virus in chick cells can be blocked by the extracellular addition of trypsin inhibitors resulting in inactive virus (Muramatsu and Homma, 1980). In another paramyxovirus, Newcastle disease virus, the cleaved F products has been demonstrated in both the intracellular smooth membranes presumably the Golgi and the plasma membranes (Nagai et al., 1976b). The conversion of the togavirus pE_2 to E_2 and E_3 is important for the association of E_2 with E_1 in the viral budding process. This cleavage event has been shown to take place predominantly on the plasma membrane (Jones et al., 1974) where virus assembly occurs (Smith and Brown, 1977; Scheefers et al., 1980). Although fractionation studies have detected some cleaved influenza virus HA in smooth membranes (Rott et al., 1978) cleavage is thought to predominantly occur extracellularly (Lazarowitz et al., 1973) and to be mediated by trypsin-like proteases abundant in the extracellular environment of the respiratory tract where the virus colonizes (Murphy and Webster, 1985). It thus appears that cleavage may occur after the synthesis of the precursors in the Golgi and/or the plasma membrane depending on the virus and host cells.

The prerequisite that glycoprotein processing occurs before cleavage has been studied with the inhibitor, tunicamycin. In the presence of tunicamycin, the env precursor of Rous sarcoma virus has been detected on the cell surface as an uncleaved, nonglycosylated polypeptide of 58K (Stohner and Hunter, 1979). Nonglycosylated pE_2, on the other hand, was found to be insoluble and hence not transported to the infected cell surface (Leavitt et al., 1977) nor cleaved (Garoff and Schwartz, 1978). With the F protein, inhibition of glycosylation

has no effect on the cleavage of F_0 or the incorporation of the F protein into viral particles. Such unglycosylated, cleaved proteins, F_1 and F_2 can be found in viral particles synthesized in the presence of tunicamycin (Strauss and Strauss, 1983).

Mechanism of Cleavage Activation of F Protein

The absolute requirement of cleavage in the activation of glycoproteins HA, F, E_2, env and HN is assumed to be due to exposure of active sites following cleavage. This is most notably demonstrated by studies performed with the F protein. UV-circular dichroism spectra analysis of the precursor and products of F showed a spectra shift with cleavage indicative of conformational change of the protein (Kohama et al., 1981; Hsu et al., 1981). Detergent binding studies by velocity sedimentation analysis of Triton X-100 F protein complexes, showed increased binding of detergent after cleavage of F_0 resulting from an increase in the exposed hydrophobic surface (Hsu et al., 1981). Such an increase in hydrophobic surface has been predicted by Blumberg et al. (1985) from their nucleic acid sequence of the F gene. The involvement of these hydrophobic regions in the fusion activity of the F protein is supported by an unusually hydrophobic polypeptide sequence uncovered by cleavage at the amino-terminal of the F_1 fragment (Gething et al., 1978; Scheid et al., 1978; Richardson et al., 1980). This hydrophobic amino acid sequence appears to be conserved among the F_1 fragments of different paramyxoviruses (Matsumoto, 1982). The importance of this sequence in fusion is supported by the finding that oligopeptide structures resembling the hydrophobic sequence can inhibit

the fusion activities of F and HA (Richardson et al., 1980). Conformational change may also be involved in the activation of other glycoproteins.

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PART II

CHARACTERIZATION OF THE SYNTHESIS AND PROCESSING OF THE HERPES SIMPLEX VIRUS TYPE 2 GLYCOPROTEIN, gG

CHAPTER I

INTRODUCTION

The glycoproteins specified by herpes simplex virus type 2 (HSV-2) can be classified into 5 antigenic groups designated gB-2, gC-2, gD-2, gE-2 and gG-2. These glycoproteins are exposed on the surface of infected cells and viral envelopes where they are important for the induction of immune responses to the virus. They also play key roles in the infectious process of the virus such as the entry of the virus into cells, cell-to-cell virus spread and influencing viral tissue tropism and host range (Spear, 1984). The study of these viral glycoproteins is thus imperative in providing a better understanding of their role in the pathogenicity of the virus as well as in providing a tool for elucidating the molecular events which occur within the cell.

Considerable progress has been made in the characterization of the synthesis of herpes simplex virus (HSV) glycoproteins, but the synthesis of certain glycoproteins of HSV-2, particularly a glycoprotein recently designated gG-2, are still poorly understood. Traditionally, the HSV-1 glycoproteins have provided a model system for studies of the glycoproteins of herpes simplex viruses. Because of the similarities of the HSV-1 and HSV-2 glycoproteins, information and reagents developed from studies of HSV-1 glycoproteins have also been applied to the investigations of HSV-2 counterparts. Unlike the glycoproteins gB-2, gC-2, gD-2 and gE-2 of HSV-2, no counterpart to gG-2 has been described in HSV-1. The pathway of maturation of gG hence remains unresolved. Preliminary observations in the laboratory

have suggested that the pathway of synthesis of HSV-2 gG to be different from that reported for the other HSV glycoproteins. Thus, it was the overall objective of this investigation to identify and characterize the various intermediates that were involved in the biosynthetic pathway of gG-2. The specific aims of this project were to:

1) Identify the intermediates of gG synthesis with the use of inhibitors of glycoprotein processing, monensin and tunicamycin.

2) Localize the different intermediates in the nuclear versus the cytoplasmic compartments.

3) Determine the time of the synthesis of gG and its intermediates.

4) Characterize the glycoprotein intermediates with glycosidic enzymes.

5) Determine the processing precursor-product relationship of different intermediates.

6) Determine the approximate size of the primary gG translation product in an _in vitro_ translation system.

CHAPTER II

MATERIALS AND METHODS

Cells and Cell Culture

Cell cultures used in these studies included human epidermoid number 2 (HEp-2), African green monkey kidney (Vero) and human embryonic lung (MRC-5) cells. Cells were propagated in Eagle's media (Auto-Pow minimum essential media, Flow Laboratories) supplemented with 10% donor calf serum (DCS) for HEp-2 cells or 10% fetal bovine serum (FBS) for Vero and MRC-5 cells, and 0.075% sodium bicarbonate. Infected cells were maintained in Eagle's medium containing 2% DCS and 0.025% sodium bicarbonate (maintenance medium) or as otherwise indicated.

Viruses

The HSV-2/186 strain was primarily used throughout this research project unless indicated otherwise. Other laboratory strains of HSV-2 used were designated G and 333 (obtained from Bernard Roizman, University of Chicago), and two recent clinical isolates designated DG and JP. The HSV-1/F strain was also used in some of these studies.

Virus stocks were prepared by infecting HEp-2 cells at a low multiplicity of infection (MOI), approximately 0.01 plaque forming units per cell (pfu/cell). Cultures were harvested when the infected cells exhibited 3+ to 4+ cytopathic effect and the virus was released by freeze-thaw and ultrasonication in maintenance medium. Virus titrations were conducted on Vero cell-monolayers (Bone and Courtney,

1974). Serial ten-fold dilutions of virus stocks were inoculated onto the monolayers and after a 1 h virus adsorption period at 37°C, excess inoculum was removed and medium containing 2% methylcellulose was added. The cells were incubated at 37°C in a 5% CO_2 humidified atmosphere. Three days after infection a solution of neutral red (1:10,000 in PBS) was added and the virus plaques were counted following an overnight incubation.

Infection and Metabolic Labelling of Cell Cultures

Confluent monolayers were used for all experiments unless otherwise noted. Cell cultures were infected with an input virus MOI of 10 to 50 pfu/cell, unless otherwise stated. Following a one hour virus adsorption at 37°C, the excess inoculum was removed, the cell monolayers washed twice with warmed phosphate buffered saline (PBS), pH 7.4 and maintenance medium with or without the appropriate inhibitors was added. The inhibitors used in this study included monensin (Calbiochem) at 10^{-6} molar (M), tunicamycin (Sigma) at 1.25 microgram/milliliter (μg/ml) or 10 μg/ml, cytosine arabinoside (Sigma) at 50 μg/ml and cycloheximide (Sigma) at 50 μg/ml final concentrations. The infected cultures were incubated in a humidified atmosphere with 5% CO_2 at 37°C.

In radiolabelling experiments (except pulse-chase) isotopic labelled precursors were added to the cultures at 4 h postinfection. When (^{3}H) fucose (ICN, specific activity of 30 curie/millimole, (Ci/mmol), (^{3}H) galactose (ICN, specific activity of 10-25 Ci/mmol), (^{3}H) glucosamine (ICN, specific activity of 40 Ci/mmol) and (^{3}H)

mannose (ICN, specific activity of 10.4 Ci/mmol) were used, maintenance medium containing 1/10 the normal concentration of glucose was used. Similarly, when (^{35}S) methionine (Amersham, specific activity of 1390 Ci/ml) or (^{3}H) amino acids [(ICN, specific activity, 273 millicurie/milligram) (mCi/mg)] were added to the cultures, medium containing 1/10 the normal concentration of methionine or amino acids, respectively, was used.

Harvest of Infected Cell Cultures

At the indicated times, cell cultures were harvested by scraping the cell monolayers into the medium with a rubber policeman. The cells were pelleted by centrifugation at 1,600 x g for 5 to 10 minutes and either fractionated into cytoplasmic and nuclear fractions or frozen immediately at -20°C. Prior to use the frozen pellets were resuspended in water at 1 x 10^7 cells/0.5 ml and disrupted by ultrasonication for 2 minutes.

Cytoplasmic and nuclear fractions were obtained using the method described by Penman (Penman, 1966) with minor modifications. Briefly, following two washings with PBS the cell pellets were resuspended at a concentration of 1 x 10^7 cells/ml in reticulocyte standard buffer (RSB) containing 10 millimolar (mM) Tris HCl (pH 7.4), 10 mM KCl and 1.5 mM $MgCl_2$ and incubated on ice for 15 minutes. The swollen cells were then disrupted by 20 strokes in a Dounce homogenizer and centrifuged at 250 x g for 5 minutes to pellet the nuclei. The supernatant (cytoplasmic fraction) was removed, brought to 1% NP-40 in RSB, incubated for 10 minutes at 37°C, and recentrifuged at 600 x g for

10 minutes to remove insoluble material. The supernatant consisting of the clarified cytoplasmic fraction was stored at -20°C. The nuclear pellet was washed twice in RSB followed by a wash in 1% NP-40 in RSB. The pellet was then washed twice in RSB, resuspended in water at a concentration of 1 x 10^7 nuclei/ml, disrupted, and frozen at -20°C until use. The purity of the nuclear fraction was verified by light microscopy.

Immunofluorescence Test

The indirect immunofluorescence test was performed essentially as described by Porter et al. (1969). HEp-2 cells were seeded and grown in 60 mm plates containing coverslips. The subconfluent monolayers were infected with HSV-2 at an input MOI of approximately 0.05 pfu/cell. After 1 h virus adsorption, the inoculum was removed and the cells were incubated in media with or without monensin (10^{-6} M) or tunicamycin (10 μg/ml). At 24 h postinfection the coverslips were removed and washed three times for 5 minutes each in PBS at room temperature. For membrane immunofluorescence the washed coverslips were incubated with 20 microliters (μl) of rabbit anti-gG serum (see below) or normal rabbit serum (each at 1:10 dilution in PBS). For internal immunofluorescence however, the coverslips were fixed in acetone for 5 minutes and then air-dried. Before staining, the cells were rehydrated in PBS for 5 minutes and then reacted with the anti-gG or normal rabbit serum. Following a 30 minute incubation, the coverslips for both membrane and internal immunofluorescence were subjected to three consecutive 5-minute washes in PBS and then reacted

with fluorescein-conjugated goat anti-rabbit antibody (GAR-FITC) for another 30 minutes. The coverslips were again washed three times in PBS, dipped once in distilled water and mounted in a glycerol solution (1:2 dilution in PBS) on microscopic slides. The coverslips were examined using a Nikon microscope with an ultra-violet light source for immunofluorescence.

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

Analytical and preparative SDS-PAGE was performed as described previously (Powell and Courtney, 1975). Samples were electrophoresed through a 7% acrylamide gel cross-linked with N,N^{1}-methylene-bis-acrylamide (BIS) throughout this study. Prior to electrophoresis samples were thawed, solubilized by sonication and then denatured by boiling for 2 minutes in an equal volume of a solution (2 x SUM) containing 2% SDS, 2% β-mercaptoethanol and 1 M urea in 0.05 Tris-HCl (pH 6.7).

Following analytical SDS-PAGE, gels of radiolabelled samples were first fixed in destaining solution (25% methanol, 7% glacial acetic acid) for 30 minutes and then immersed in Enlightening (New England Nuclear) for 15 minutes before drying under vacuum onto Whatman grade 2 paper in a gel-dryer (Hoefer Scientific). The gels were exposed to Kodak XAR-5 X-omat film at -70°C.

Appropriate peaks from preparative SDS-PAGE were pooled and concentrated using an Amicon ultrafiltration cell equipped with a YM-10 membrane (Amicon) and the concentrate stored at -20°C until use for SDS-HTP chromatography.

Immunoblotting

The immunoblotting procedure used was essentially as originally described by Towbin et al. (1979) with modifications described in detail by Compton and Courtney (1984). Briefly, proteins were first analyzed by SDS-PAGE and the resolved proteins were electrophoretically transferred onto nitrocellulose paper (Schleicher and Schuell) at 100 volts for 2 1/2 to 3 h in an electrophoretic blotting unit (Hoeffer). The nitrocellulose paper (blot) was then incubated for 1 h with a NT buffer [0.01 M Tris-HCl (pH 7.4), 0.9% NaCl] containing 3% bovine serum albumin (wt/vol) to bind free sites on the paper. Next, the blot was washed with deionized water and reacted with polyclonal or monoclonal anti-gG serum or polyclonal anti-pgG serum, diluted 1:50 in NETG buffer 0.05 M Tris-HCl (pH 7.4), 0.15 M NaCl, 5 mM EDTA, 0.25% gelatin (wt/vol) with 0.05% NP-40. After a 2 h reaction at room temperature the blot was washed 10 times with deionized water followed by the addition of (^{125}I)-protein A (diluted in NETG containing 0.05% NP-40, to a concentration of 10^6 counts per minute/ml). Following a 2 h incubation at room temperature, the blot was washed 10 times with 0.5% Triton X-100 and 0.1% SDS in NETG buffer and then rinsed with deionized water. The immunoreactive proteins were visualized following autoradiography using Kodak XAR-5 X-omat film.

Solubilization of Antigens for Immunoprecipitation and Endo H Digestion

Cell samples were first solubilized in 1% SDS solution by boiling for 2 minutes. The free SDS was then removed by dilution into 4

volumes of a solution containing 1.25% Triton X-100 in TNE [10 mM Tris-HCl (pH 7.4), 10 mM NaCl, 1 mM EDTA] (Anderson and Blobel, 1983). The solubilized extract was then centrifuged at 100,000 x g for 30 minutes to remove particulate material. Following centrifugation, the supernatant was saved and served as "solubilized antigen" preparation for the procedures described below.

SDS-hydroxylapatite (SDS-HTP) Chromatography for Fractionation of HSV-2 Glycoproteins

The SDS-HTP chromatography was performed as essentially reported by Moss and Rosenblum (1972) and has been described in detail elsewhere (Eberle and Courtney, 1980). Briefly, HTP (Bio-Rad) was washed in 0.01 M sodium phosphate buffer (pH 6.4) containing 0.01% SDS to remove the fines. The washed HTP was poured into a column (Glenco), allowed to settle and washed with a 0.01 M sodium phosphate buffer (pH 6.4) containing 0.01% SDS and 1 mM dithiothreitol. Pooled fractions from preparative SDS-PAGE and concentrated by ultrafiltration were boiled in 0.1% SDS-1 mM dithiothreitol for 30 seconds and then loaded onto the HTP column. Proteins were then eluted by a linear gradient of sodium phosphate.

Purification of HSV-2 Glycoproteins

The glycoproteins were purified as described by Eberle and Courtney (1980). Briefly, HSV-2 infected cells were metabolically labelled with (^{3}H) glucosamine from 4 to 24 h after infection, harvested and solubilized for SDS-PAGE. The samples were electrophoresed on preparative SDS-PAGE and fractions of a peak

containing gG, pgG, gB or pgB were pooled, concentrated and dialyzed with an Amicon ultrafiltration cell equipped with a YM-10 membrane (Amicon). The purified proteins were further resolved using SDS-HTP chromatography. Following HTP chromatography, peaks of radioactivity representing glycoproteins gG, pgG and the gB were obtained. The gG and pgG peak fractions were each pooled, concentrated and dialyzed, and rechromatographed until homogenous peaks of gG and pgG were obtained as determined by SDS-PAGE. The purified glycoproteins were resuspended in 0.1% SDS and stored at -20°C.

Antibody Preparation

Monoclonal gG antiserum (13 C5) was a kind gift from N. Balachandran (Balachandran et al., 1982). Rabbit anti-gG and anti-pgG antisera were prepared previously by Richard Eberle in our laboratory (Eberle and Courtney, 1980). The purified peaks of gG or pgG from preparative SDS-PAGE followed by SDS-HTP chromatography just described were each electrophoresed on cylindrical gels. Segments containing these glycoproteins were cut out from the gels, emulsified with Freund's adjuvant and each injected into rabbits to produce the anti-gG or anti-pgG sera.

Immunoprecipitation

Immunoprecipitations were performed as previously described (Eberle and Courtney, 1980). A 100 µl volume of solubilized antigen was reacted with 50 µl of antiserum incubated overnight at 4°C, followed by a 4 to 6 h incubation with 50 µl of either Staphylococcus aureus cells (Pansorbin, La Jolla, CA) or 100 µl Protein A conjugated

Sepharose CL-4B beads (Pharmacia). The immunoprecipitates were pelleted by centrifugation at 1600 x g and washed 3 times in TNE containing 0.1% Triton X-100. After the final wash the immunoprecipitates were solubilized in 60 µl of 2 x SUM and analyzed by SDS-PAGE.

Endo H Digestion

Removal of high-mannose carbohydrate chains was accomplished with the use of the endo-β-N-acetylglucosaminidase H (endo H) as described by Wenske et al. (1982). Solubilized samples were made 0.2% and 50 mM with respect to SDS and sodium citrate (pH 5.5), respectively. Treated samples were reacted with endo H (purchased from Division of Laboratories and Research, New York State Dept. of Health, Albany, NY) at a final concentration of 0.025 units/ml in a 120 µl volume. An appropriate amount of buffer was added to the control-untreated samples. After 6 h incubation at 37°C the proteins were precipitated with 1 ml of acetone at -20°C and pelleted at 130,000 x g. The protein pellets were resuspended in 2 x SUM and analyzed by SDS-PAGE followed by immunoblotting.

Endo F Digestion

The reaction with endo-β-N-acetylglucosaminidase F (endo F) to cleave high-mannose and complex oligosaccharide chains was carried out essentially as described by Elder and Alexander (1982) and Clark Edson (personal communication). To a 33 µl purified gG sample (approximately 10 ng/ml), 1 µl of mercaptoethanol, 1 µl of 5% SDS and 10 µl of a 5X buffer [0.25 M Tris HCl (pH 8.5), 0.25 M EDTA and 0.25% NaN_3] were

added and the mixture was then boiled for 5 minutes. After cooling, 5 µl of 10% NP-40 was mixed with the sample, followed by the addition of a 5 µl endo F (NEN) containing solution at a final concentration of 15 or 30 units of enzyme/ml. The reaction mixture was incubated overnight at 37°C in the presence of 1 mM PMSF (Sigma). The products were precipitated with acetone and analyzed by SDS-PAGE and immunoblotting as described above.

Neuraminidase Digestion

HSV-2 infected HEp-2 cells cultured in the presence or absence of monensin (10^{-6} M) were solubilized in a solution of 1% Tween-40 and 1% deoxycholate (DOC). The solubilized extracts were adjusted to 0.1 M sodium acetate (pH 5.5) and 0.005 M $CaCl_2$. Neuraminidase (Sigma) at 4 units/ml was added to the treated samples to give a final concentration of enzyme of 1 unit/ml. After a 2 h incubation at 37°C, the products were precipitated with acetone and analyzed by SDS-PAGE followed by immunoblotting with anti-gG serum.

Pulse-chase Experiments

Confluent HEp-2 cell cultures in 35 mm dishes were infected with HSV-2/186 strain at an input MOI of approximately 50 pfu/cell and grown in maintenance medium containing 1/10 the normal concentration of methionine or glucose, for (^{35}S) methionine or (^{3}H) mannose labelling, respectively. At 10 h postinfection, the cells were pulsed for 20 minutes or 4 h (as indicated) with 200 Ci/ml of (^{35}S) methionine or (^{3}H) mannose in a 1 ml volume. At the end of the pulse, the cells were washed twice with maintenance medium and further

incubated. At varying times after the pulse, the samples were harvested, solubilized and immunoprecipitated with the anti-gG serum. The immunoprecipitates were then analyzed by SDS-PAGE.

Isolation of RNA

HEp-2 cells infected with HSV-2 were harvested at 18 h post infection and total cellular RNA was isolated by the guanidinium thiocyanate-CsCl extraction method essentially as described by Glisin et al. (1974). The cell pellet was solubilized in a 4 M guanidinium thiocyanate solution containing 25 mM sodium citrate and 0.2% Sarkosyl at concentration of 1 x 10^7 cells/ml. RNA was isolated by pelleting through an 18 ml CsCl solution [5.7 M CsCl, 100 mM EDTA (pH 7.5)] by centrifugation in a Beckman SW-27 rotor at 24,000 revolutions per minute for 20 h at 15°C. The pelleted RNA was next resuspended in TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] at 200 µl/10^8 cells equivalent. The RNA was then washed by ethanol precipitation, following addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.2 volumes of absolute ethanol, at -20°C and recovered by centrifugation at 12,000 x g at -10°C for 10 minutes. The RNA was washed twice more and stored in 70% ethanol until use.

Selection of Poly A^+ RNA

Before poly A^+ RNA selection, the total RNA was pelleted, dried and resuspended in water containing 100 units/ml of RNasin (P-L Biochemicals) at a RNA concentration of 0.5-1 mg/ml. The method for selection of mRNA was performed following basically that described by Maniatis et al. (1982). Total RNA, suspended in loading buffer [20 mM

Tris HCl (pH 7.6) 0.5 M NaCl, 1 mM EDTA and 0.1% SDS] was added to a 1 ml column of oligo (dT) cellulose type 7 (P-L Biochemicals) followed by washing with 5 to 10 mls of loading buffer. Elution of the poly A^+ RNA was carried out with 2 to 3 mls of a low salt elution buffer [10 mM Tris HCl (pH 7.5), 1 mM EDTA, 0.05% SDS]. The selected poly A^+ RNA was made 0.3 M with respect to sodium acetate (pH 5.4) and precipitated in 2.2 volumes of ethanol at -20°C.

In vitro Translation

Isolated poly A^+ RNA was resuspended in water at an approximate concentration of 100 µg/ml in the presence of 100 units/ml of RNasin, heated at 65°C for 5 minutes and then added to a rabbit reticulocyte translation kit (BRL) containing 125 mM potassium acetate and 2 mM magnesium acetate. 0.05 or 1 µg of poly A^+ RNA was added to the translation mixture in a total volume of 120 µl. At the end of a 2 h incubation at 30°C, the mixture was solubilized in 1% SDS, boiled for 2 minutes and centrifuged at 13,000 x g for 5 min to remove insoluble material. The supernatant was then diluted in 4 volumes of 1.25% Triton X-100, TNE buffer and immunoprecipitated with anti-gG serum. The immunoprecipitates were analyzed by immunoblotting with anti-gG serum. Control globin mRNA was incubated in the presence of 5 µCi/ml of (^{35}S) methionine in place of water (under otherwise identical conditions), in a total translation mixture volume of 30 µl, solubilized and analyzed on the immunoblot without prior immunoprecipitation.

CHAPTER III

RESULTS

Introduction

The primary objective of this research was to characterize the synthesis and processing of the glycoprotein, gG of HSV-2. The focus of this study was to provide the identification and characterization of the intermediates involved in the synthetic pathway of gG. The synthesis of gG is assumed to occur by the membrane glycoprotein synthetic pathway described previously. Translation of gG messenger RNA is presumed to occur on polyribosomes associated with the ER where the nascent polypeptide is translocated into the lumen. Following translocation, the nascent polypeptide is probably glycosylated in the ER and then further processed through passage in the ER and Golgi before the mature gG is transported to the plasma membrane or incorporated into the mature virus particles.

The approach used in this study is depicted in Figure 1. Throughout this study, the detection and identification of gG and its intermediates were carried out by the use of monospecific anti-gG antibodies. These antibodies were first extensively characterized to determine their gG-specific reactions. To facilitate the detection of gG intermediates involved in the different synthetic steps in the ER and the Golgi, two inhibitors of glycoprotein processing were used. Tunicamycin, an inhibitor of N-linked glycosylation in the ER would allow the study of intermediates whose processing was inhibited at the level of the ER. Any unglycosylated intermediates detected in the

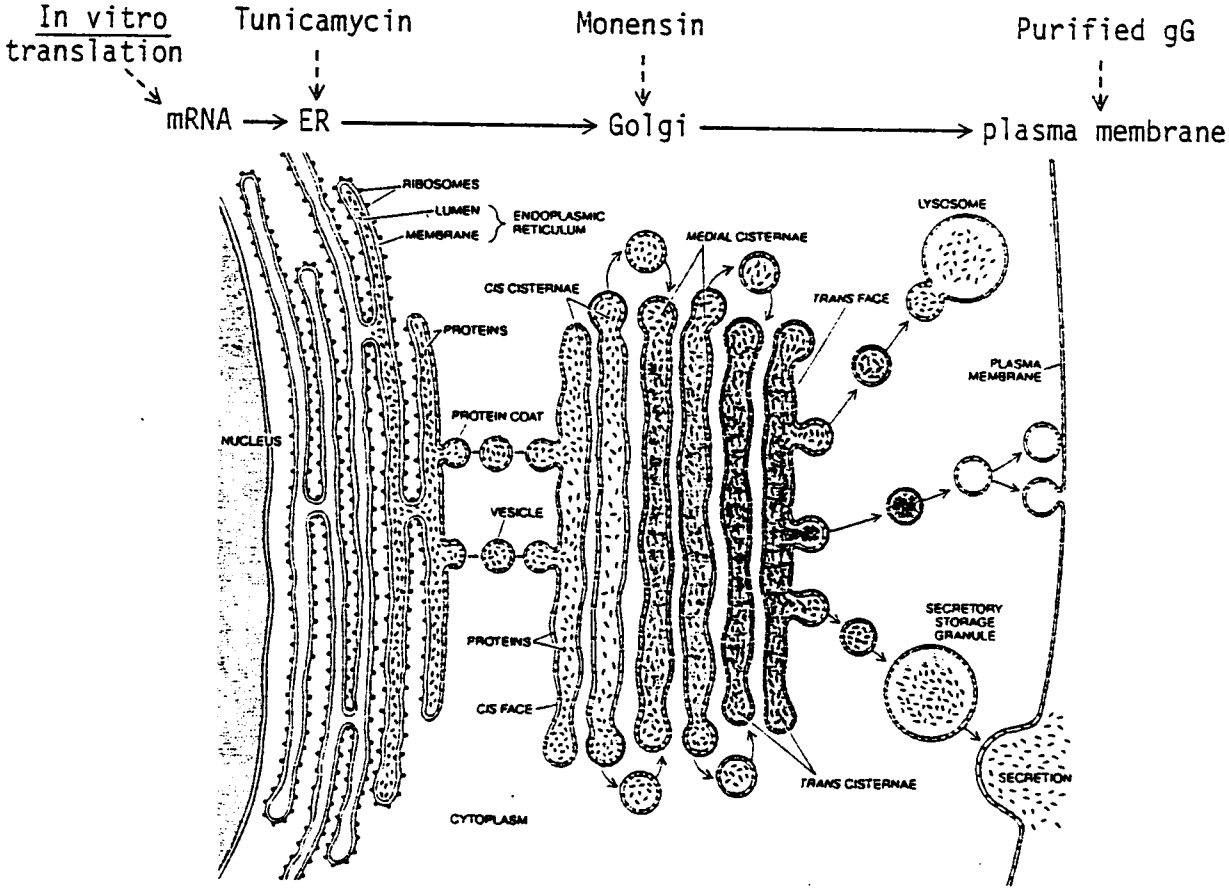


Figure 1. Strategy for the study of gG synthesis. Characterization of the different stages of gG processing are indicated (↓). (Diagram is modified from Rothman, J. E., 1985.)

presence of tunicamycin were also compared with the primary translation product of gG in an in vitro translation system. On the other hand, monensin which disrupts the normal transport and processing functions of the Golgi would hence allow the detection of intermediates that were to be processed in the Golgi. The intermediates identified were then further characterized by glycosidic enzymes specific for glycoprotein precursors to determine the extent and type of glycosylation on each of these intermediates. The cellular localization of these different intermediates were also examined by cell-fractionation and immunofluorescence experiments. Finally, how these different glycoprotein intermediates were related to each other in gG synthesis was determined by kinetic studies of their synthesis and in pulse-chase experiments. From these results, a model for the pathway of gG synthesis is presented.

Isotopic Labelling of HSV-2 Glycoproteins and the Effect of Inhibitors on Their Synthesis

Isotopic Labelling of HSV-2 Glycoproteins. From the previous discussion on the processing of oligosaccharide chains, it has been shown that different sugar residues are incoporated into oligosaccharide structures in varying amounts at different stages of processing. Mannose residues are most abundant on high-mannose structures whereas fucose and galactose residues are added on complex oligosaccharides. Additionally, galactose residues are also found on O-linked oligosaccharides. On the other hand, N-acetylglucosamine residues are present in a variety of oligosaccharide structures. The

incorporation of different sugar radiolabels into HSV-2 glycoproteins was studied to provide an idea of the kinds of oligosaccharide structures associated with HSV-2 glycoproteins. Cultures of HEp-2 cells were infected with HSV-2 and labelled from 4 to 24 hours (h) postinfection with either [^{3}H]-glucosamine, galactose, fucose or mannose at 20 μCi/ml. The cultures were harvested at 24 h postinfection and extracts from these cultures were then analyzed by SDS-PAGE followed by autoradiography. Since glucosamine residues are present in a variety of oligosaccharide structures, the components detected by radiolabelling with [^{3}H]-glucosamine in Figure 2 probably represent the different glycosylated proteins synthesized in HSV-2 infected cells. Of these glycoproteins, the majority were also labelled with [^{3}H]-galactose and [^{3}H]-fucose suggesting that they contain mature oligosaccharides. However, [^{3}H]-mannose labelling was observed in the minor, faster migrating components (indicated by dots in the figure) and these presumably represent high-mannose containing glycoproteins.

Effect of Tunicamycin on HSV-2 Glycoprotein Synthesis.

Tunicamycin has been previously shown to inhibit the formation of lipid-linked oligosaccharide precursors and hence is a specific inhibitor of N-linked glycosylation (Takasuki and Tamura, 1971; Hanover and Lennarz, 1978). In contrast, the synthesis of O-linked oligosaccharides are not inhibited by tunicamycin. To study the effect of tunicamycin on the synthesis of HSV-2 glycoproteins, HEp-2 cells were infected with HSV-2 and after the 1 h adsorption period, were

Figure 2. Isotopic labelling of HSV-2 glycoproteins with different sugars. HEp-2 cells were infected HSV-2 and isotopically labelled with either (^{3}H) mannose (Man), glucosamine (GluN), galactose (Gal), fucose (Fuc), or amino acids (A A) at 20 μCi/ml from 4 to 24 h postinfection. At 24 h postinfection, the cells were harvested, solubilized and analyzed by SDS-PAGE followed by autoradiography.

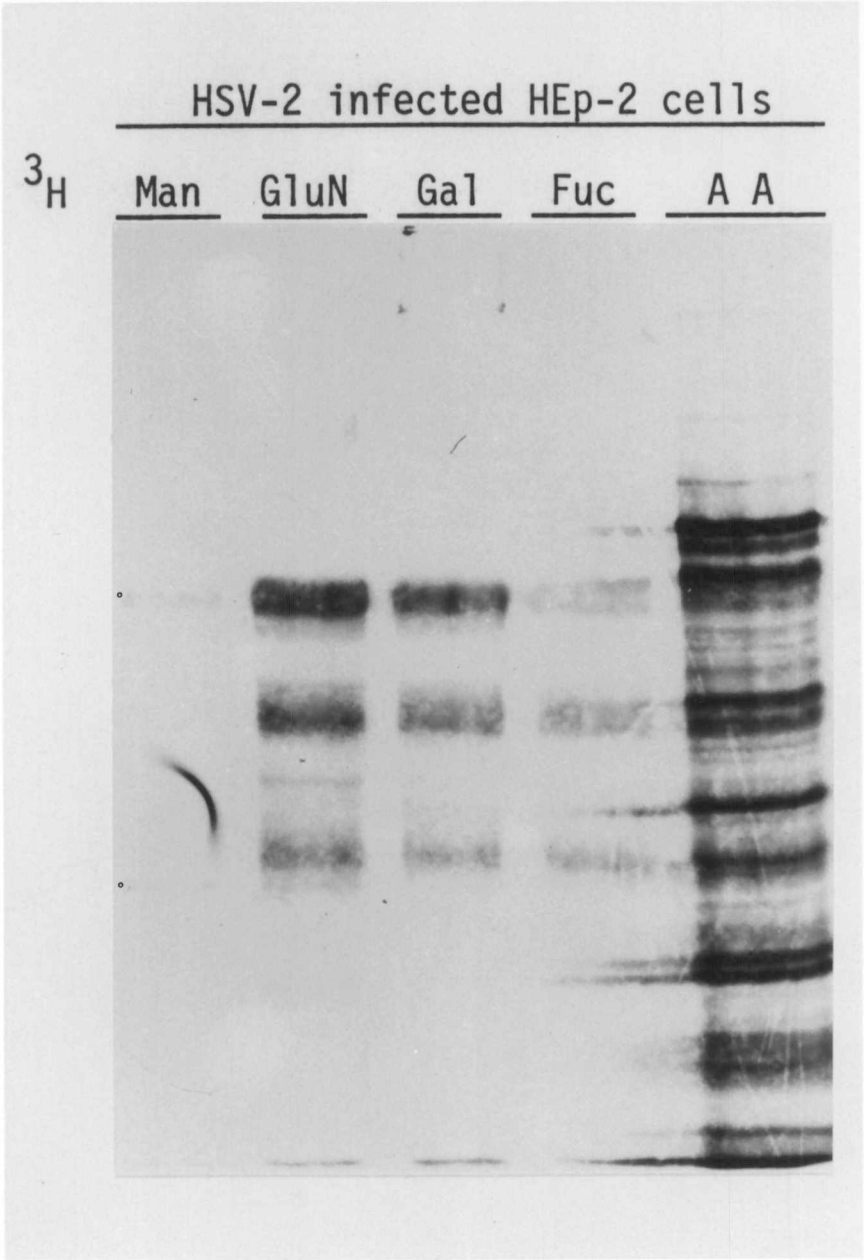


Figure 2

incubated with or without various amounts of tunicamycin. The cells were then isotopically labelled with either [^{3}H] amino acids or [^{3}H]-glucosamine at 20 μCi/ml from 4 to 24 h postinfection. At 24 h postinfection the cells were harvested and analyzed on SDS-PAGE followed by autoradiography. At a concentration of 10 μg/ml of tunicamycin, virtually complete inhibition of glycosylation was observed (Figure 3). Similar inhibition was also observed in cells labelled with [^{3}H]-galactose (data not shown). At this concentration of inhibitor, there was no apparent effect on the protein synthesis as shown by the [^{3}H] amino acid incorporation profiles in the same figure. This inhibition of glycosylation in the presence of tunicamycin therefore suggests that predominantly N-linked oligosaccharides are present on these glycoproteins with the absence of any detectable O-linked carbohydrates.

Specific Effect of Tunicamycin on the Synthesis of HSV-2 Glycoproteins. The inhibition of glycosylation of HSV-2 glycoproteins by tunicamycin suggests the absence of detectable O-linked glycosylation on HSV-2 glycoproteins in the presence of this inhibitor. This observation was somewhat surprising since in HSV-1 infected cells cultured in the presence of tunicamycin, glycosylation by the addition of O-linked oligosaccharides has been reported (Pizer et al., 1980; Wenske and Courtney, 1983). In order to further confirm this inhibition of glycosylation of HSV-2 glycoproteins, the effect of tunicamycin on HSV-1 and HSV-2 glycoprotein synthesis under the same conditions was compared. MRC-5 cell cultures were infected with HSV-1,

Figure 3. Effect of tunicamycin on the synthesis of HSV-2 glycoproteins. HEp-2 cells were infected with HSV-2 and after 1 h virus adsorption the cells were maintained in the presence of 0, 1.25 or 10 μg/ml of tunicamycin (TM). The infected cultures were incubated with 20 μCi/ml of either (^{3}H) glucosamine (^{3}H GluN) or (^{3}H) amino acids (^{3}H AA) from 4 to 24 h postinfection. The cells were harvested at 24 h postinfection, solubilized and analyzed by SDS-PAGE followed by autoradiography.

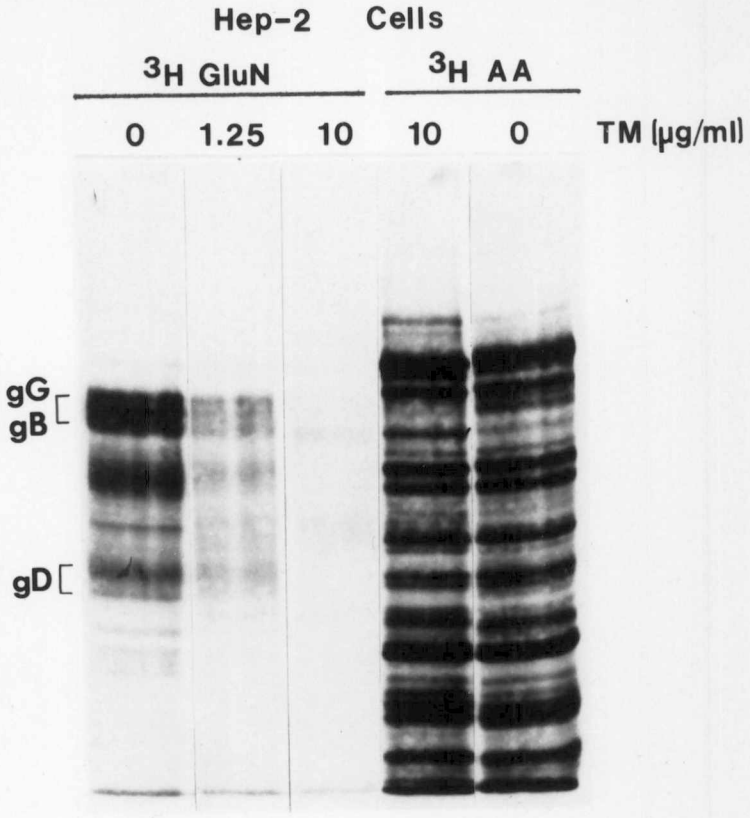


Figure 3

HSV-2 or doubly infected with both virus types and cultured in the presence or absence of tunicamycin. The cultures were then labelled with [^{3}H]-glucosamine from 4 to 24 h postinfection and the cell extracts were analyzed by SDS-PAGE followed by autoradiography as shown in Figure 4. In HSV-1 infected cells in the presence of tunicamycin, the O-glycosylated HSV-1 glycoprotein, gC, whose synthesis is insensitive to tunicamcyin (Pizer et al., 1980; Wenske and Courtney) was readily detected. However, under the same conditions, HSV-2 glycoprotein synthesis was inhibited. Similarly, in cultures doubly infected with both HSV-1 and HSV-2, in the presence of tunicamycin, no HSV-2 glycoproteins were observed even though HSV-1 O-linked glycoproteins were detected.

Therefore, this study suggests that unlike HSV-1 glycoproteins, no detectable O-linked glycosylation occurs on HSV-2 glycoproteins in the presence of tunicamycin. The absence of glycosylation is not due to HSV-2 specific inhibition of O-linked glycosylation machinery since HSV-1 glycoproteins can be glycosylated in the same cell doubly infected with both viruses.

Effect of Monensin on the Synthesis of HSV-2 Glycoproteins. Since monensin disrupts the normal transport and processing functions of the Golgi (Uchida et al., 1979; Johnson and Schlesinger; Kaariainen et al., 1980; Tartakoff, 1983), it was employed to facilitate the detection of HSV-2 glycoprotein processing intermediates that might accumulate as a result of the disruption of the Golgi functions. Cultures of HEp-2 cells were infected with HSV-2 and incubated with or without 10^{-6}

Figure 4. Synthesis of the glycoproteins of HSV-1 versus HSV-2 in the presence of tunicamycin. MRC-5 cells were infected with HSV-1, HSV-2 or both HSV-1 and HSV-2, or mock infected and cultured in the presence (+) or absence (-) of 10 μg/ml of tunicamycin (TM). The cultures were incubated in the presence of 20 μCi/ml of (^{3}H) glucosamine from 4 to 24 h postinfection. At 24 h postinfection, the cells were harvested and the solubilized extracts were analyzed by SDS-PAGE followed by autoradiography.

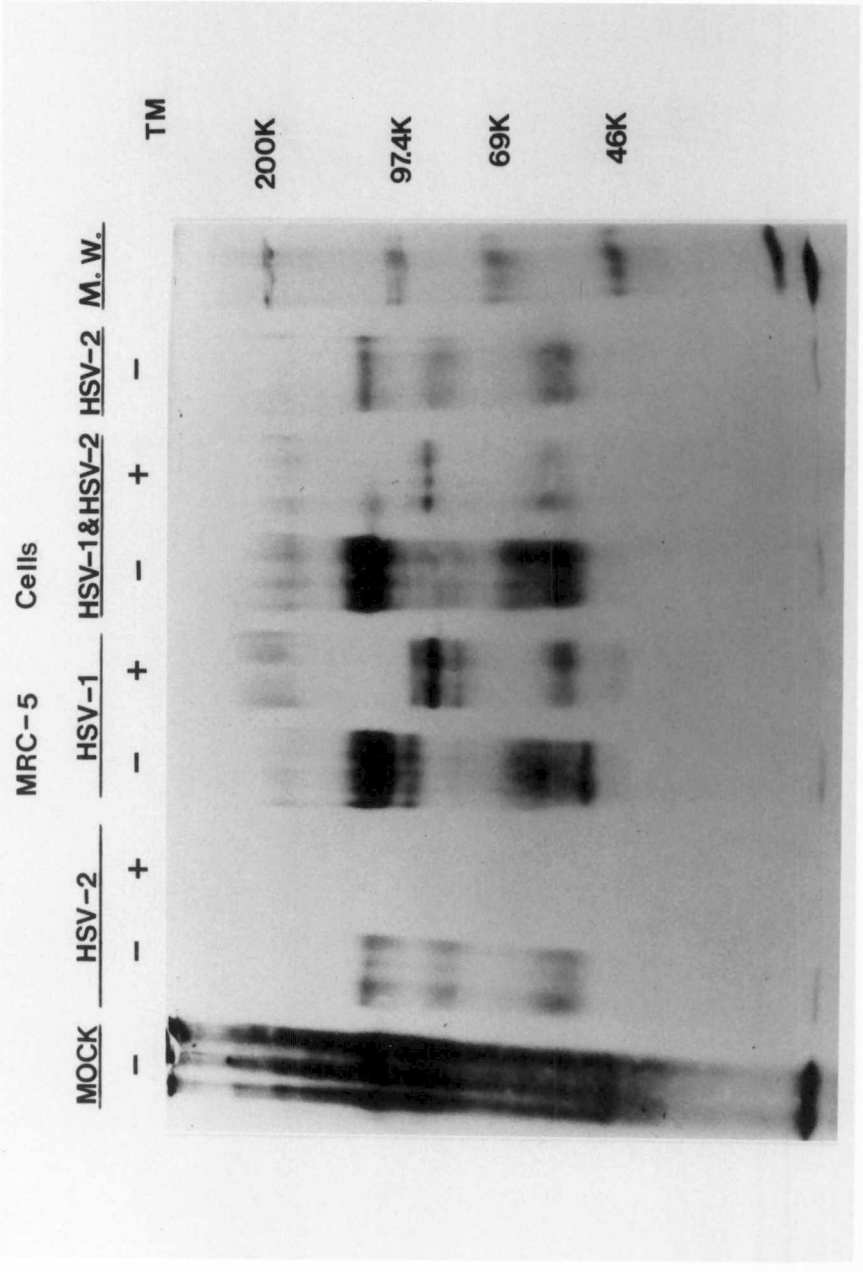

MRC-5 Cells
MOCK
HSV-2
HSV-1
HSV-1&HSV-2
HSV-2
M.W.
TM
200K
97.4K
69K
46K

Figure 4

molar (M) monensin (previously determined to be the effective dose in data not shown). After the 1 h virus-adsorption period, the cells were labelled from 4 to 24 h postinfection with 20 μCi/ml of [^{3}H]-glucosamine, mannose or amino acids. The SDS-PAGE profile of the glycoproteins synthesized in these cultures is shown in Figure 5. In the presence of monensin, the synthesis of the mature forms of HSV-2 glycoproteins was inhibited. At the same time, there was an apparent accumulation of faster migrating glycosylated polypeptides labelled with [^{3}H]-glucosamine and [^{3}H]-mannose. At 10^{-6} M monensin the synthesis of viral proteins such as the major viral capsid polypeptide was unaffected as shown by the [^{3}H] amino acid incorporation profile. These data suggest that at a concentration of monensin that did not affect protein synthesis, there was an accumulation of presumably high-mannose intermediates of the mature HSV-2 glycoproteins. The identity of these intermediates will be discussed in greater detail below.

Detection of the Intermediates of HSV-2 gG Synthesis

Immunoprecipitation Analysis of HSV-2 gG and Its Intermediates Synthesized in the Presence of Monensin and Tunicamycin. Because of the rapid processing of glycoproteins in HSV infected cells (Honess and Roizman, 1975), inhibitors of glycoprotein processing were employed to facilitate the identification of potential intermediates of gG synthesis. Since monensin disrupts the transport and processing of glycoproteins in the Golgi (Tartakoff, 1983), it was used to reveal intermediates of gG processing. Tunicamycin, an inhibitor of N-linked

Figure 5. Effect of monensin on the synthesis of the HSV-2 glycoproteins. HSV-2 infected HEp-2 cells were cultured with (+) or without (-) 10^{-6} M monensin (MN) in the presence of 20 µCi/ml of (^{3}H) amino acids (^{3}H A A), (^{3}H) glucosamine (^{3}H GluN) or (^{3}H) mannose (^{3}H Man) from 4 to 24 h postinfection. The infected cells were harvested at 24 h postinfection and the solubilized cell-extracts were subjected to analysis on SDS-PAGE and autoradiography. Faster migrating components are indicated by the circles (•) while the viral capsid protein is indicated by the arrow (›).

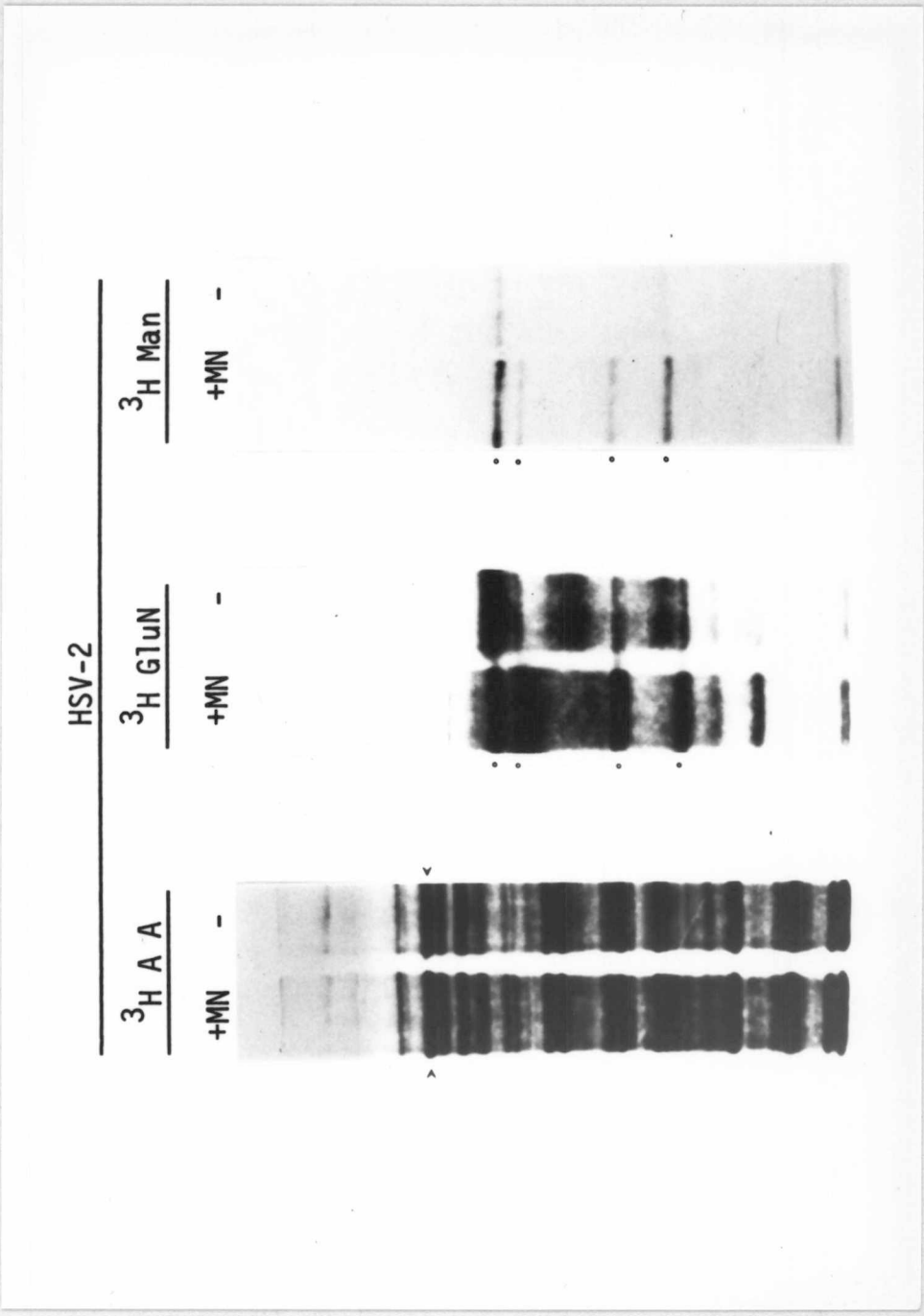


Figure 5

glycosylation, was also employed to promote the detection of possible O-linked intermediates that are insensitive to tunicamycin or nonglycosylated precursors resulting from the inhibition by the drug. These gG intermediates were identified with monospecific anti-gG antibodies. HSV-2 infected HEp-2 cells cultured in the presence or absence of monensin (10^{-6} M) and tunicamycin (10 μg/ml) were labelled with [^{3}H]-glucosamine from 4 to 24 h postinfection. At 24 h postinfection the cells were harvested, solubilized and immunoprecipitated with either a monoclonal anti-gG, designated 13 C5 (kindly provided by Dr. N. Balachandran), or a rabbit polyclonal monospecific anti-gG serum. Following the addition of Pansorbin, the immunoprecipitates were solubilized and analyzed by SDS-PAGE (Figure 6). In the absence of inhibitors the prominent mature gG, designated 108K (108,000 apparent molecular weight), and a less prominent 72,000 molecular weight component (designated 72K) were detected. In the presence of monensin, the synthesis of gG was altered. Partially glycosylated intermediates designated as 104K, 96K and 72K (apparent molecular weights of 104,000, 96,000 and 72,000 respectively), were detected suggesting that processing of precursors to the mature gG is inhibited in the Golgi. In cultures containing tunicamycin no [^{3}H]-glucosamine-labelled gG components were detected implying that gG contains mainly N-linked oligosaccharides and no significant O-linked structures.

The monoclonal anti-gG serum immunoprecipitated the same molecular weight glycoproteins as the polyclonal anti-gG suggesting both

Figure 6. Immunoprecipitation analysis of HSV-2 gG and its intermediates synthesized in the presence of monensin or tunicamycin. HSV-2 infected HEp-2 cells cultured in the presence (+) or absence (-) of monensin (MN, 10^{-6} M) or tunicamycin (TM, 10 μg/ml) were labelled with (^{3}H) glucosamine from 4 to 24 h postinfection. At 24 h after infection, the cells were harvested, solubilized and immunoprecipitated with either monoclonal anti-gG, 13αC5 (a kind gift from N. Balachandran), or polyclonal anti-gG rabbit antiserum. Following addition of Pansorbin, the immunoprecipitates were solubilized and analyzed by SDS-PAGE.

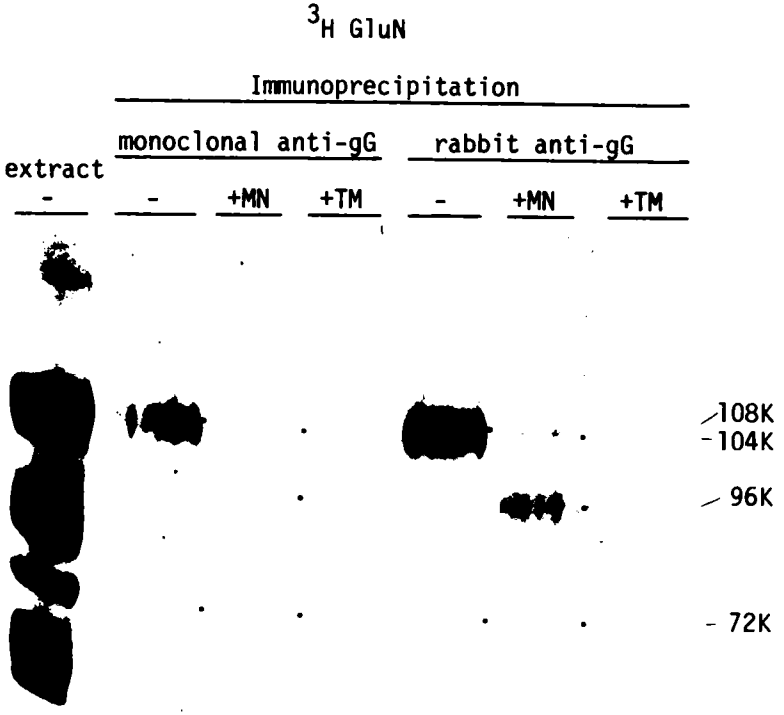

^{3}H GluN
Immunoprecipitation
monoclonal anti-gG
rabbit anti-gG
extract
-
-
+MN
+TM
-
+MN
+TM
108K
104K
96K
72K

Figure 6

antibodies recognized the same proteins. These data also support the proposal that the rabbit anti-gG serum was specific for gG.

Immunoblot Analysis of HSV-2 gG and Its Intermediates Synthesized in the Presence of Monensin and Tunicamycin. The immunoblot technique or Western blot, developed by Towbin et al. (1979) provides a more rapid and sensitive method for the detection and analysis of proteins with specific antibodies. Since this technique is not dependent on the incorporation of metabolic radiolabels into the proteins under investigation (see Materials and Methods), it was used to detect gG precursors that might be poorly radiolabelled or present in too low amounts to be readily observed by radiolabelling. The presence of the potential gG precursors in the presence or absence of monensin or tunicamycin was further investigated by immunoblotting using the anti-gG antiserum. HSV-2 infected HEp-2 cells cultured in the presence of monensin (10^{-6}) or tunicamycin (10 μg/ml) were harvested at 24 h postinfection, solubilized and resolved by SDS-PAGE. The resolved proteins in the polyacrylamide gel were electrophoretically transferred onto nitrocellulose paper which was then reacted with the polyclonal rabbit anti-gG antibody followed by ^{125}I-labelled protein A. The reactive components were visualized by autoradiography shown in Figure 7. In the absence of inhibitors, the fully glycosylated (108K) as well as the partially glycosylated 104K and 72K components were clearly detected. In the presence of monensin, the 104K, 96K and 72K components were also observed. Only one component of apparent molecular weight of 100K was detected in the presence of tunicamycin

Figure 7. Detection of HSV-2 gG and its intermediates synthesized in the presence of monensin or tunicamycin using monospecific antiserum to gG on the immunoblot. HSV-2 infected HEp-2 cells were cultured with (+) or without (-) 10^{-6} M monensin (MN) or 10 μg/ml tunicamycin (TM). At 24 h postinfection, the cultures were harvested and the solubilized proteins were first resolved by SDS-PAGE and then electrophoretically transferred to nitrocellulose paper. The nitrocellulose paper containing the resolved proteins was then reacted with rabbit anti-gG serum followed by ^{125}I-labelled protein A and autoradiography.

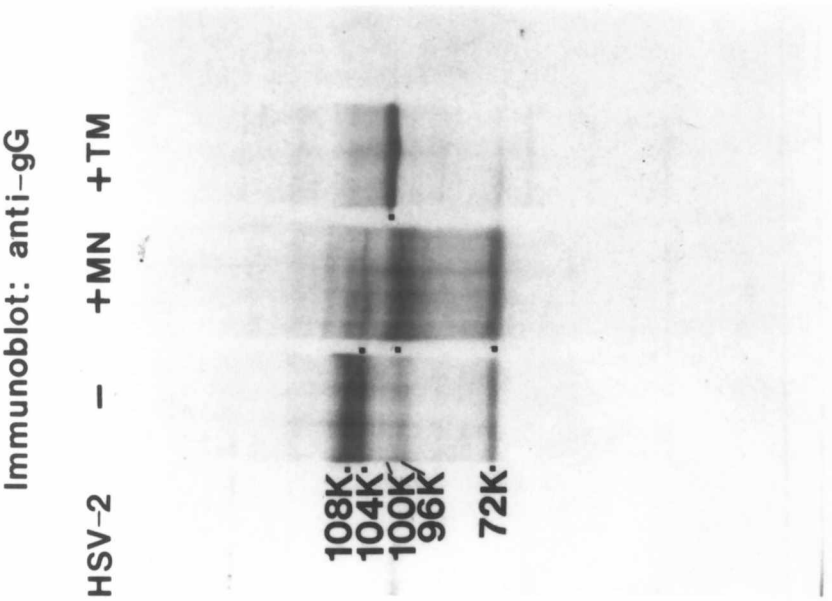


Figure 7

and it may represent the unglycosylated precursor of gG. The immunoblot hence allows for the detection of the same proteins as well as proteins that are poorly detected by immunoprecipitation.

In order to determine if the components detected represent proteins containing gG specific antigenic determinants and not contaminating reactivities of the polyclonal anti-gG antibody, reactivity of the monoclonal anti-gG 13αC5 to these components was assayed. Since monoclonal antibodies recognize only a single antigenic determinant, components reacted with the monoclonal anti-gG antibody would indicate the presence of this gG-specific determinant on the proteins.

Solubilized extracts of HSV-2 infected HEp-2 cultures grown in the presence or absence of monensin and tunicamycin were first immunoprecipitated with either the polyclonal rabbit anti-gG, the monoclonal anti-gG, 13αC5, or normal rabbit serum. After extensive washing, the immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nitrocellulose paper and then reacted with the rabbit anti-gG serum or the monoclonal, 13αC5. The reactive proteins were again visualized after the addition of ^{125}I-protein A by autoradiography (Figure 8). The same proteins that reacted first with the polyclonal rabbit anti-gG antibody (108K, 104K, 96K, 72K and the 100K components detected previously in Figure 6), also reacted with the monoclonal anti-gG antibody and vice versa. Each of these components detected appeared to be antigenically related to gG and probably represents intermediates of gG processing.

Figure 8. Immunoblot analysis of HSV-2 gG and its intermediates synthesized in the presence of monensin or tunicamycin. HSV-2 infected HEp-2 cells cultured in the presence or absence of the inhibitors monensin (10^{-6} M) and tunicamycin (10 μg/ml) were harvested at 24 h postinfection. The solubilized cell extracts were first immunoprecipitated with either monoclonal anti-gG, rabbit anti-gG or normal rabbit serum (NRS). After extensive washings, the immunoprecipitated proteins were resolved by SDS-PAGE, transferred onto nitrocellulose paper and then reacted with (A) rabbit anti-gG sera or (B) monoclonal anti-gG. After addition of ^{125}I-labelled protein A, the reactive proteins were visualized by autoradiography.

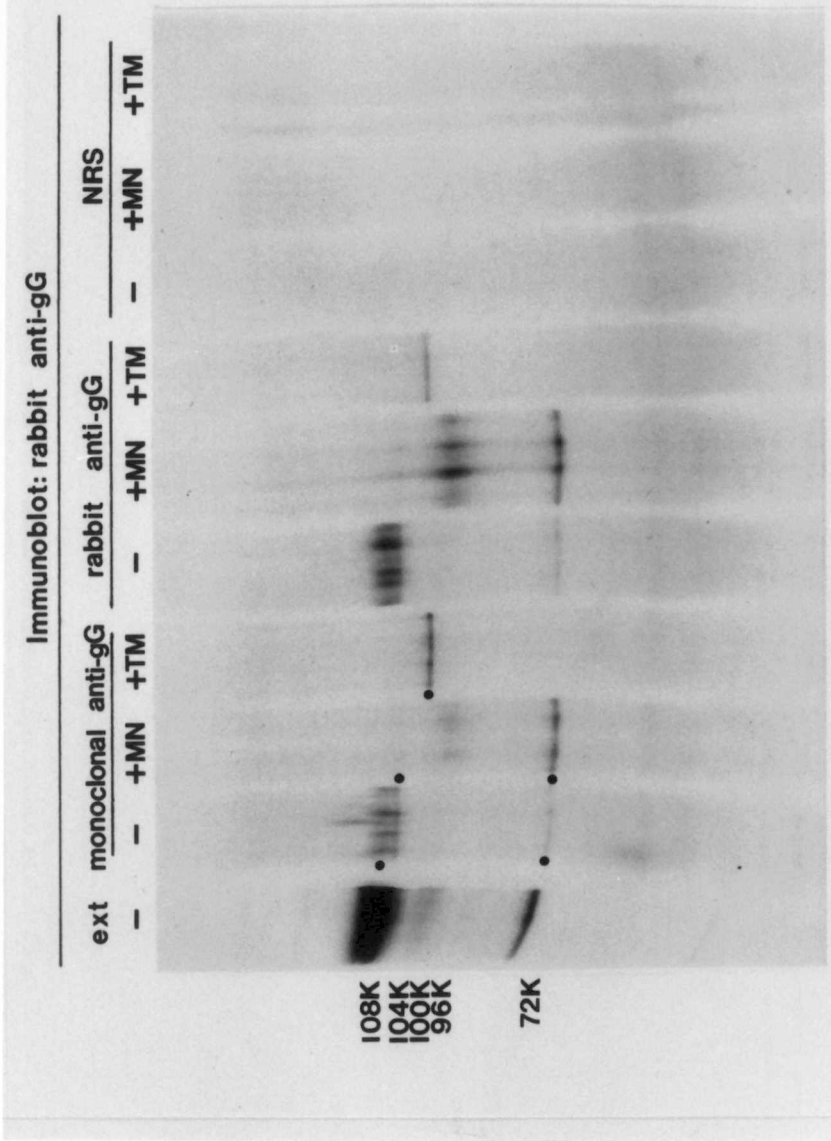

Immunoblot: rabbit anti-gG
ext
monoclonal anti-gG
rabbit anti-gG
NRS
–
–
+MN
+TM
–
+MN
+TM
–
+MN
+TM
108K
104K
100K
96K
72K

Figure 8A

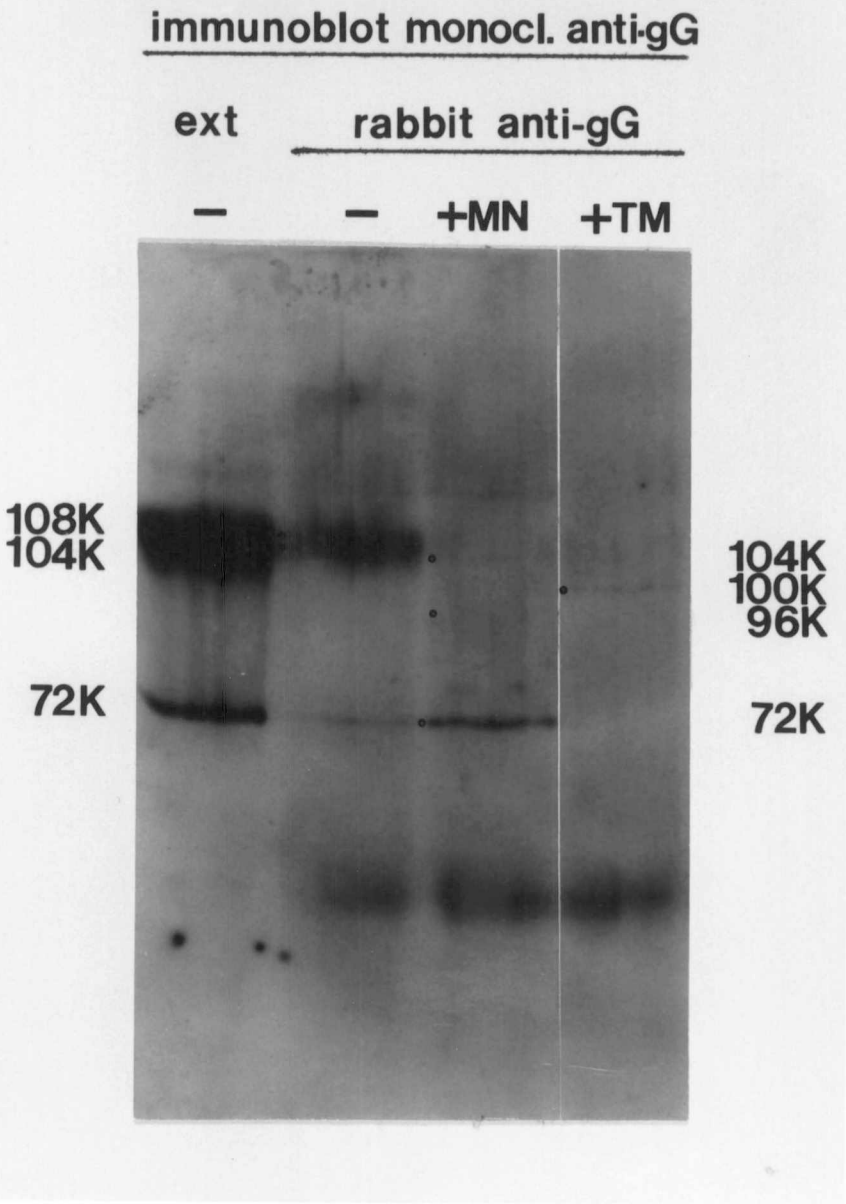

immunoblot monocl. anti-gG
ext
rabbit anti-gG
–
–
+MN
+TM
108K
104K
72K
104K
100K
96K
72K

Figure 8B

HSV-type-specific Reactivity of Polyclonal Anti-HSV-2 gG Serum. In an effort to determine if any of the proteins detected by the polyclonal anti-gG serum represented non-gG-related proteins such as host cell products, the anti-gG antiserum was also reacted with HSV-1 infected or mock-infected cell proteins. HEp-2 cells infected with HSV-1, HSV-2 or both as well as mock-infected cells were solubilized and the proteins analyzed by immunoblotting with the polyclonal rabbit anti-gG serum. No HSV-1 infected or mock-infected cell proteins were reactive with the polyclonal antiserum (Figure 9). Only the HSV-2 specific gG 108K, 104K and 72K components were detected by the polyclonal anti-gG serum suggesting that this rabbit antiserum reacts type-specifically and that all anti-gG reactive proteins probably represent products of the gG gene. Later studies will provide evidence to show that all these components represent different processing intermediates of gG synthesis.

Comparison of the Intermediates Detected in gG Synthesis in Different HSV-2 Laboratory Strains and Clinical Isolates. Previous studies have shown that differences can exist among various strains of HSV-1 concerning the rate of migration of glycoproteins on SDS-gels. In order to determine if the same components seen in the synthesis of HSV-2 strain 186 (in the presence or absence of inhibitors) were also associated with other HSV-2 strains, the synthesis of gG in different laboratory strains and clinical isolates of HSV-2 were examined. HEp-2 cells were infected with different laboratory and clinical HSV-2 strains and cultured in the presence or

Figure 9. HSV type-specific reactivity of the rabbit anti-gG serum. HEp-2 cells infected with HSV-1, HSV-2 or both as well as mock-infected cells were harvested at 24 h postinfection. The solubilized cell extracts were resolved on SDS-PAGE and the separated proteins were then transferred onto nitrocellulose paper. The blots were then reacted with anti-gG rabbit antiserum followed by ^{125}I-labelled protein A.

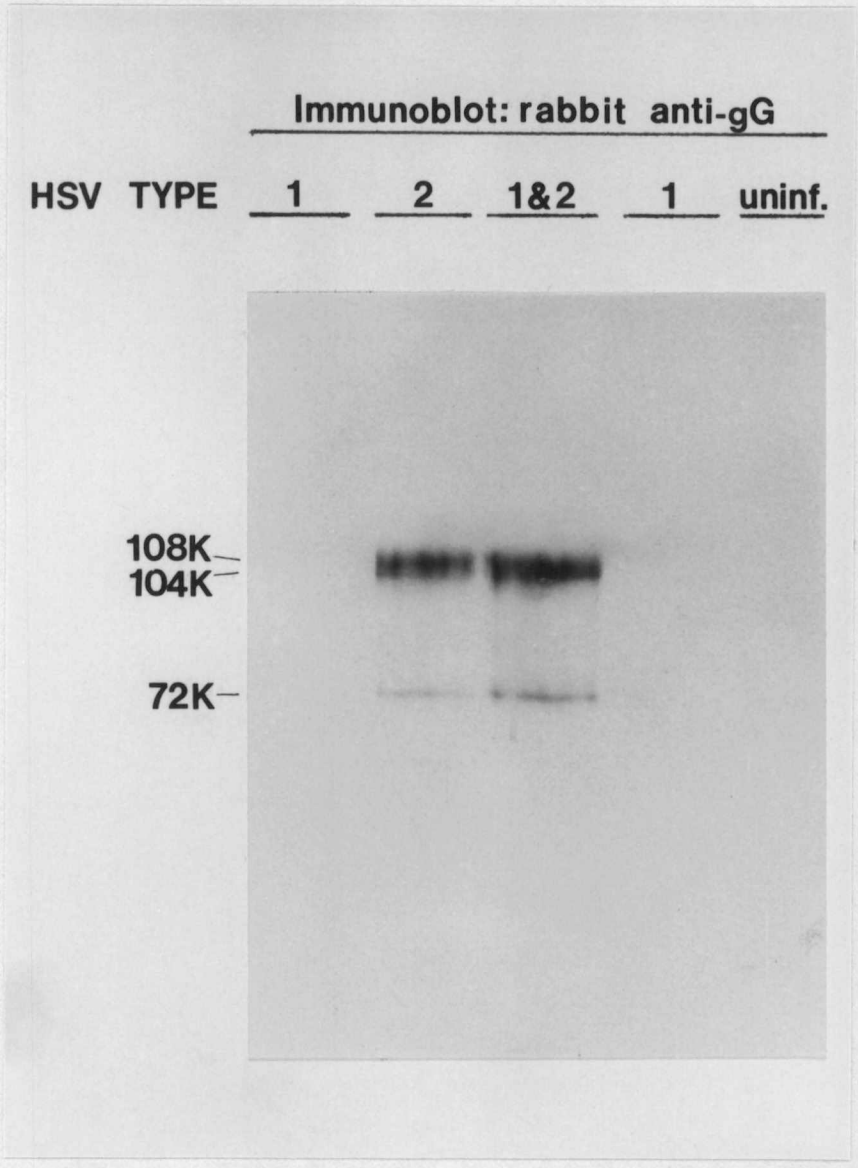

Immunoblot: rabbit anti-gG
HSV TYPE
1
2
1&2
1
uninf.
108K
104K
72K

Figure 9

absence of monensin or tunicamycin until harvesting at 24 h postinfection. The solubilized cell extracts from these cultures were then analyzed by immunoblotting with the rabbit anti-gG serum (Figure 10). In cultures infected with different HSV-2 strains (clinical isolates DG and JP, and laboratory strains 333, G and 186), identical gG-related intermediates were detected. These components synthesized by the various strains of HSV-2 in the presence or absence monensin or tunicamycin were identical in their electrophoretic mobilities. Similar findings were observed in 10 other clinical isolates studied (in data not shown). These results suggest that the pathway of synthesis of gG in the different HSV-2 strains is conserved.

Reactivity of a Monospecific Anti-gG Rabbit Serum with the Precursors of gG. Since the polyclonal rabbit anti-G serum was obtained by immunizing rabbits with the mature gG as antigen, only precursors possessing the same antigenic sites present on the mature gG can be detected with this anti-gG antiserum. In order to study potential intermediates of gG processing (such as cleavage product(s) of gG precursors) that do not possess mature gG determinants, an anti-serum against a precursor of gG (see Materials and Method) designated anti-pgG was employed. The reactivities of the anti-pgG were compared with that of the anti-gG serum. HSV-2 infected HEp-2 cells cultured in the presence or absence of monensin or tunicamycin were harvested at 24 h postinfection and solubilized. The solubilized antigens were then immunoprecipitated with either anti-gG, anti-pgG or normal rabbit serum and the immunoprecipitates were resolved on

Figure 10. Synthesis of gG and its intermediates in cells infected with different strains of HSV-2 in the presence or absence of monensin or tunicamycin. HEp-2 cells were infected with (A) HSV-2 strains, JP, DG, G or 186, or (B) HSV-2 strains 333 or 186 and maintained in the presence (+) or absence (-) of monensin (MN, 10^{-6} M) or tunicamycin (TM, 10 μg/ml). At 24 h after infection, the cells were harvested and the solubilized extracts were analyzed on SDS-PAGE followed by immunoblotting with anti-gG rabbit serum.

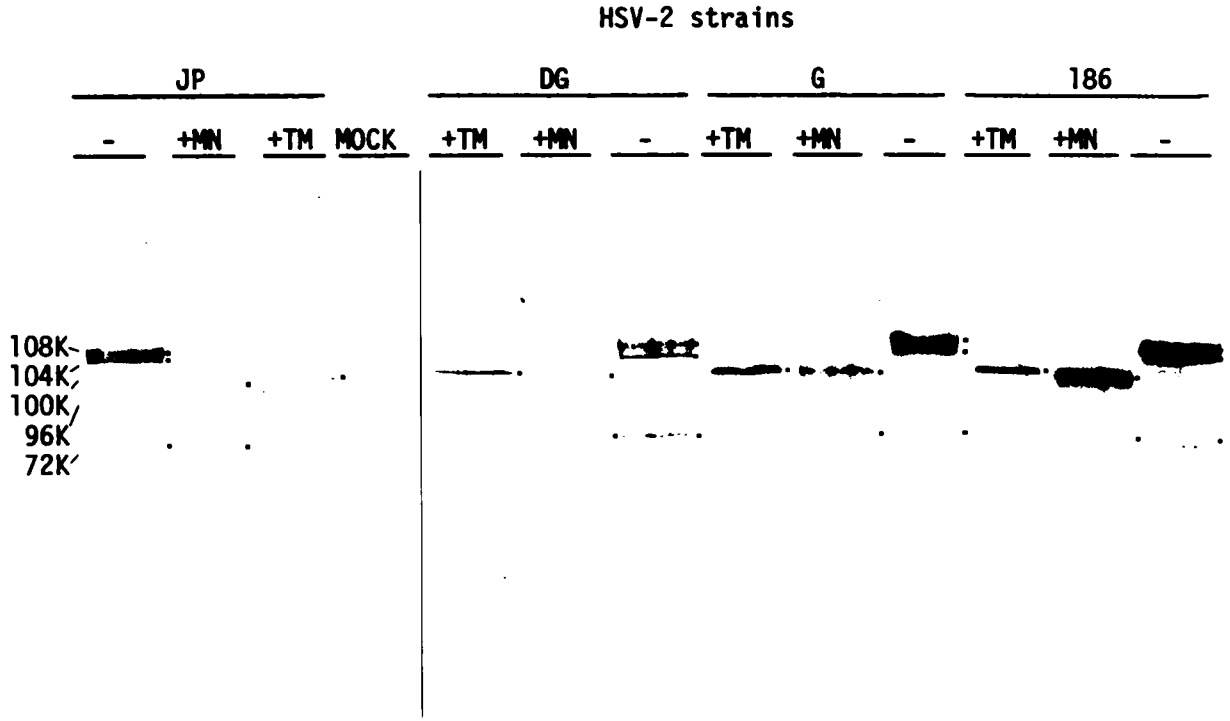

HSV-2 strains
JP
DG
G
186
-
+MN
+TM
MOCK
+TM
+MN
-
+TM
+MN
-
+TM
+MN
-
108K
104K
100K
96K
72K

Figure 10A

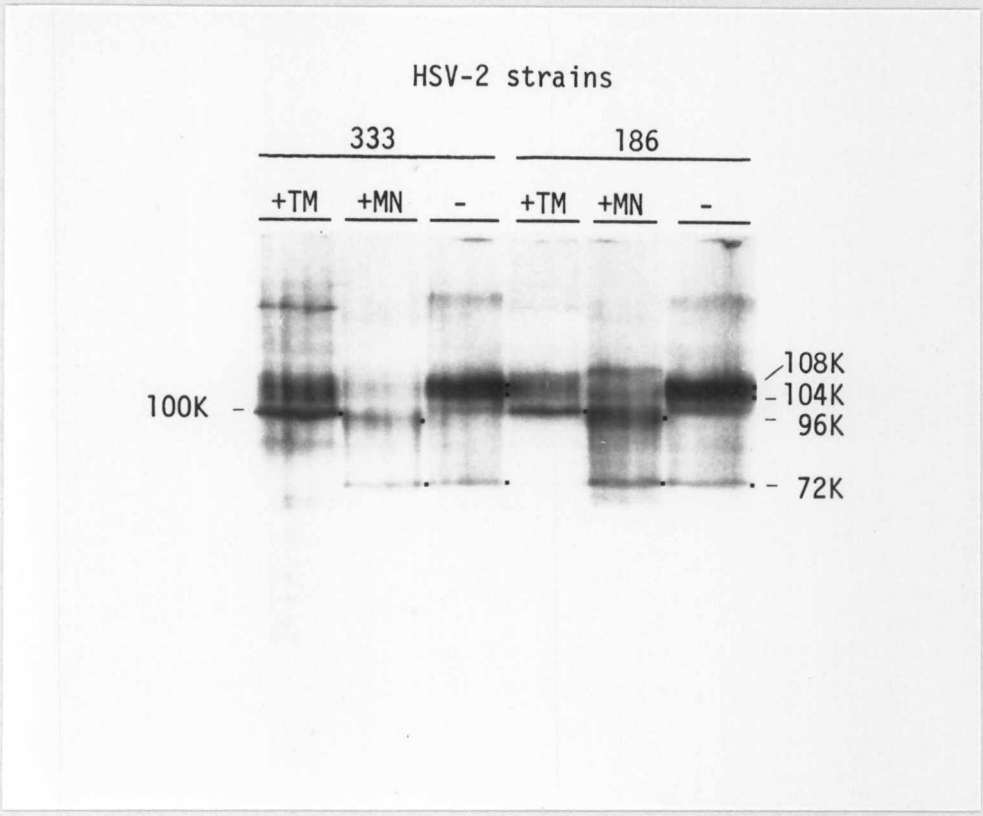


Figure 10B

SDS-PAGE followed by immunoblotting with anti-gG or anti-pgG (Figure 11). On the anti-gG immunoblot, the 104K and 72K components (synthesized in the presence and absence of monensin), and the 100K (synthesized in the presence of tunicamycin) that were first immunoprecipitated by anti-pgG also reacted with the anti-gG. The 96K which accumulated in the presence of monensin also reacted with anti-gG serum. Of the components immunoprecipitated by anti-gG, the 108K, 104K, 100K, 96K and 72K components (seen on the immunoblot stained with anti-gG), once again, only the 104K, 100K and 72K components were detected by anti-pgG on the immunoblot. These data suggest that the anti-pgG serum reacts with antigenic determinants that are present on gG intermediates 104K, 72K and 31K and absent from the more processed forms of gG, the 96K and 108K. It will be shown that the 72K and 104K contained high-mannose oligosaccharides while presumably complex structures are present on the 96K and the mature gG (108K). It will also be shown below that anti-pgG also reacted with a high-mannose containing component of 31,000 (designated as 31K).

Identification and Analysis of gG Intermediates

Effect of the Endoglycosidase, Endo H Treatment on the Intermediates of HSV-2 gG.

The enzyme, endo H, originally described by Tarentino and Maky (1974), specifically cleaves at a site between the first two N-acetylglucosamine residues of high-mannose oligosaccharide chains from glycoproteins. Removal of these high-mannose structures by endo H results in an increased electrophoretic mobility of the glycoprotein products. Such an approach was used to identify the

Figure 11. Reactivity of a monospecific anti-pgG rabbit serum with the precursors of gG. HSV-2 infected cell antigens prepared from cultures grown in the presence (+) or absence (-) of monensin (MN) or tunicamycin (TM) were first immunoprecipitated with either anti-gG, anti-pgG or normal rabbit serum (NRS). The immunoprecipitates were then resolved by SDS-PAGE followed by immunoblotting with either anti-gG or anti-pgG.

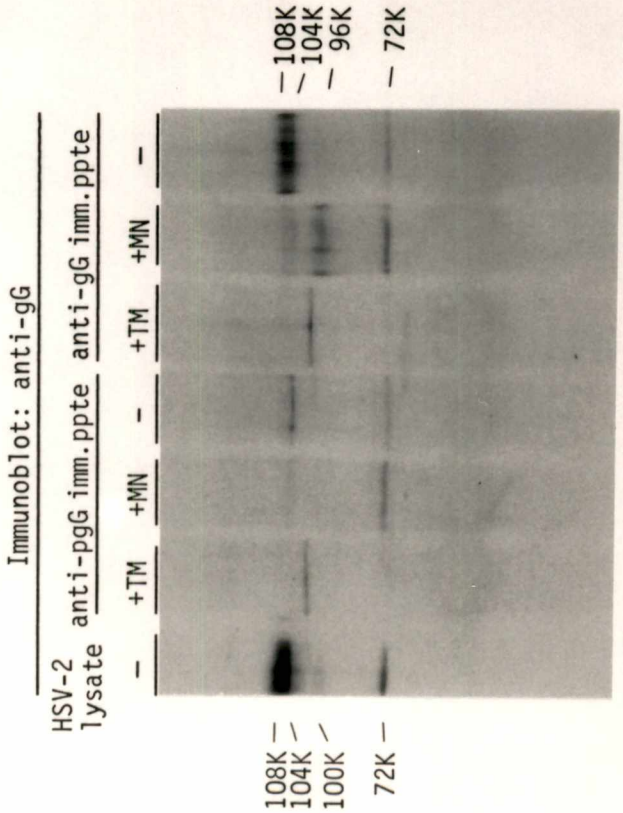

Immunoblot: anti-gG
HSV-2 lysate
anti-pgG imm.ppte
anti-gG imm.ppte
–
+TM
+MN
–
+TM
+MN
–
108K
104K
100K
72K
108K
104K
96K
72K

Figure 11A

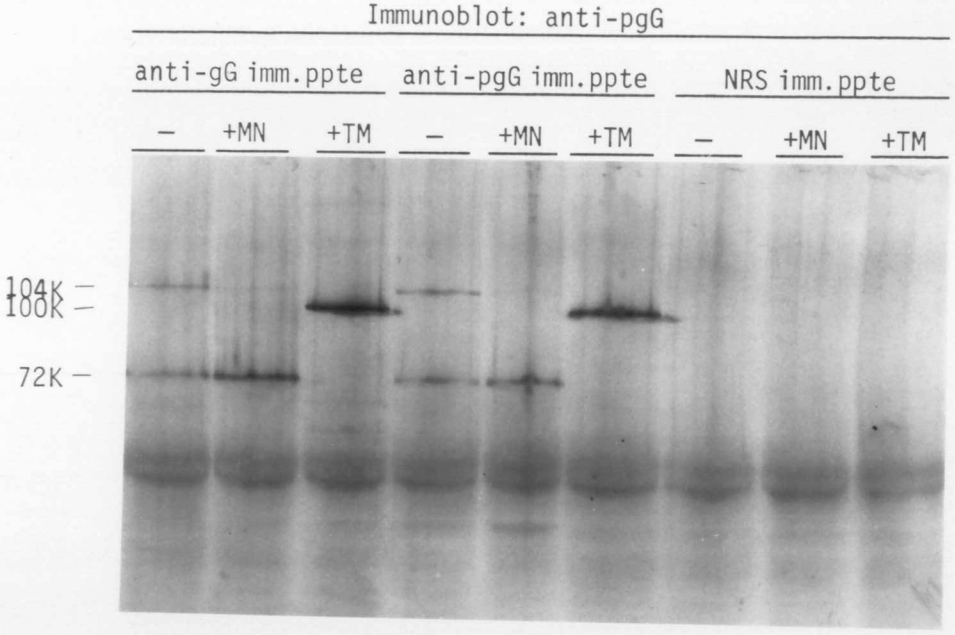

Immunoblot: anti-pgG
anti-gG imm.ppte
anti-pgG imm.ppte
NRS imm.ppte
–
+MN
+TM
–
+MN
+TM
–
+MN
+TM
104K
100K
72K

Figure 11B

high-mannose containing precursor components in the synthesis of gG. HSV-2 infected HEp-2 cells were cultured in the presence or absence of monensin or tunicamycin and harvested at 24 h postinfection. The solubilized extracts from these samples were subjected to endo H digestion and the products were then resolved on SDS-PAGE followed by immunoblotting with anti-gG serum. As seen in Figure 12, the 96K and the 108K gG, synthesized in the presence and absence of monensin, respectively, were not affected with respect to their electrophoretic mobility following endo H treatment suggesting high-mannose oligosaccharides are absent. In contrast, both the 72K and the 104K components resolved from cultures grown with or without monensin, were converted into faster migrating components by endo H suggesting the presence of high-mannose intermediates. The digestion of the 72K gives rise to a 69,000 apparent molecular weight species (designated as 69K) while the 104K gives rise to a 100K protein which has a similar molecular weight as the 100K component synthesized in the presence of tunicamycin. These observations further support the suggestion that the 100K represents the unglycosylated precursor polypeptide of gG.

Reactivity of Anti-pgG with Endo H Digests of gG Precursors Associated with the Nuclear and Cytoplasmic Fractions. The enzyme endo H, was similarly used to study the distribution of the high-mannose intermediates that were identified by the anti-gG antiserum. HSV-2 infected cells grown in the presence or absence of monensin were fractionated into nuclear or cytoplasmic fractions and then subjected

Figure 12. Effect of the endo-β-N-acetylglucosaminidase H (endo H) treatment on the intermediates of HSV-2 gG. HSV-2 infected HEp-2 cells were cultured in the presence or absence of tunicamycin (TM, 10 μg/ml) or monensin (MN, 10^{-6} M) and harvested at 24 h postinfection. The solubilized proteins were then treated with (+) or without (-) endo H (as described in Materials and Methods) and the digestion products were resolved by SDS-PAGE followed by immunoblotting with rabbit anti-gG serum.

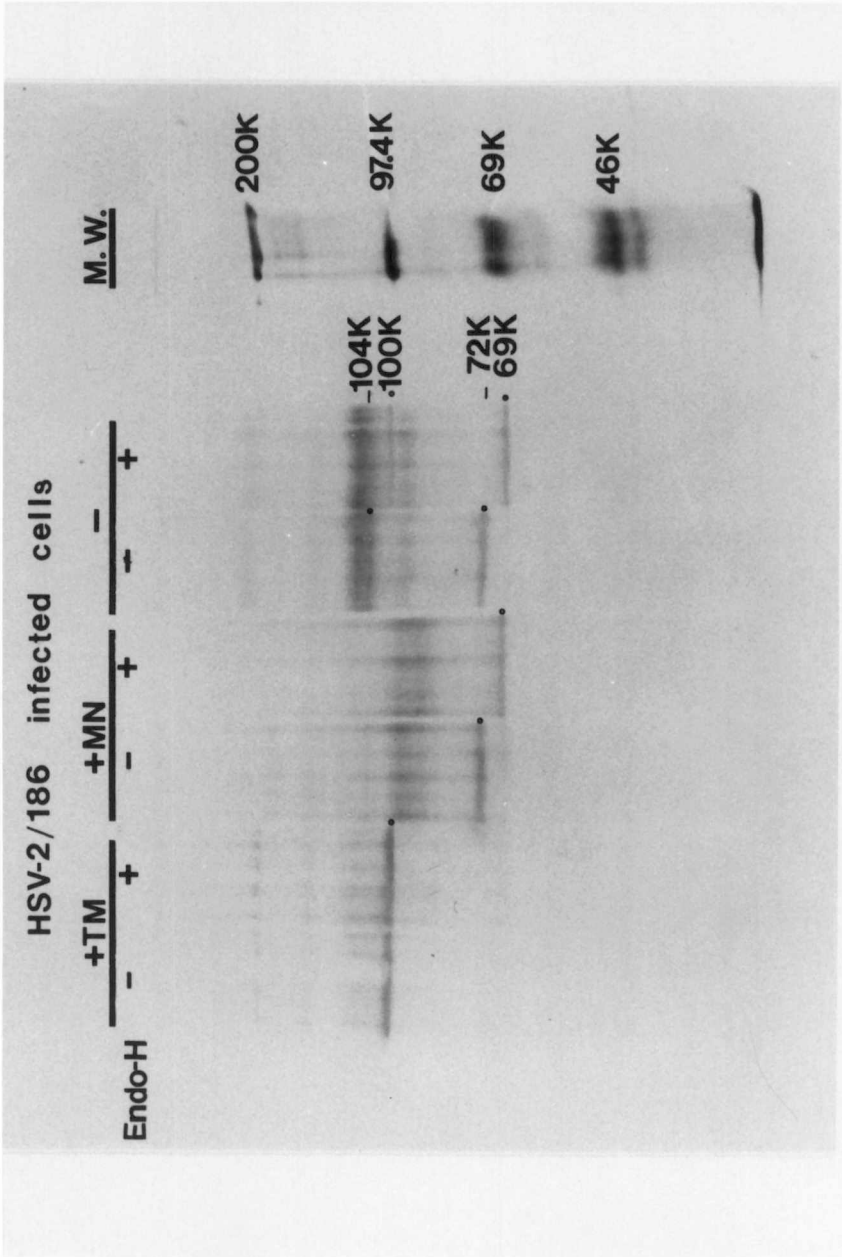

HSV-2/186 infected cells
+TM
+MN
Endo-H
M.W.
200K
97.4K
69K
46K
104K
100K
72K
69K

Figure 12

to endo H treatment. Following endo H digestion, the samples were analyzed by immunoblotting with anti-pgG. As shown in Figure 13, gG precursors, 104K and 72K, detected in the nuclear or cytoplasmic fractions from cultures grown with or without monensin were sensitive to endo H and probably represent the high-mannose intermediates. A 31K high-mannose containing component detected by anti-pgG primarily in the nuclear fraction of cultures grown in the presence of monensin may represent a cleavage product of the 104K which as shown later, also yields the 72K component. High molecular weight components detected primarily in nuclear fractions may represent possible oligomers of gG and its intermediates. The importance of these high molecular weight components to the synthesis of gG is unclear at present and is under further investigation. These observations indicate that the high-mannose containing components 104K, 72K and 31K are primarily associated with the nuclear fraction infected cells.

Effect of Neuraminidase Treatment on HSV-2 gG and Its Intermediates. Since sialic acid residues are added as terminal sugars on N-linked oligosaccharide chains, this has been used as an indication of the presence of fully processed oligosaccharide structures. Neuraminidase, an enzyme which catalyzes the removal of terminal sialic acid residues from these structures was employed to characterize the degree of processing of gG and its intermediates. HSV-2 infected cells cultured in the presence or absence of monensin were solubilized and reacted with or without neuraminidase. The products of neuraminidase digestion were analyzed by immunoblotting with anti-gG (Figure 14).

Figure 13. Reactivity of anti-pgG with endo H digests of gG precursors associated with the nuclear and cytoplasmic fractions. HSV-2 infected HEp-2 cells grown in the presence or absence of monensin were fractionated into nuclear and cytoplasmic fractions (as described in Materials and Methods) and then subjected to endo H treatment. Following endo H digestion, the samples were analyzed by SDS-PAGE and immunoblotting with rabbit anti-pgG serum.

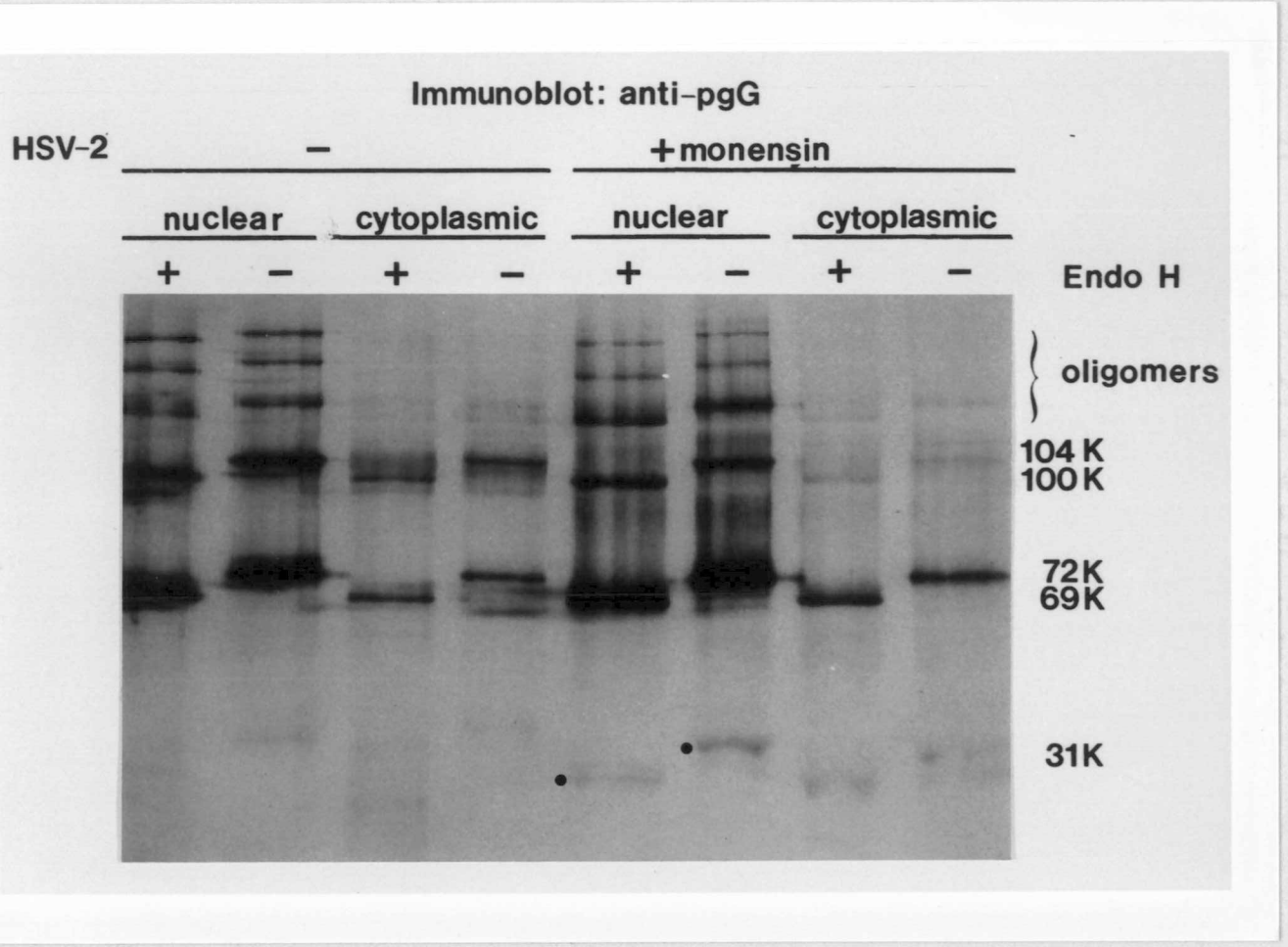

Immunoblot: anti-pgG
HSV-2
–
+monensin
nuclear
cytoplasmic
nuclear
cytoplasmic
+
–
+
–
+
–
+
–
Endo H
oligomers
104 K
100 K
72K
69K
31K

Figure 13

Figure 14. Effect of neuraminidase treatment on HSV-2 gG and its intermediates. HSV-2 infected HEp-2 cells were cultured in the presence or absence of monensin (MN, 10^{-6} M) and harvested at 24 h postinfection. The cell extracts were resuspended in 0.005 M $CaCl_2$ and 0.1 M sodium acetate pH 5.5 and were mixed with (+) or without (-) neuraminidase (1 unit/ml). After neuraminidase digestion the components of gG were analyzed by SDS-PAGE followed by immunoblotting with anti-gG serum.

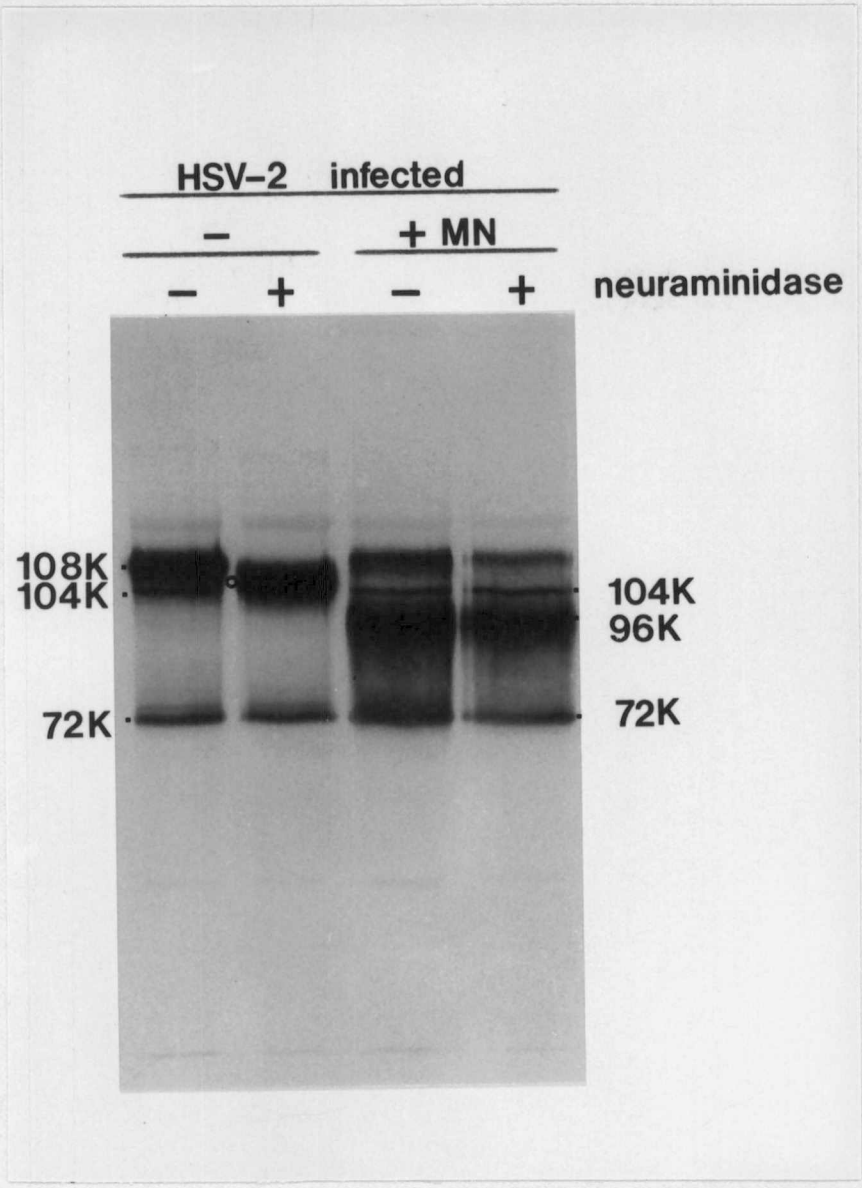


Figure 14

Treatment with the enzyme resulted in increased electrophoretic mobility of the 108K gG from samples grown without inhibitors, indicating the removal of sialic acid residues. The migration of the partially glycosylated intermediates 104K, 96K and 72K were unaffected by the enzyme suggesting the absence of sialic residues. This result suggests that endo H resistant 96K protein accumulated in the presence of monensin appears to lack terminal processing and may represent incomplete complex glycoproteins.

Determination of the Apparent Molecular Weight of Purified gG Following Deglycosylation with the Endoglycosidase, Endo F. From the endo H studies described above, it was found that two high-mannose intermediates, the 104K and the 72K components were involved in the synthesis of gG. In order to determine which of the two high-mannose intermediates represents the direct precursor to gG, the size of the deglycosylated gG was examined. This was achieved by the treatment of gG with the endoglycosidase, endo F, which cleaves N-linked glycans of both high-mannose and complex types from glycoproteins (Elder and Alexander, 1982). gG was purified from HSV-2 infected cells by preparative gel electrophoresis followed by elution from a hydroxylapatite chromatograph column (see Materials and Methods) and subjected to digestion with 15 or 30 units/ml of endo F. The products of the digestion were then analyzed by immunoblotting with anti-gG serum as shown in Figure 15. Reaction of purified 108K gG with increasing concentrations of enzyme resulted in a parallel increase of a product with an average molecular weight of 69,000. This product has

Figure 15. Effect of endo-β-N-acetylglucosaminidase F, endo F on purified gG. gG purified from HSV-2 infected HEp-2 cells (described in the Materials and Methods) was subjected to treatment with 15 or 30 units/ml of endo F. The products of endo F digestion were then analyzed on SDS-PAGE followed by immunoblotting with anti-gG serum.

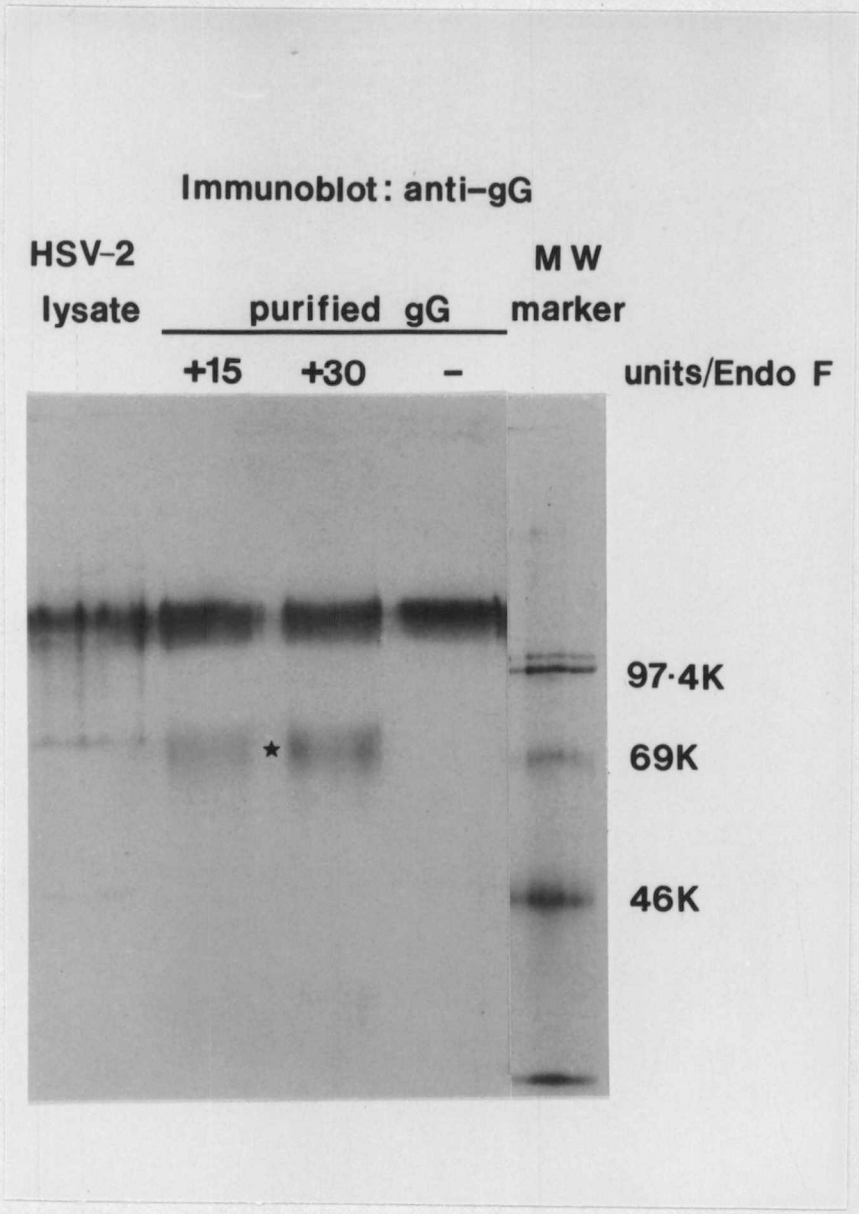

Immunoblot: anti-gG
HSV-2
lysate
purified gG
+15
+30
-
M W
marker
units/Endo F
97·4K
69K
46K

Figure 15

similar migration properties to the 69K product resulting from endo H digestion of the 72K high-mannose intermediate. This finding therefore suggests that gG is derived from the further processing and glycosylation of the 72K high-mannose precursor.

Cellular Localization of gG and Its Intermediates

Cellular Localization of HSV-2 gG Components Synthesized in the Presence or Absence of Tunicamycin by Cell-Fractionation Procedure. It has been previously reported that HSV glycoprotein precursors tend to be associated with the nuclear fraction of the cell while the more mature forms were associated with the cytoplasmic fraction (Compton and Courtney, 1984). The distribution of gG and its intermediates were similarly investigated. Infected HEp-2 cells cultured in the presence or absence of monensin or tunicamycin were fractionated into nuclear and cytoplasmic fractions. Following Dounce homogenization, the solubilized proteins were analyzed by immunoblotting with the anti-gG antiserum (Figure 16). The fully glycosylated gG 108K was predominantly associated with the cytoplasmic fraction. In contrast, the high-mannose 104K and 72K components appeared to be primarily associated with the nuclear fraction. In cells cultured in the presence of tunicamycin, the nonglycosylated 100K component was also associated with the nuclear fraction. This result is consistent with the idea that the fully glycosylated 108K gG localized in the cytoplasmic fraction is processed from the unglycosylated 100K. In addition, the 72K and the 104K high-mannose precursor represent intermediates to the cytoplasmic 108K gG. Additionally, this result

Figure 16. Cellular localization of HSV-2 gG components synthesized in the presence or absence of tunicamycin. HEp-2 cells infected with HSV-2 were maintained in the presence (+) or absence (-) of tunicamycin and harvested at 24 h postinfection. The cytoplasmic and nuclear fractions were obtained by Dounce homogenization (see Materials and Methods), solubilized and the proteins were analyzed by immunoblotting with the anti-gG rabbit serum.

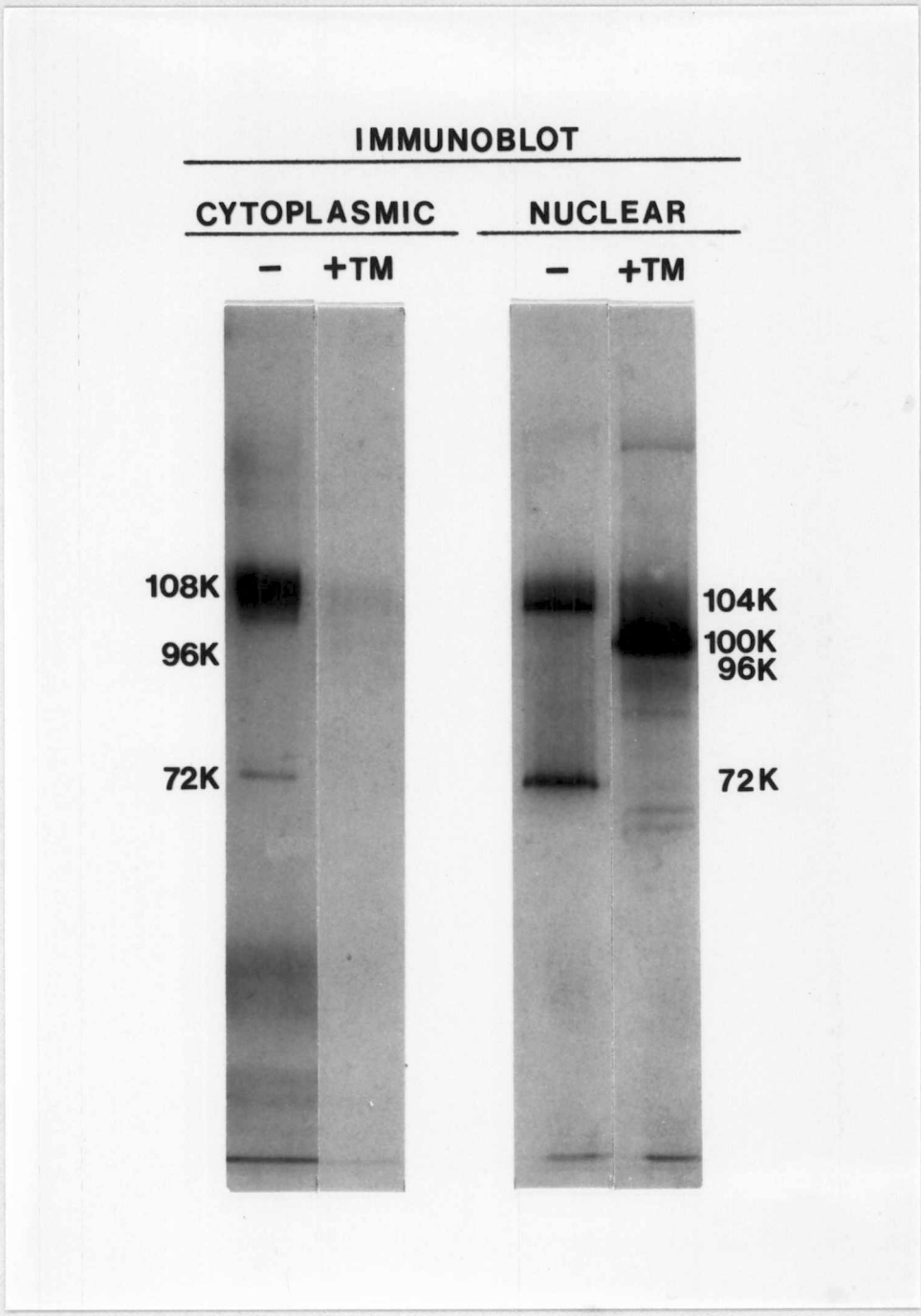

IMMUNOBLOT
CYTOPLASMIC
NUCLEAR
–
+TM
–
+TM
108K
96K
72K
104K
100K
96K
72K

Figure 16

also suggests that the transport of the intermediates synthesized in the presence of monensin or tunicamycin to the cytoplasmic compartments was retarded in the presence of these inhibitors.

Immunofluorescence Studies of HSV-2 gG Synthesis in the Presence or Absence of Monensin or Tunicamycin. The localization of these gG specific antigens in the HSV-2 infected cells was also studied by immunofluorescence. Infected HEp-2 cells cultured in the presence or absence of monensin or tunicamycin were harvested at 24 h postinfection and prepared for internal or membrane immunofluorescence (as described in Materials and Methods). The cells were then stained with either the rabbit anti-gG serum or normal rabbit serum followed by the reaction with fluorescein-conjugated goat anti-rabbit serum for the immunofluorescent detection of gG antigens (Figure 17). Whereas no specific immunofluorescence was detected with normal rabbit serum, specific internal as well as membrane immunofluorescence was observed with the rabbit anti-gG serum. HSV-2 gG antigens were detected in the perinuclear region and in the cytoplasmic compartments of infected cells cultured in the absence of inhibitors. In monensin and tunicamycin treated cells, the gG antigens appeared to be primarily distributed in the perinuclear region. In membrane immunofluorescence studies, gG-specific fluorescence was readily observed on HSV-2 infected cells grown in the absence of inhibitors. However, this fluorescence appeared to be reduced in intensity in monensin-treated cultures and no significant specific fluorescence was observed in tunicamycin-treated cultures. The perinuclear staining of gG specific

Figure 17. Immunofluorescence studies of HSV-2 gG synthesis in the presence or absence of monensin or tunicamycin. HEp-2 cultures grown on coverslips were infected at a low multiplicity of infection, with HSV-2 and maintained in the presence (+) or absence (-) of monensin (MN) or tunicamycin (TM) until harvest at 24 h postinfection. The coverslips were then prepared for either (A) internal or (B) membrane immunofluorescence tests with rabbit anti-gG serum or normal rabbit serum (NRS), as described in the Materials and Methods.

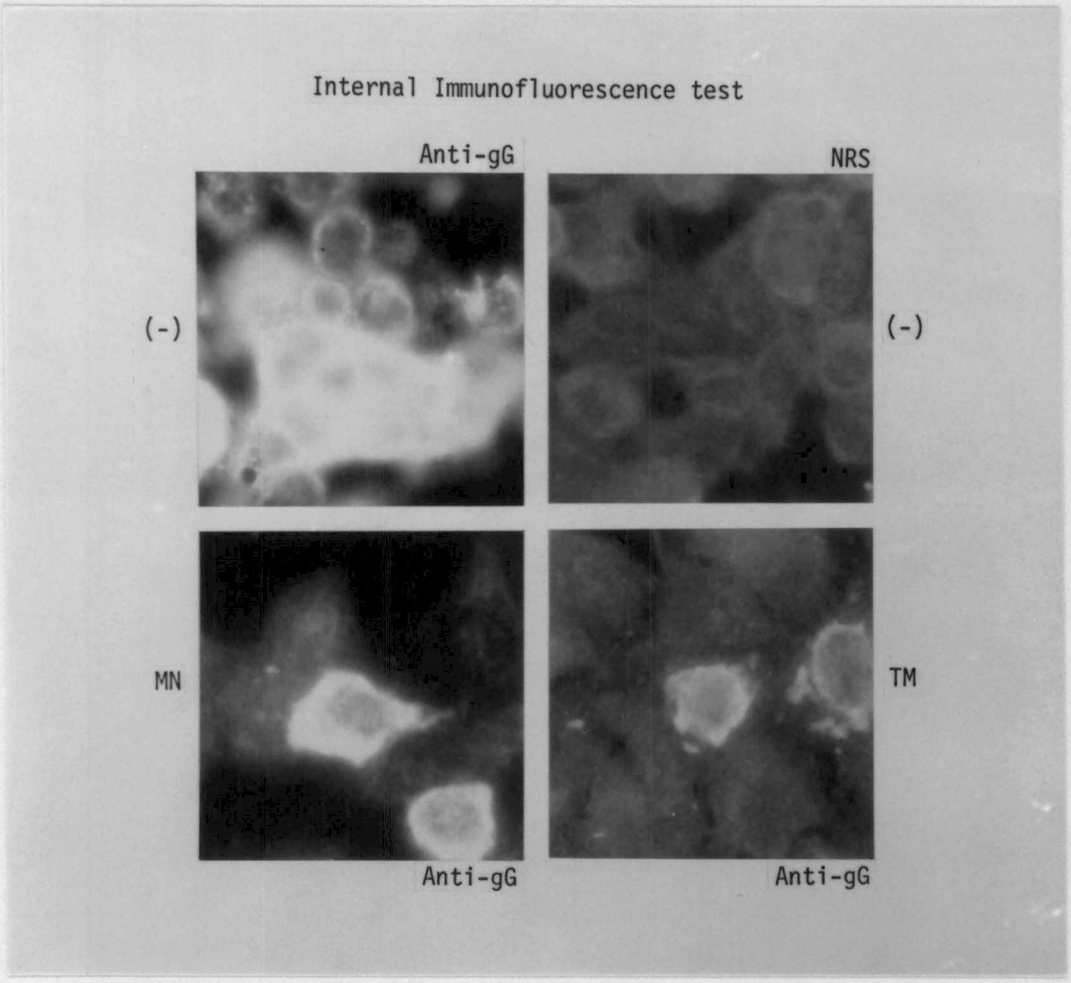

Internal Immunofluorescence test
Anti-gG
NRS
(-)
(-)
MN
TM
Anti-gG
Anti-gG

Figure 17A

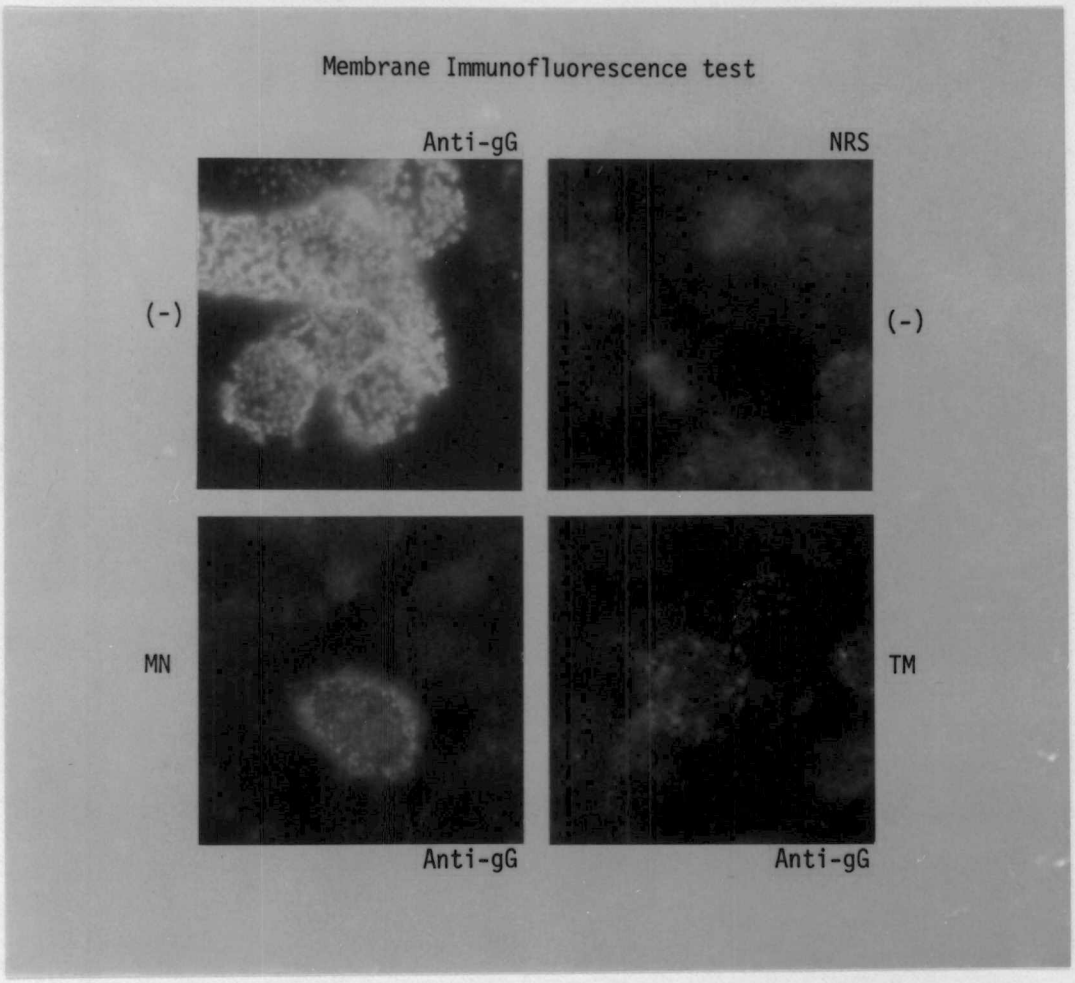

Membrane Immunofluorescence test
Anti-gG
NRS
(-)
(-)
MN
TM
Anti-gG
Anti-gG

Figure 17B

antigens and the lack of membrane gG specific fluorescence in cells grown in the presence of monensin and tunicamycin support the results described above concerning the nuclear localization of gG intermediates in the presence of these inhibitors. In the presence of monensin or tunicamycin, only single-cell gG-specific fluorescence was observed. This is in agreement with the lack of virus-spread in the presence of these inhibitors.

Synthesis of gG and Its Intermediates

Kinetics of Synthesis of HSV-2 gG and Its Intermediates. In an effort to determine the time appearance of gG intermediates in the infected cells, HEp-2 cultures were infected with HSV-2 and maintained in the presence or absence of monensin or tunicamycin. At various times after infection, these cultures were harvested and analyzed on SDS-PAGE followed by immunoblotting with anti-gG serum (Figure 18). At early time periods such as 1 and 4 h postinfection, only antigens originating from the inoculum were observed. The high-mannose 104K component was first detectable at 8 h postinfection. Specific increase in the 72K high-mannose component and the fully glycosylated 108K gG was observed at 9 to 10 h after infection. In HSV-2 infected cells cultured in the presence of monensin (Figure 19), the 104K component was again first detected at 8 h postinfection while the 72K and the 96K were observed 9 h postinfection. The 100K unglycosylated component detected in cultures containing tunicamycin, was initially detected at 8 h postinfection and was present in increased amounts at later times as shown in Figure 20. According to the cascade regulation of HSV

Figure 18. Kinetics of synthesis of HSV-2 gG and its intermediates in the absence of inhibitors. HEp-2 cell cultures were infected with HSV-2 and incubated in the absence of inhibitors of glycosylation. At various times postinfection (P.I.), the different cultures were harvested and solubilized for analysis on SDS-PAGE. The resolved proteins were identified on the immunoblot with anti-gG rabbit serum.

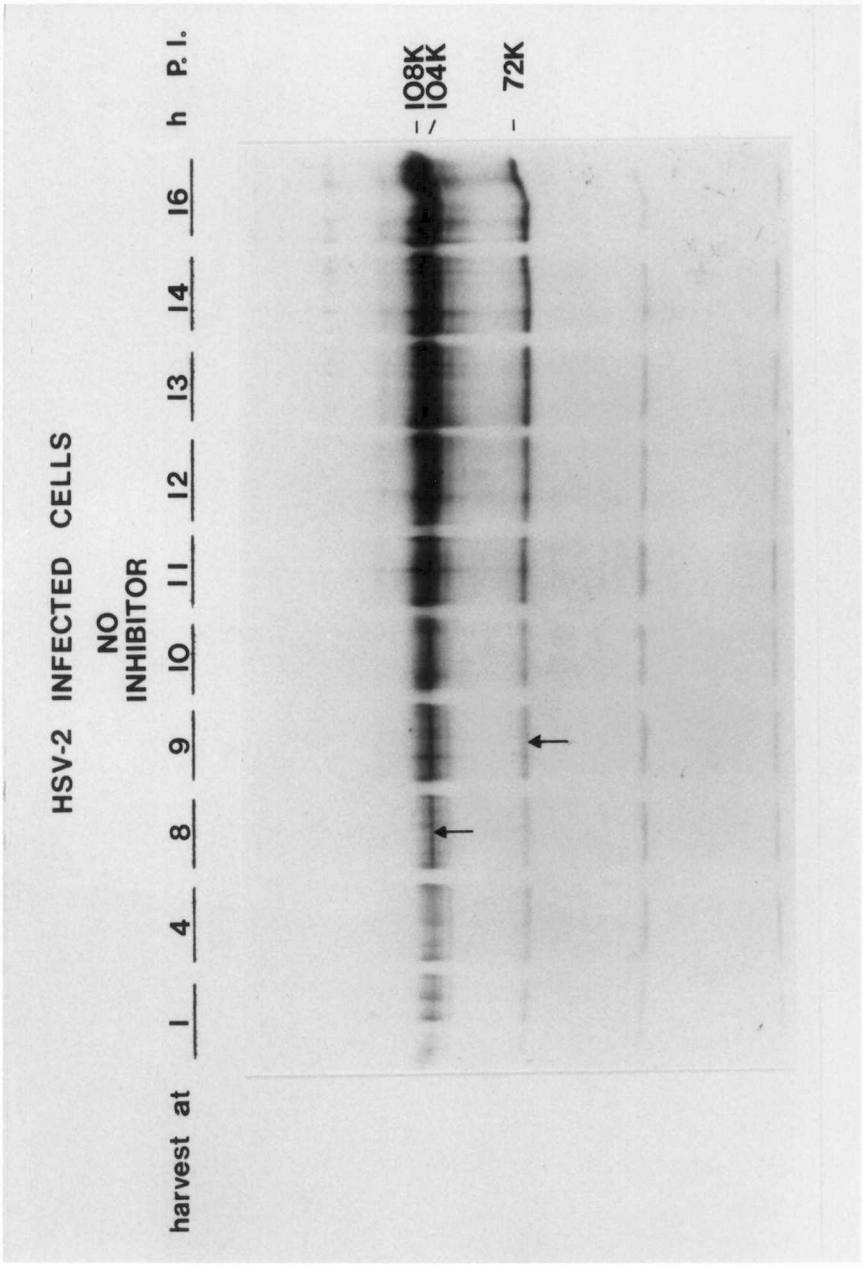

HSV-2 INFECTED CELLS
NO
INHIBITOR
harvest at 1 4 8 9 10 11 12 13 14 16 h P. I.
108K
104K
72K

Figure 18

Figure 19. Kinetics of synthesis of HSV-2 gG and its intermediates in the presence of monensin. HSV-2 infected HEp-2 cultures were maintained in the presence of 10^{-6} M monensin. At the indicated times postinfection (P.I.), the cultures were harvested and solubilized for SDS-PAGE followed by immunoblotting with anti-gG serum.

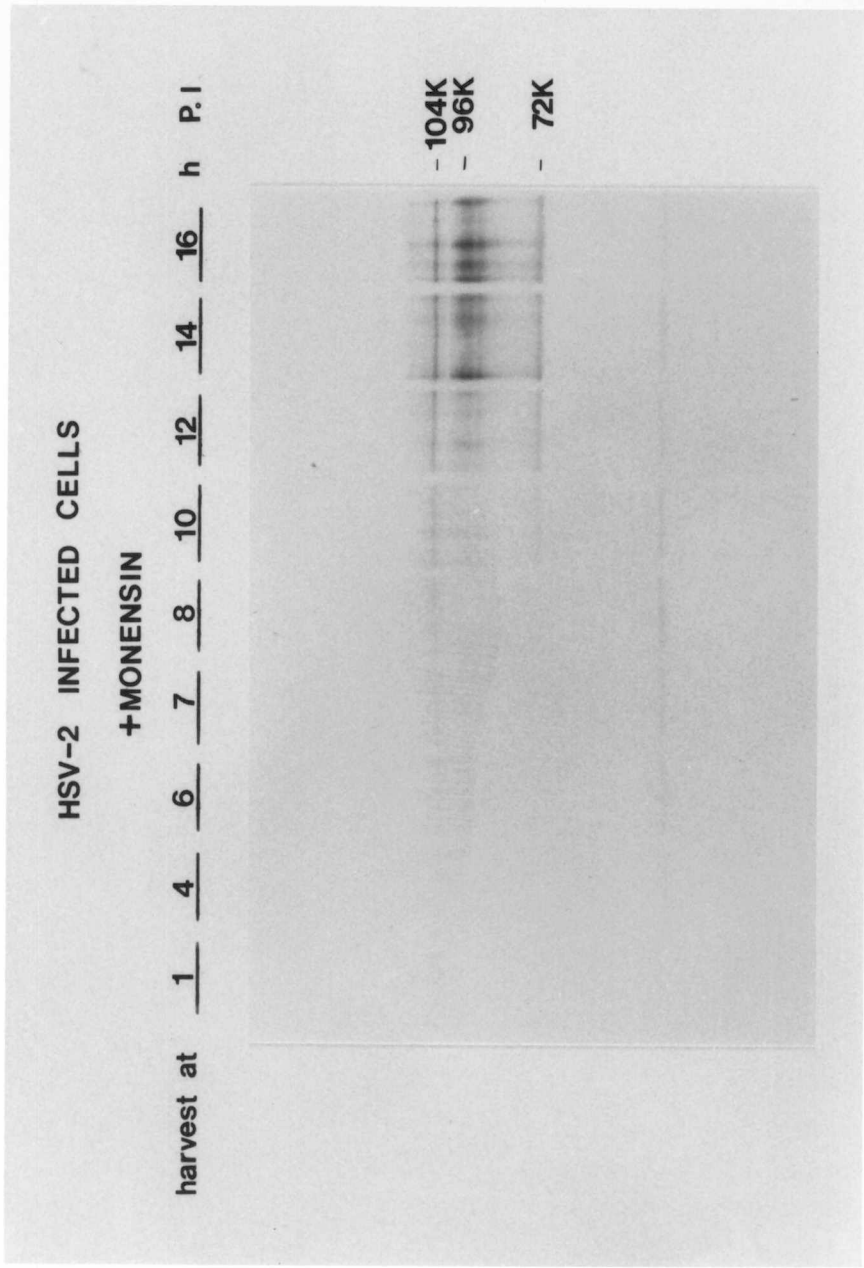

HSV-2 INFECTED CELLS
+MONENSIN
harvest at 1 4 6 7 8 10 12 14 16 h P.I
104K
96K
72K

Figure 19

Figure 20. Kinetics of synthesis of HSV-2 gG and its intermediates in the presence of tunicamycin. HSV-2 infected HEp-2 cultures were maintained in the presence of tunicamycin and harvested at the indicated times postinfection. The solubilized cultures were analyzed by SDS-PAGE followed by immunoblotting with anti-gG serum.

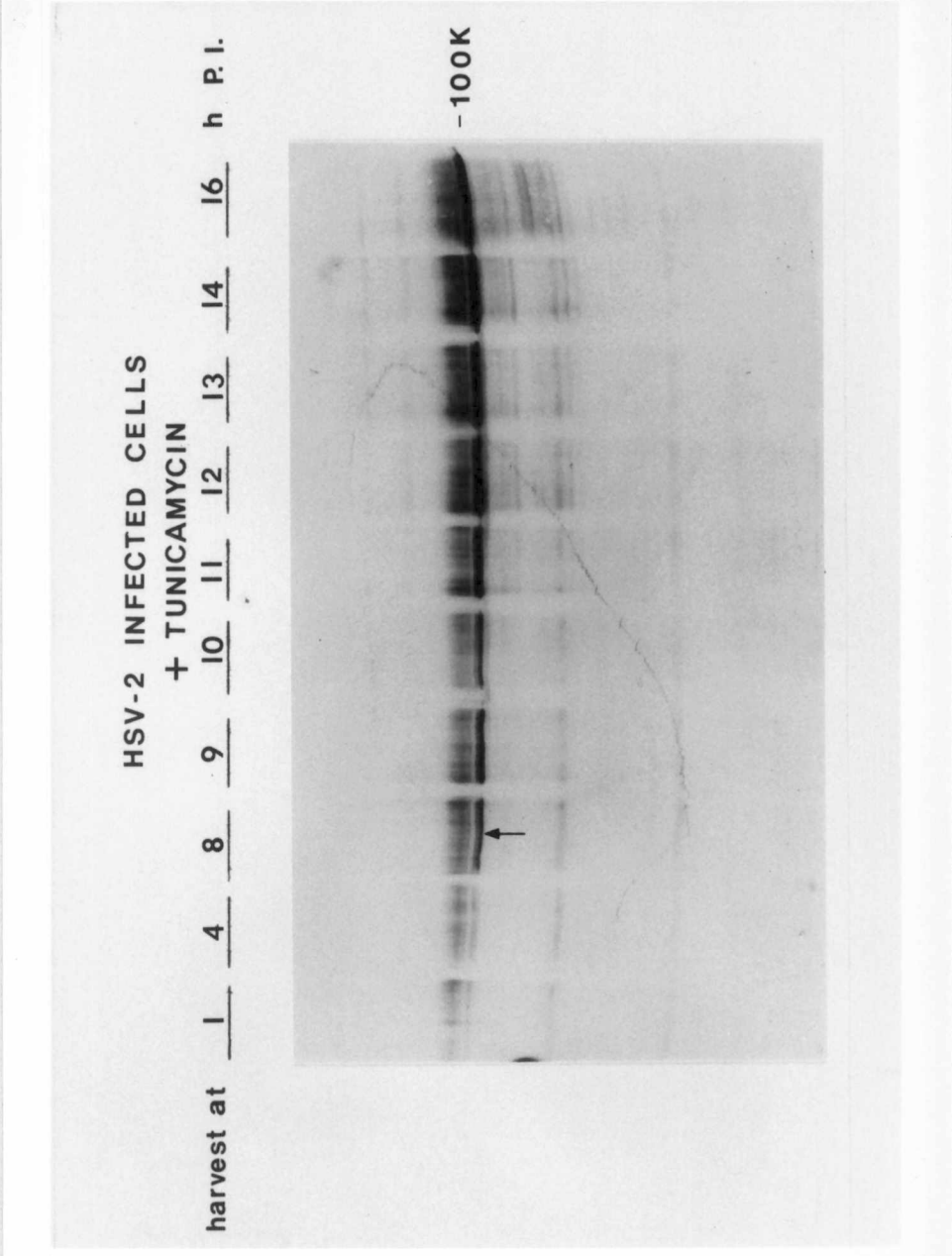

HSV-2 INFECTED CELLS
+ TUNICAMYCIN
harvest at 1 4 8 9 10 11 12 13 14 16 h P.I.
-100K

Figure 20

protein synthesis proposed by Honess and Roizman (1974), the kinetics of synthesis of gG appear to fall into the late or gamma-gene class since gG was detected after the times of maximal synthesis of early or beta genes (5 h to 7 h postinfection). Increased synthesis of gG was observed at late times (12 to 18 h postinfection) which is characteristic of "true late" gene products within HSV-infected cells.

Determination of the Requirement for DNA Replication for the Synthesis of gG. Previous studies have shown that "true late" proteins such as gC of HSV-1 are not synthesized in the absence of viral DNA synthesis whereas the synthesis of immediate early and early gene-products remain unaffected (Powell et al., 1975; Compton and Courtney, 1985). In order to further verify the observation that gG is a late gene product, the requirement for viral DNA replication for the induction of gG synthesis was examined.

HSV-2 infected cells were incubated in the presence or absence of an inhibitor of HSV-DNA synthesis, cytosine arabinoside (Ara-C), and harvested at either 1 h or 24 h postinfection. The solubilized proteins were then analyzed by immunoblotting with anti-gG (Figure 21). Only background levels (compare to the 1 h harvest) of HSV-2 gG synthesis were detectable in cultures containing Ara-C even though normal levels of gB, a beta gene product, was not affected by Ara-C (data not shown). In contrast, significant amounts of the 108K, 104K and 72K components were detected in the culture containing no inhibitor. These data therefore suggest that gG is not synthesized in

Figure 21. Effect of inhibition of HSV-DNA synthesis on the expression of HSV-2 gG. HSV-2 infected HEp-2 cells were maintained in the presence (+) or absence (-) of cytosine arabinoside (Ara-C, 50 µg/ml) and harvested at either 1 h or 24 h postinfection. The solubilized proteins were then resolved by SDS-PAGE followed by immunoblotting with anti-gG rabbit serum.

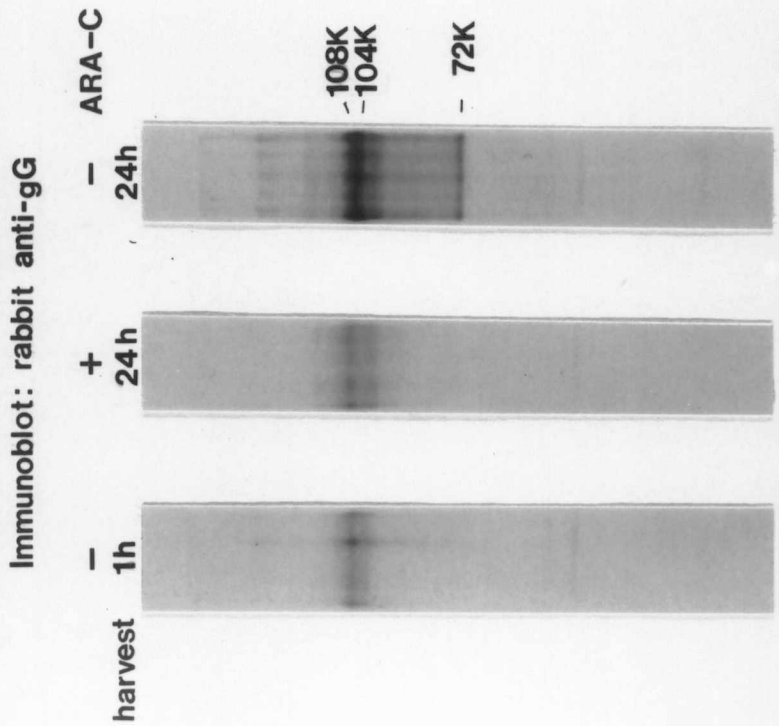

Immunoblot: rabbit anti-gG
ARA-C
–
24h
+
24h
–
1h
harvest
108K
104K
72K

Figure 21

the absence of viral DNA synthesis and hence represents a "true late" gene product.

Kinetics of gG Synthesis Following Reversal of Protein Synthesis. From the kinetics of synthesis the 104K high-mannose component appears to be synthesized prior to the synthesis of other gG specific components. To determine whether this 104K component represents the primary precursor to the 72K and 108K gG detected later, the relative times of appearance of gG and its intermediates following synchronization of protein synthesis was determined. Parallel HEp-2 cell cultures were infected with HSV-2 and prior to significant amounts of synthesis of gG (at 9 h postinfection), cycloheximide, an inhibitor of protein synthesis was added to the culture. In order to allow any precursor molecules already synthesized at 9 h to mature, cycloheximide was then maintained in these cultures for 3 h until 12 h postinfection. At 12 h postinfection cycloheximide was removed and the different cultures were harvested at the indicated times for analysis by immunoblotting using the anti-gG antiserum (Figure 22). After the release from inhibition of protein synthesis new specific gG synthesis was detected at 13 h postinfection with the increase in appearance of the 104K high-mannose component. However, specific increase in the 72K and the 108K gG was not detected until 2 h later. This finding further suggests the 104K to be the primary precursor to the 72K and the mature gG.

Synthesis of gG and Its Intermediates Following the Inhibition of Protein Synthesis. To further determine the processing of the gG

Figure 22. Kinetics of synthesis of gG and its precursors following the addition of cycloheximide. To achieve synchronous gG synthesis, parallel cultures of HEp-2 cells were infected with HSV-2 and at 9 h postinfection (P.I.) cycloheximide (CX) at 50 μg/ml was added to the cultures. Cycloheximide was maintained in the cultures of 3 h until 12 h postinfection and then removed. Following removal of the drug, samples were harvested at the various times indicated and analyzed by immunoblotting with anti-gG serum.

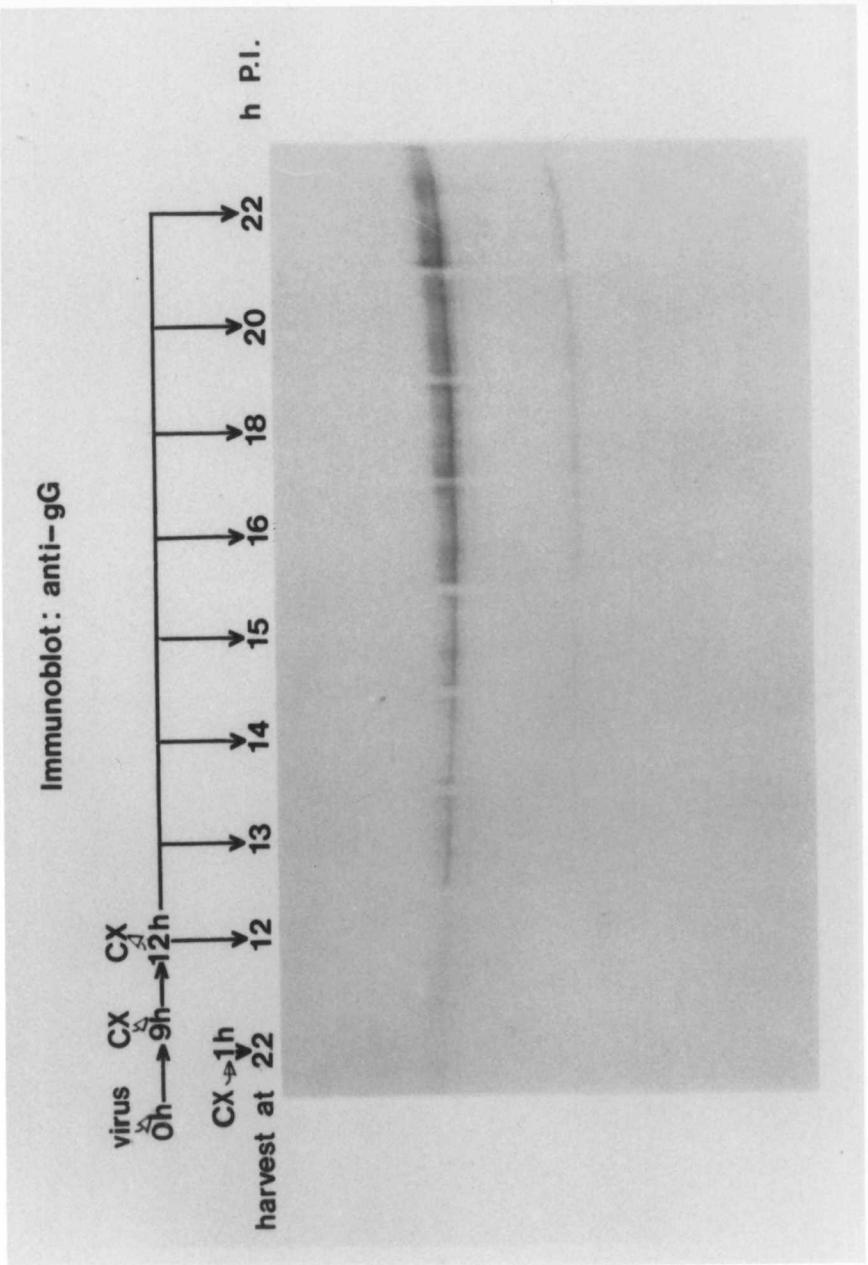

Immunoblot: anti-gG
virus CX CX
0h 9h 12h
CX 1h
harvest at 22 12 13 14 15 16 18 20 22
h P.I.

Figure 22

intermediates, the synthesis of the intermediates after the inhibition of new protein synthesis was studied. In the absence of new protein synthesis, the processing of the existing intermediates (after cycloheximide addition) into their product(s) could be followed.

HEp-2 cells infected with HSV-2 were cultured at 1 h postinfection in the presence of monensin and at 10 h, cycloheximide was added to the cultures. The cultures were then harvested at various indicated times after the addition of cycloheximide and analyzed by immunoblotting with anti-pgG. Control cultures maintained with cycloheximide from 1 to 18 h post infection (to show input virus material), or grown without cycloheximide (to indicate steady-state synthesis), were also included. The apparent processing of the 104K and 72K components (synthesized prior to 10 h postinfection) to other intermediates are shown in Figure 23. In the absence of new protein synthesis, the amounts of the 104K component was observed to decrease with time. A steady-state level of the 72K (compared with the lane at 18 h harvest without cycloheximide) was observed approximately 2 h after the addition of cycloheximide (at which time the 104K was no longer detectable). During this time a slight decrease of the 72K was observed. Since anti-pgG serum reacts poorly with more processed forms, no 96K was detectable in this experiment. A significant increase in the detectable levels of the 31K was also observed. The increased detection of the 108K mature gG was also observed (data not shown). These findings suggest the 104K to be processed into the 72K and the 31K high-mannose intermediates, and eventually to the mature 108K gG.

Figure 23. The synthesis of gG and its intermediates following the inhibition of protein synthesis. Parallel cultures of HEp-2 cells were infected with HSV-2 and after 1 h virus-adsorption period, the cultures were maintained in the presence of 10^{-6} M monensin. At 10 h after infection, cycloheximide (CX, 50 μg/ml) was added to the cultures and maintained in these cultures until harvest at the indicated times. Control cultures were maintained in the presence or absence of cycloheximide from 1 to 18 h postinfection. The solubilized cell extracts from these different cultures were analyzed by immunoblotting with anti-pgG rabbit antiserum.

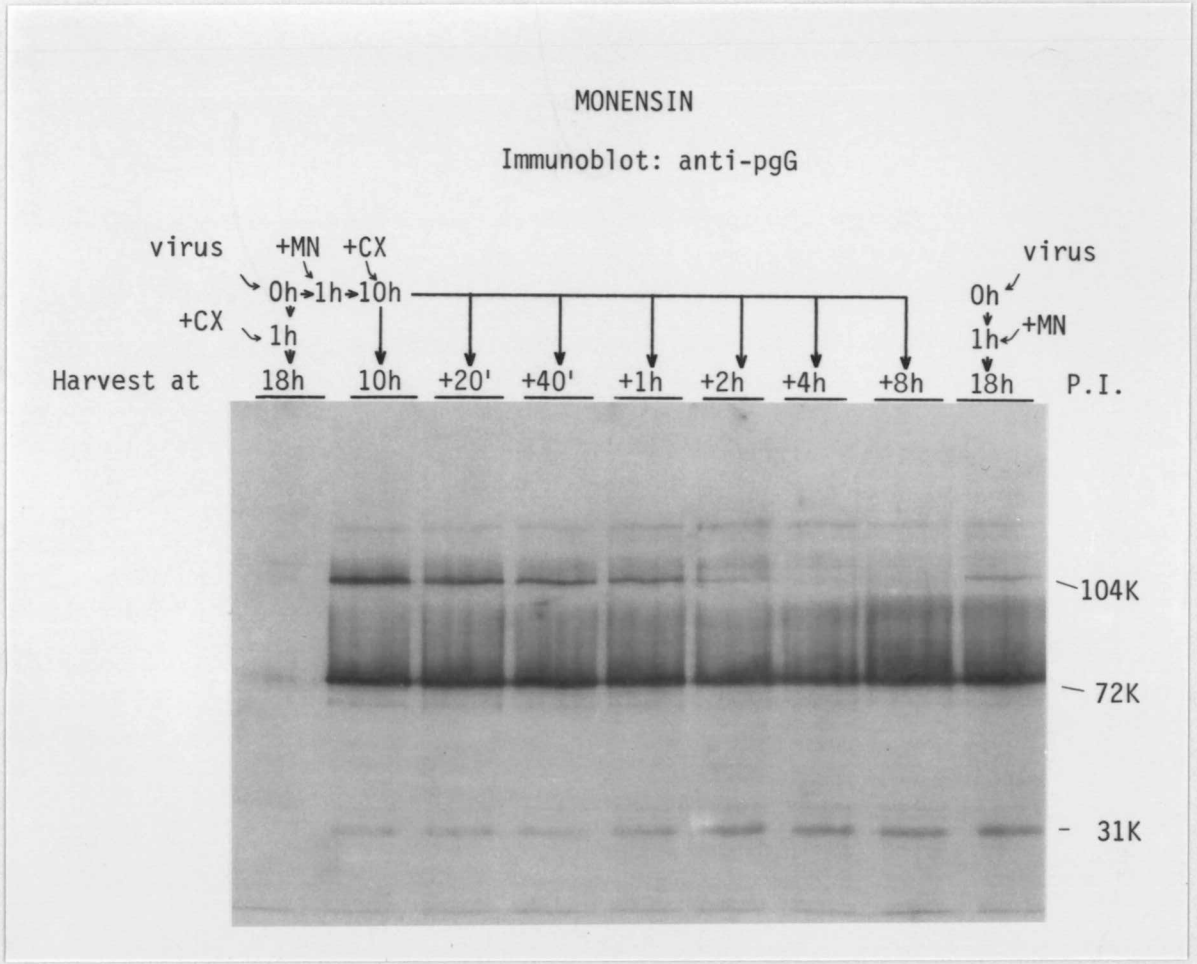

MONENSIN
Immunoblot: anti-pgG
virus
+MN
+CX
0h
1h
10h
+CX
1h
virus
0h
1h
+MN
Harvest at
18h
10h
+20'
+40'
+1h
+2h
+4h
+8h
18h
P.I.
104K
72K
31K

Figure 23

Determination of Precursor-product Relationship of gG Intermediates in Pulse-chase Experiments. The precursor-product relationship between these gG intermediates was investigated by pulse-chase experiments. Parallel HEp-2 cultures were infected with HSV-2 and pulsed at 10 h postinfection for 20 minutes with either [^{35}S]-methionine or [^{3}H]-mannose (each at 200 μCi/ml). At the end of the 20 minutes pulse the radioisotopes were removed and the cultures were chased for the indicated times before harvest. Antigens prepared from these samples were immunoprecipitated with either anti-gG serum or NRS and then analyzed on SDS-PAGE (Figure 24). The major component seen after the 20 minute pulse with [^{35}S]-methionine was the 104K component with faint amounts of the 72K detectable. With chase, this 104K band was observed to diminish with a concomitant increase in the 72K and the mature 108K gG indicating that the 104K may represent the precursor of the 72K and the 108K. A continued increase of the 72K was not observed since we suggest that the 72K is probably processed into the mature 108K gG. To specifically determine the processing relationship between the two high-mannose intermediates, 104K and 72K, the cultures were pulsed for 20 minutes with [^{3}H]-mannose instead of [^{35}S]-methionine (Figure 25). At the end of the 20 minute pulse with [^{3}H]-mannose, only the 104K high-mannose intermediate was detectable. Again, this component can be chased into the 72K component suggesting that the 72K results from the processing of the 104K. Similar levels of the 72K were observed in the 3 h chase and the 4 h continuous pulse suggesting steady-state levels of the 72K are attained at 3 h; i.e. between the generation of the 72K from 104K

Figure 24. Pulse-chase studies of precursor-product relationship between gG precursors labelled with ^{35}S-methionine. Parallel HEp-2 cultures were infected with HSV-2 and pulsed at 10 h postinfection for 20 minutes or 4 h with 200 μCi/ml of (^{35}S)-methionine. At the end of the 20 minute pulse, the radioisotopes were removed and the cultures were chased for the indicated times before harvest. Antigens prepared from these samples were immunoprecipitated with either anti-gG serum or normal rabbit serum (NRS) and then analyzed on SDS-PAGE.

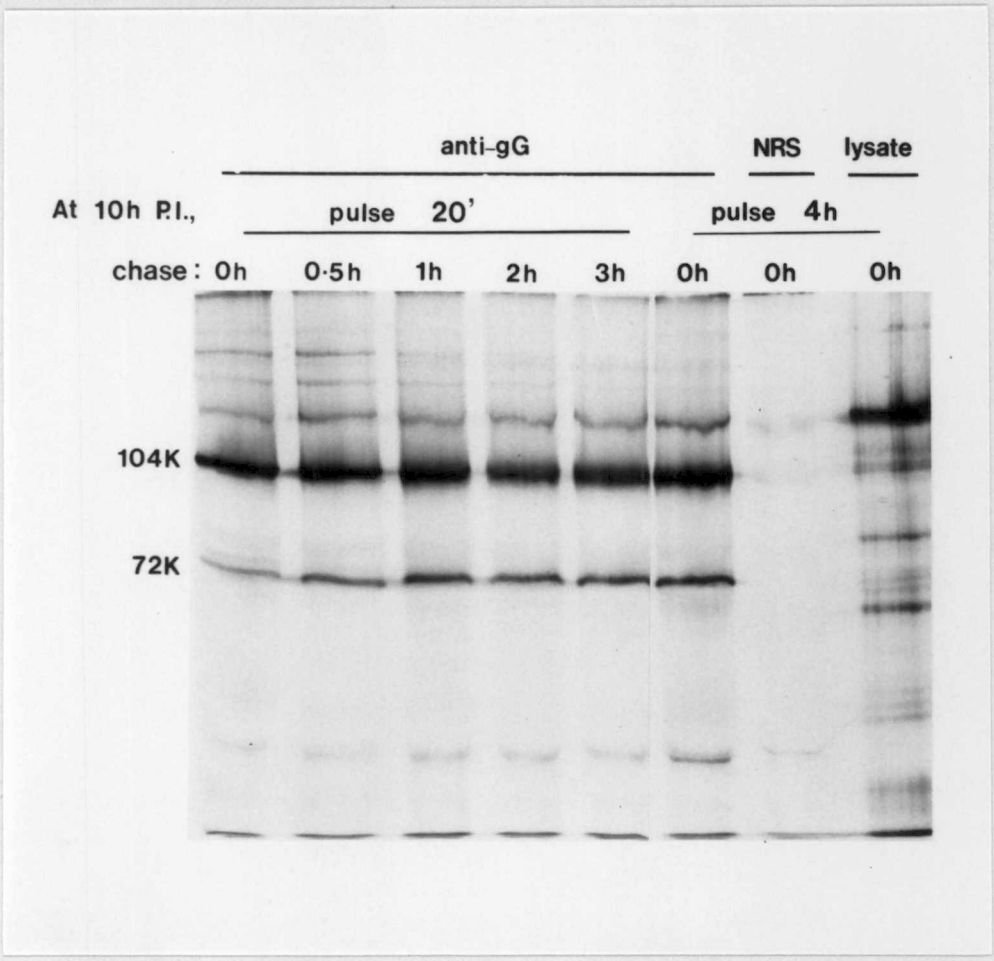

anti-gG
NRS
lysate
At 10h P.I.,
pulse 20'
pulse 4h
chase: 0h
0·5h
1h
2h
3h
0h
0h
0h
104K
72K

Figure 24

Figure 25. Pulse-chase experiments of precursor-product relationship between gG precursors labelled with ^{3}H mannose. Parallel HEp-2 cultures were infected with HSV-2 and pulsed at 10 h postinfection for 20 minutes or 4 h with 200 µCi/ml of (^{35}S) mannose. At the end of the 20 minute pulse, the radioisotopes were removed and the cultures were chased for the indicated times before harvest. Antigens prepared from these samples were immunoprecipitated with either anti-gG or normal rabbit serum (NRS) and then analyzed on SDS-PAGE.

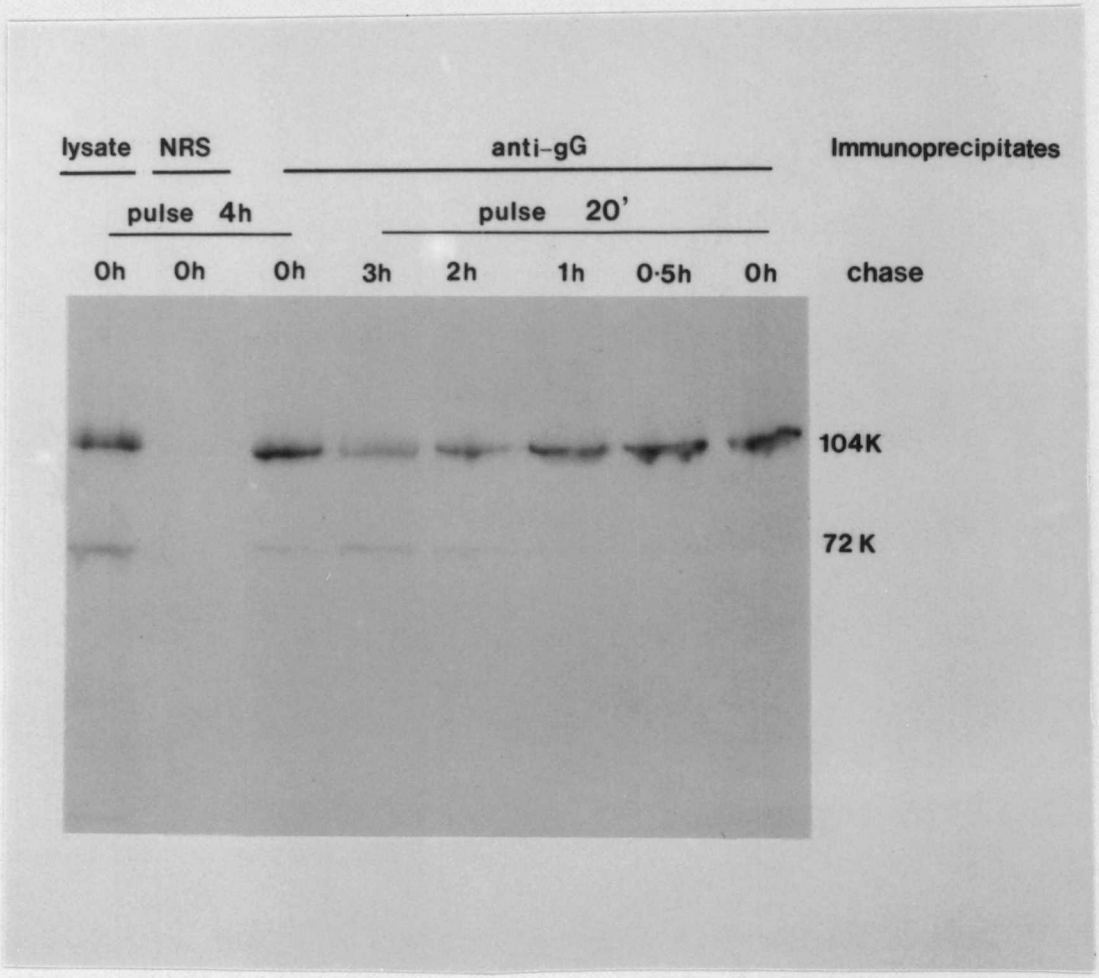

lysate
NRS
anti-gG
Immunoprecipitates
pulse 4h
pulse 20'
0h
0h
0h
3h
2h
1h
0·5h
0h
chase
104K
72 K

Figure 25

on one hand and the processing of the 72K to the mature 108K on the other. Since the gG contained predominantly mature complex oligosaccharides, it was not easily detectable when [^{3}H]-mannose was used as the radiolabel.

Determination of the Approximate Size of the Primary gG Translation Product

Since the 104K high-mannose intermediate has been shown to be the primary gG precursor, the unglycosylated 100K component detected in the presence of tunicamycin and resulting from endo H digestion of the 104K, may represent the unglycosylated precursor of the 104K. To verify this possibility, the primary translation product of gG synthesis was determined by in vitro translation. The extraction and isolation of the poly A$^+$ RNA has been described in Materials and Methods. Poly A$^+$ RNA from HSV-2 infected cells harvested at 18 h postinfection was translated in a rabbit reticulocyte translation system. Following a 2 h incubation at 30°C, the translation products were solubilized and immunoprecipitated with the anti-gG serum and the immunoprecipitates then analyzed by immunoblotting with anti-gG. Control globin mRNA as shown in Figure 26, was translated in the presence of [^{35}S]-methionine and analyzed on the same immunoblot without immunoprecipitation. The synthesis of [^{35}S]-methionine-labelled globin was clearly detected. In vitro translation of poly A$^+$ RNA from HSV-2 infected cells encoded a single polypeptide of an approximate apparent molecular weight of 100K. This supports the

Figure 26. In vitro translation of poly A^+ RNA from HSV-2 infected HEp-2 cells. HSV-2 infected cells were harvested at 18 h postinfection and poly A^+ RNA was isolated as described in Materials and Methods. Various (0, 1x or 2x) amounts of poly A^+ RNA were translated in a rabbit reticulocyte translation kit (BRL) for 2 h at 30°C. The in vitro translated products were solubilized and immunoprecipitated with the anti-gG rabbit antiserum. The immunoprecipitates were then solubilized and analyzed by the immunoblot with the anti-gG serum. Control globin mRNA were translated under identical conditions except in the presence of 5 μCi of [^{35}S] methionine and analyzed without immunoprecipitation on the immunoblot. Solubilized lysates from HSV-2 infected HEp-2 cells cultured in the presence or absence of tunicamycin were also analyzed on the same immunoblot to indicate the relative positions of the gG intermediates. The arrow indicates the detection of gG-specific translation product.

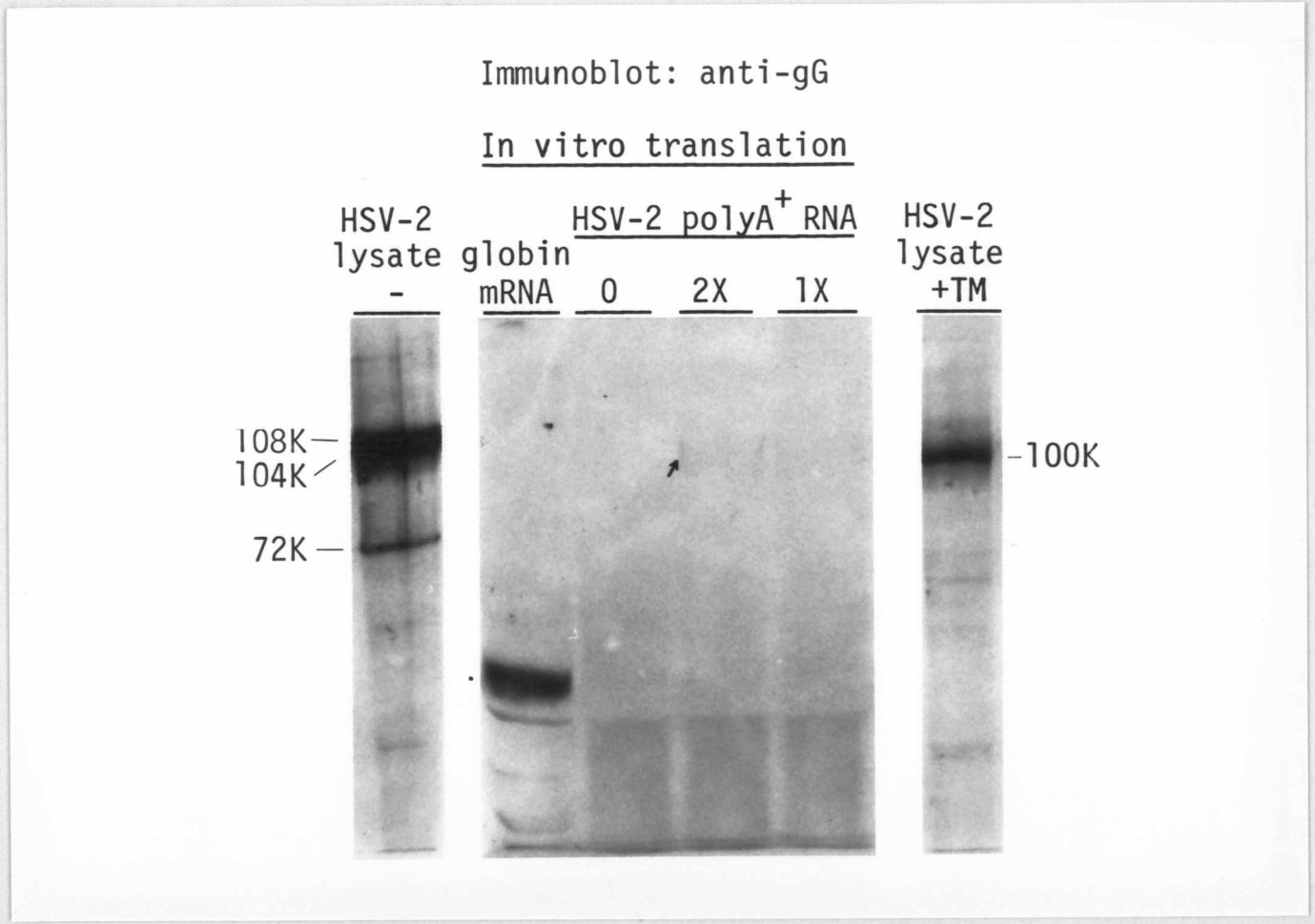

Immunoblot: anti-gG
In vitro translation
HSV-2 lysate -
HSV-2 polyA+ RNA
globin mRNA
0
2X
1X
HSV-2 lysate +TM
108K
104K
72K
100K

Figure 26

suggestion that the 100K is the primary unglycosylated precursor of gG synthesis.

CHAPTER IV

DISCUSSION

Introduction

The glycoproteins of HSV have been the focus of numerous studies because of the important role these components play in the various aspects of the infectious cycle of the virus. The HSV glycoproteins are major components of the viral envelopes as well as of the surface membranes of infected cells (Heine et al., 1972; Spear and Roizman, 1972; Spear, 1976). They hence provide major targets for the host's humoral and cellular immune responses to the virus infection. Because of their location within the envelope of mature virus particles, these glycoproteins are also implicated to play a functional role in the attachment, fusion, and penetration of the virus into the host cell. Additionally, these glycoproteins may also be involved in the virus budding (from the inner nuclear membrane) and eventual release from the cell. However, the precise function of each of these HSV glycoproteins has not been established.

The overall goal of the research program within our laboratory has been to study the synthesis and processing of the HSV glycoproteins within the infected cell in order to obtain some insights into the roles of the glycoproteins within the infectious cycle of the virus. Such information is vital for the development of a rational effective treatment and preventive strategies against the virus. Moreover, the study of the HSV glycoprotein processing may also provide new clues

into the biosynthetic mechanisms associated with processing of eucaryotic cell glycoproteins.

This investigation has focused on unravelling the biosynthetic pathway that is involved in the synthesis of the HSV-2 glycoprotein designated gG. Because of the predominant use of HSV-1 as a model system for studying the synthesis of HSV glycoproteins (see review by Courtney, 1984) and since gG of HSV-2 has no known HSV-1 counterpart, the synthesis of the gG glycoprotein of HSV-2 is poorly understood. The availability of monospecific and monoclonal antibodies reactive with gG, as well as inhibitors of glycoprotein processing and specific endoglycosidases were all of major importance in enabling the identification of the glycoprotein precursors and intermediates involved in the synthesis of gG. The mechanisms associated with the processing of gG appear to differ from that observed in the processing of the other HSV glycoproteins. These studies have demonstrated the involvement of a cleavage event in the scheme of processing of gG and a biosynthetic pathway of gG is proposed. The information obtained in these studies will provide a foundation for the further characterization of the functional role of gG within the HSV-2 infected cell. Insight into the cellular mechanisms associated with the synthesis and processing of glycoproteins is also obtained.

Synthesis of HSV-2 Glycoproteins in the Presence of Tunicamycin

In these studies tunicamycin was used to define the types of glycosidic linkage that might be present on the glycoproteins of HSV-2. Since tunicamycin is a specific inhibitor of N-linked glycosylation

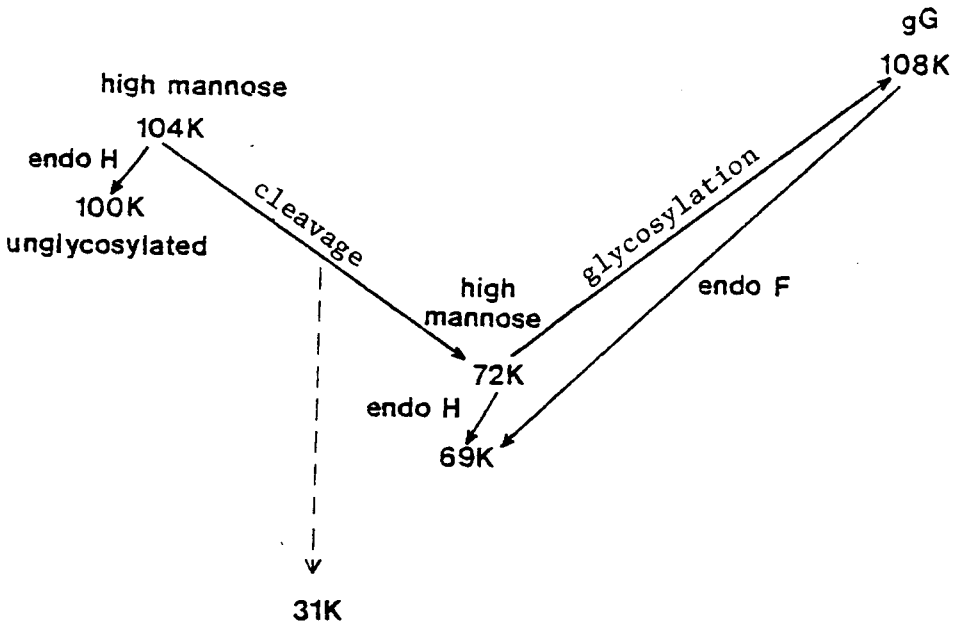
(Tkacz and Lampen, 1975), absence of detectable glycosylation in the presence of the inhibitor suggests the presence of N-linked carbohydrates on the glycoproteins. In contrast, since the synthesis of O-linked glycosylated proteins is not affected by tunicamcyin, O-linked glycosylation would be detected in the presence of the inhibitor.

Studies in which tunicamycin was used suggest that predominantly N-linked oligosaccharide structures are associated with the glycoproteins of HSV-2. In the presence of tunicamycin, the glycosylation of the HSV-2 glycoproteins was completely inhibited even though under identical culture conditions, glycosylation of the HSV-1 glycoprotein, gC (known to contain O-linked oligosaccharides) was readily detected. These results suggest that no detectable O-linked glycosylation is associated with the HSV-2 glycoproteins when the virus-infected cultures are maintained in the presence of tunicamycin. The possibility that the O-linked glycosylation events of the cell may be inhibited by some effect of the HSV-2 infection was ruled out since in cells doubly infected with HSV-1 and HSV-2, O-linked glycosylation of the HSV-1 gC was observed in the presence of tunicamycin. Additionally, it has been suggested that perhaps in some proteins, the presence of high-mannose structures may be required for the proper configuration for the addition of O-linked oligosaccharides (Spear, 1985). Inhibition of the formation of high-mannose structure in the presence of tunicamycin would hence result in the absence of O-glycosylation which is believed to occur in the Golgi. Therefore, the presence or absence of O-linked oligosaccharides on the

glycoproteins of HSV-2 would have to be further determined by the analysis of the carbohydrate chains on the mature glycoproteins.

With respect to the effect of tunicamycin on gG synthesis, the presence of predominantly N-linked oligosaccharide structures on gG is supported from the observation that there is a complete inhibition of glycosylation in the presence of the drug. However, the possibility that the presence of high-mannose structures may be required in order to achieve the proper confirmation of the protein for the addition of O-linked sugars on gG cannot be ruled out completely. This is deemed unlikely if one considers the apparent molecular weights of the various intermediates in proposed pathway for gG synthesis (Figure 27). For example, the removal of the high-mannose structures from the 104K intermediate (detected in the absence of tunicamycin), generates a 100K component that comigrates with the unglycosylated 100K detected in the presence of tunicamycin. We suggest that the 100K component represents the unglycosylated precursor of gG based on the data generated with endo H digestion, tunicamycin and in vitro translation. This evidence, together with the fact that no O-linked carbohydrate are present on the 104K intermediate, further supports the conclusion that significant amounts of O-linked oligosaccharides are not associated with gG. In addition, it can be deduced that only high-mannose sugars are present on the 72K and 31K components since both are endo H sensitive components and as their combined apparent molecular weights approximate that of their precursor, the 104K high-mannose intermediate. Finally, the mature 108K gG derived from processing of the 72K component, also lacks O-linked structures since the molecular weight of the 108K gG

Figure 27. Proposed scheme of gG synthesis and processing.


gG
108K
high mannose
104K
endo H
100K
unglycosylated
cleavage
glycosylation
endo F
high
mannose
72K
endo H
69K
31K

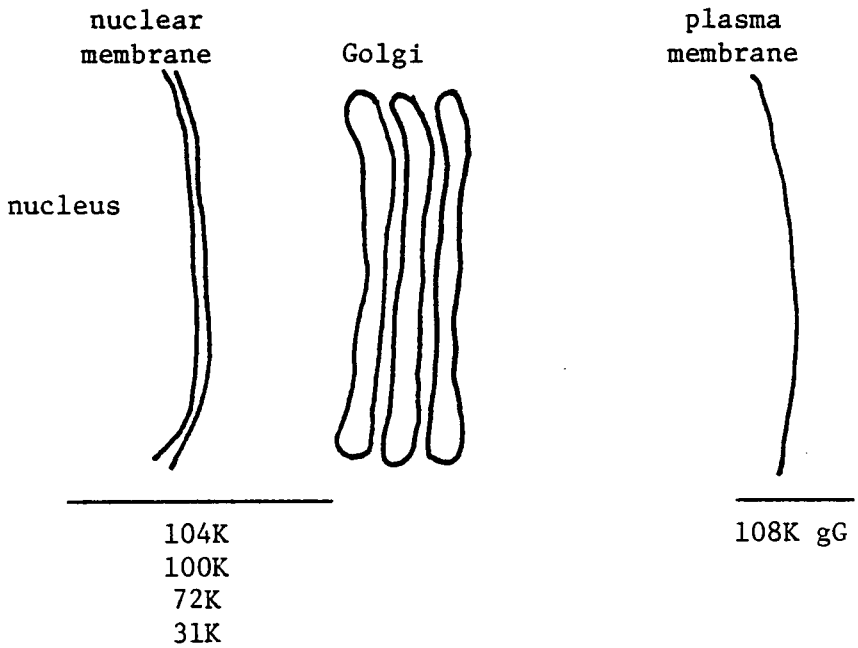

nuclear
membrane
Golgi
plasma
membrane
nucleus
104K
100K
72K
31K
108K gG

Figure 27

without the N-linked complex sugars (following endo F digestion to remove N-linked high-mannose and complex oligosaccharides), comigrates with the 69K component which is generated following the removal of the high-mannose oligosaccharides from the 72K component by endo H. From the results obtained, it appears that gG contains predominantly N-linked carbohydrates.

The finding of O-linked glycosylation associated with gG in the presence of tunicamycin has been recently reported by Balachandran and coworker (Balachandran and Hutt-Fletcher, 1985). These workers were able to detect relatively small amounts (compared to the controls cultured in the absence of tunicamycin) of an anti-gG reactive protein labelled with [^{3}H]-glucosamine or galactose (100 μCi/ml) for a labelling period from 3 to 20 h postinfection in the presence of tunicamycin. Under such radiolabelling conditions, detection of unglycosylated proteins in the presence of tunicamycin which are isotopically labelled with amino acids that are derived from conversion of some of the oligosaccharide radiolabels (see review by Warren, 1972) cannot be excluded. From the difference in radiolabelling intensities of gG in cultures grown in the presence versus the absence of tunicamycin (from data of these workers), comparatively little, if any O-linked sugars can be deduced to be present on gG.

Synthesis of gG in the Presence of Monensin

The processing and transport of viral glycoproteins synthesis in the presence of monensin have been shown to be inhibited at the level of the Golgi. In this study monensin was used to facilitate the

detection of the gG intermediates which were dependent on the Golgi for their processing.

Incubation of infected cells with monensin resulted in the inhibition of the synthesis of the mature gG (108K). Instead, a 72K and 104K glycoprotein, also present in infected cells cultured without monensin, were detected together with the accumulation of a diffuse 96K glycoprotein band. The endo H digestion experiment suggested the 72K and 104K to contain high-mannose sugars. Such detection of high-mannose containing intermediates in the presence of monensin has been previously described in HSV-1 infected cells (Wenske et al., 1983; Johnson and Spear, 1982). The 96K glycoprotein, however, was not of the high-mannose type being insensitive to endo H digestion. Since mature gG contains sialic acid residues, the resistance of 96K to neuraminidase together with the observation that the product of neuraminidase digestion of gG was still larger than 96K seemed to imply that the complex carbohydrate chains on 96K glycoprotein were incomplete. The diffuse nature of this 96K component can then be explained by the presence of a heterogenous population of glycosylated polypeptides resulting from varying degrees of complex sugar maturation, arrested prior to terminal sialic acid addition by the disruption of normal Golgi function. Synthesis and processing of polypeptides containing incomplete complex oligosaccharides in the presence of monensin has been previously reported by Pesonen and Korasiainen (1982).

Identification of gG Related Components

The use of monospecific anti-gG antisera has facilitated the detection of multiple anti-gG reactive proteins. Before each of these proteins was considered an intermediate or precursor component of gG several criteria were used to establish such a relationship. First, each of the proteins was reactive with both polyclonal and monoclonal anti-gG sera by radioimmunoprecipitation and on the immunoblot where the proteins were denatured, reduced to monomeric forms and separated by electrophoresis on polyacrylamide gels. This clearly suggested that each of the components detected by the anti-gG antibodies possess gG-specific antigenic determinants. The polyclonal anti-gG serum was shown to react with the same gG-specific proteins as the monoclonal antiserum since molecules first precipitated by the monoclonal anti-gG also reacted with the polyclonal anti-gG on the immunoblot and vice versa. Additionally, these anti-gG reactive components were detected only in HSV-2 infected cells and not in mock or HSV-1 infected cells suggesting these components to represent HSV-2 specific proteins.

Synthesis of gG

The primary goal of this study was to determine the synthesis and processing of gG and its intermediates. From the results of this investigation a model for the synthesis and processing pathway of gG is suggested (Figure 1). In this model, gG is proposed to be synthesized first as a high-mannose intermediate designated the 104K component which is then processed by a cleavage event into the 72K high-mannose intermediate. The 72K is then further glycosylated by the synthesis of complex oligosaccharides to generate the mature 108K gG.

Studies were conducted to determine the relationship between the gG-related proteins detected by the monoclonal and polyclonal anti-gG sera during the synthesis of gG. In kinetic studies designed to determine the time of synthesis of these components after virus infection, or the processing after the reversal of inhibition of protein synthesis in the presence of cycloheximide, the 104K high-mannose component was consistently observed to be the first component synthesized. Additionally, after the addition of cycloheximide and in pulse-chase experiments, the decrease in detection of the 104K was followed subsequently by an increase in the appearance 72K and 108K gG. These observations thus suggest that 104K component represents the primary precursor glycoprotein component synthesized in the overall scheme of gG synthesis.

The synthesis of the 104K, high-mannose intermediate in the absence of detectable unglycosylated precusors suggests that the synthesis of gG to occur by a contranslational addition of high-mannose oligosaccharide structures (see review by Kornfeld and Kornfeld, 1985). Endo H digestion of the 104K yielded a 100K protein similar in apparent molecular weight to the 100K unglycosylated component synthesized in the presence of tunicamycin. Such results strongly suggest that the 100K component represents the unglycosylated gG precursor. This is further supported by the identification of a single 100K gG-specific product in the <u>in vitro</u> translation experiments. The synthesis of gG therefore occurs by the cotranslational addition of high-mannose structures to the 100K polypeptide backbone.

Our data supports the hypothesis that the 104K high-mannose intermediate is processed to the 72K high-mannose component which is further processed into the mature gG. Since only a single gG-specific comonent of an apparent molecular weight of 100,000 was synthesized in in vitro translation experiments as well as in the presence of tunicamycin, the 72K component is probably not synthesized from a separate mRNA. In addition, evidence from the kinetic studies and pulse-chase experiments are consistent with the interpretation that the 72K is a non-specific breakdown product of 104K is unlikely in light of the evidence that it is processed to the 108K gG. In pulse-chase studies, a decrease in the amount of the 72K was observed concomitant with an increase in the 108K. More importantly, deglycosylation of the 108K following endo F digestion resulted in the appearance of a 69K component. This component comigrated with the 69K component generated following the removal of high-mannose structures from the 72K component by endo H digestion. Further glycosylation of the 72K component therefore results in the formation of the mature complex 108K gG.

Processing by Cleavage

The conversion of the 104K high-mannose component to the 72K high-mannose intermediate is suggested to occur by a cleavage processing event. Since both molecules contain high-mannose structures, trimming of the high-mannose structures from the 104K could not account for the 32,000 apparent molecular weight difference between 104K and 72K. The involvement of proteolytic cleavage of the 104K to generate the 72K product is thus suggested. Supporting evidence for

this cleavage processing is shown by the detection of a 31K high-mannose containing protein with the anti-pgG serum. The time of appearance of the 31K component in the kinetic experiment following cycloheximide addition (Figure 23) is consistent with this protein being the other cleavage-product originating from the 104K component.

The detection of the 31K only by the anti-pgG and not the anti-gG sera is also in agreement with the 31K being a cleavage product of the 104K. The anti-gG antiserum was made to the mature gG-antigen which according to this proposed model contains only the 72K portion of the 104K and hence cannot recognize the 31K product. However, the anti-pgG was raised in rabbits against the pgG molecule (shown to be the 104K in this study) which contains the 72K and the 31K antigenic determinants. Therefore, both the 72K, and the 31K are recognized by the anti-gG antiserum. The poor reaction of the anti-pgG with the complex glycosylated gG and the 96K probably reflects changes in exposed antigenic sites which may occur following the processing of the high-mannose to the complex structures.

Experiments were also conducted to confirm this cleavage event by attempting to inhibit the conversion of the 104K intermediates to the 72K and the 31K. These studies were performed by culturing HSV-2 infected cells in the presence of amino acids analogs, canavanine and azetidine (analogs of arginine and proline, respectively), or inhibitors of proteases, PMSF, TLCK, TPCK and pepstatin A (specific for serine proteases, trypsin, chimotrypsin and pepsin, respectively). However, specific inhibition of this cleavage event was not observed. Whether this is due to the possibility that inappropriate experimental

conditions were used in these studies or perhaps that the cleavage enzyme of the 104K intermediate is insensitive to the incorporation of the amino acid analogs or the presence of these inhibitors, needs to be further determined.

The similarity of the cleavage processing of gG to that described for ortho- and paramyxoviruses, togaviruses and retroviruses is not clear at present. Unlike the cleavage of these viruses which takes place both intracellularly and extracellularly, the cleavage of gG appears to occur at a pre-Golgi site (possibly the endoplasmic reticulum) within the infected cell. The 104K precursor and cleaved products 72K and 31K were detected predominantly in association with the nuclear fraction while mainly the matured cleavage product, the 108K gG was associated with the cytoplasmic fraction. Unlike the cleavage of the F protein of paramyxoviruses which is cleaved even in the presence of tunicamycin (Strauss and Strauss, 1983), no cleavage of the unglycosylated precursor was detected in the presence of the inhibitor. Further studies are needed to determine if a similar cleavage sequence to that seen in the other viruses described, were involved in the processing of gG.

Possible Functions of gG

The functional role of gG is currently unclear due to the lack of virus mutants in the production of gG. From the similarities in antigenic determinants as well as processing pathways in gG synthesis associated with various laboratory strains and clinical isolates of HSV-2, one could suggest that a highly conserved function for gG in

HSV-2 which may be important for the survival of the virus. The absence of gG in HSV-1 indicates either that the function of this molecule is not crucial for the production of the HSV-1 or that some as yet unidentified functional counterpart of gG (not necessarily antigenically similar) may be present. Studies employing genetically engineered HSV-2 mutants in the gG gene may provide the means to address these questions.

Although no definite functional role of gG can be inferred from the results of this investigation, a possible involvement of gG in the maturation of the HSV-2 virus can be suggested. One could speculate that since gG is synthesized as a late gene product, i.e. after viral DNA synthesis, the involvement of gG in the virus the maturation event, possibly virus budding is suggested. What triggers this budding process is not known but it presumably requires the interaction of viral membrane protein(s) with the viral capsid components. The true-late glycoproteins probably are good candidates for such a function. Of these late gene products of HSV, gC has been shown to be a nonessential gene for virus production of HSV-1 in tissue culture since HSV-1 strains lacking the production of this glycoprotein replicates normally in such cultures (Spear, 1976). Since the virus matures by budding from the nuclear membrane, the cleavage of the 104K gG precursor associated with the nuclear fraction may provide the activation for this budding process. This role may be similar to the cleavage-dependent budding of pE_2 to E_2 and E_3 in the maturation of togavirus from the plasma membrane (Bracha and Schlesinger, 1976; Jones et al., 1977). According to the popular model for virus egress

(Johnson and Spear, 1980; Compton and Courtney, 1983) the virus containing the 72K cleavage product would then be further glycosylated by passage through the Golgi to the 108K gG in the mature virus.

Counterpart of HSV-2 in HSV-1

Numerous studies, including this investigation have demonstrated, using monospecific polyclonal and monoclonal anti-gG sera, the absence of any detectable antigenic counterpart to the gG of HSV-2 in the type 1 virus. However, of the four other HSV-2 glycoproteins gB, gC, gD and gE, each has a counterpart glycoprotein in HSV-1 sharing common antigenic determinants (also demonstrated by genetic sequence homology), colinear map locations on their respective viral genomes, common biochemical properties and presumably analogous functions. Considering such remarkable similarities between the glycoproteins of the two virus types, the absence of a counterpart of HSV-2 gG in HSV-1 is surprising.

Two possible explanations may account for the lack of detection of a HSV-1 gG. The first possibility is that the function of gG is no longer essential for the survival of HSV-1 virus in its host. Since the natural occurrence of the two virus types in humans is genital for HSV-2 and oral-facial for HSV-1, gG may provide a selective advantage for growth of HSV-2 in the genital regions. A different function may be required for the HSV-1 oral-facial replication.

Another possible explanation for the absence of HSV-2 gG counterpart in HSV-1 may be that in the type 1 virus a different protein (as yet unidentified) may have evolved that performs an

analogous function to gG in the replication of the virus but is no longer antigenically related to gG. Recent studies reported at the 10th International Herpesvirus Workshop by several investigators (Lee et al., 1985) have suggested the identification of a possible gG counterpart designated gG-1 in HSV-1 based on colinear gene mapping of the 55,000 molecular weight protein and HSV-2 gG to the left of the gD gene in their respective genomes. However, no antigenic similarities were demonstrated between HSV-2 gG and gG-1. Whether gG-1 represents a HSV-2 functional counterpart has yet to be confirmed.

CHAPTER V

CONCLUSION

The data generated from this investigation are focused on the events associated with the synthesis and processing of the HSV-2 glycoprotein, gG. The following conclusions have been drawn from the series of experiments described in this dissertation. These include:

1. The glycoprotein contains predominantly N-linked oligosaccharide structures of the complex type. The complex type structures appear to be fully processed including the presence of terminal sialic acid residues.

2. The time of the synthesis of gG indicates that the glycoprotein would be classified as a late or gamma class relative to the cascade pathway of HSV protein synthesis. HSV-2 DNA synthesis is required for the expression of gG synthesis.

3. The gG glycoprotein is synthesized as a cotranslationally glycosylated high-mannose intermediate of 104K. The 104K is processed to the 72K high-mannose intermediate which is then further glycosylated to generate the mature gG.

4. Unlike the synthesis of the other HSV glycoproteins, the processing of gG involves cleavage of the 104K intermediate into the 72K and 31K high-mannose containing glycoproteins. Additionally, this cleavage event requires the presence of high-mannose carbohydrate structures on the 104K. This cleavage processing of gG primarily occurs prior to further processing in the Golgi.

5. The high-mannose precursors of gG are primarily associated with isolated nuclei. The mature gG glycoprotein is localized mainly in the cytoplasmic fraction of the cell. Transport of gG antigens to the plasma membrane is inhibited in the presence of monensin or tunicamycin.

6. The unglycosylated gG precursor and the primary translation product of gG synthesis has an apparent molecular weight of approximately 100,000.

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