Herpesvirus-induced expression of sLe^x and related O-linked glycans in the infected cell

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Abstract

Lewis antigens constitute a family of fucosylated carbohydrate antigens (glycotopes), involved in leukocyte homing and related immunological phenomena. These glycotopes are only expressed restrictedly in normal cells, but are induced at appropriate occasions. It is well established that many tumors "hijack" Lewis glycotopes for e.g. extravasation and metastasis, and recent data indicate that also human retroviruses may use a similar strategy for colonization of distal tissues. The overall goal of the present thesis was to explore the prerequisite for this phenomenon to occur in cells infected with herpesviruses, a virus family where persistent infections and immune evasion are important hallmarks.

Using confocal immunofluorescence, neo-expression of Lewis antigens was found on cells infected with herpes simplex virus type-1 (HSV-1), varicella-zoster virus (VZV), and cytomegalovirus (CMV). However, whereas the neurotropic viruses VZV and HSV-1 induced sialyl Lewis x (sLe^x), CMV induced Lewis y (Le^y) at the surface of the infected cells. Real time RT-PCR methods for transcriptional analysis of all known human fucosyltransferase genes (FUT) were developed to determine the mechanism behind virus-specific induction of different glycotopes. The herpesviruses investigated were all able to induce transcription of FUT3, FUT5 and FUT6 relevant for sLe^x and Le^y synthesis whereas only CMV induced FUT1, necessary for Le^y expression. In most cases the transcriptional activity of these genes was several orders of magnitude larger in virus-infected cells compared to uninfected cells.

The viral factors causing neo-expression of glycotopes were explored using FUT5 and the HSV-1 infected cell as a model system. It was found that the transcripts of the immediate early viral gene, designated ICP0, was able to induce FUT5 transcription without assistance of the translated gene product. This finding explained the extremely early occurrence of host FUT5 RNA, detectable as early as one hour post infection. However, several other viral factors were engaged in regulation of the FUT5 transcription downstream the ICP0 induction. The viral glycoprotein gC-1 was identified as a probable candidate as a carrier of O-linked glycans and important regulatory elements of the O-glycosylation sequon of gC-1 were characterized. These regulatory elements were decisive for the social behavior of virus-infected cells in culture.

The conclusion of the present work is that herpesviruses possess powerful mechanisms for viral control of the expression of selectin ligands and similar glycotopes, of relevance for tumor metastasizing and tissue invasion of human transforming retroviruses. sLe^x and Le^y constitute targets for development of cancer chemotherapy, but further investigation is necessary to determine whether this approach is applicable also for treatment of herpesvirus-infections.

List of Publications

I. Mårdberg K, **Nyström K**, Tarp MA, Trybala E, Clausen H, Bergström T, Olofsson S. Basic amino acids as modulators of an O-linked glycosylation signal of the herpes simplex virus type 1 glycoprotein gC: functional roles in viral infectivity. *Glycobiology 2004; 14(7):571-81.*

II. Nyström K, Biller M, Grahn A, Lindh M, Larson G, Olofsson S. Real time PCR for monitoring regulation of host gene expression in herpes simplex virus type 1-infected human diploid cells. *J Virol Methods 2004; 118(2):83-94.*

III. **Nyström K**, Grahn A, Lindh M, Brytting M, Mandel U, Larson G, Olofsson S. Virus-induced transcriptional activation of host FUTgenes associated with neo-expression of Le^y in cytomegalovirus- and sialyl-Le^x in varicella-zoster virus-infected diploid human cells. *Glycobiology 2007; 17(4): 355-366.*

IV. Nyström K, Elias P, Larson G, Olofsson S. Herpes simplex virus type 1 ICP0-activated transcription of host fucosyltransferase genes resulting in neo-expression of sialyl-Le^x in virus-infected cells*. In Manuscript.*

Table of Contents

List of Abbreviations and Acronyms

CHX	Cycloheximide
CMV	Cytomegalovirus
CS	Chondroitin sulfate
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific ICAM-3-grabbing nonintegrin
*dl*1403	HSV-1 mutant with deletion in ICP0
E	Early
FITC	Fluorescein Iso-thiocyanate
Fuc	fucose
Fuc-TI	fucosyltransferase 1
FUT	Fucosyltransferase gene
Gal	galactose
GalNAc	N-acetyl galactosamine
gC, gD	glycoprotein C, D
GlcNAc	N-acetylglucosamine
Glycotope	Acronym for carbohydrate epitopes, defined by a specific antibody or lectin
GMK	Green monkey kidney cells
HEL	Human embryonic lung fibroblasts
HS	Heparan sulfate
HSV-1	Herpes simplex virus type-1
$HSV_{114,117}$	HSV-1 mutated at gC aminoacid 114 and 117
HTLV-1	Human T-cell leukemia virus type-1
ICP0	Infected cell protein 0
IE	immediate early
L	Late
LAT	Latency associated transcript
Le^a	Lewis a
Le^b	Lewis b
Le^x	Lewis x
Le^y	Lewis y
MOI	Multiplicity of infection
ND10	Nuclear domain 10
PCR	Polymerase chain reaction
pgC	Precursor gC
sLe^a	sialyl Lewis a
sLe^x	sialyl Lewis x
TRITC	Tetramethyl Rhodamine Iso-Thiocyanate
tsS	HSV-1 temperature sensitive mutant in ori binding protein
tsK	HSV-1 temperature sensitive mutant in ICP4
VHS	Virion host shut-off
VZV	Varicella-zoster virus

Introduction

Lewis antigens, carbohydrates closely related to the ABO histo-blood group antigens, are important ligands for selectins, whose interactions are of relevance for leukocyte homing. Selectin binding of leukocytes via sialyl Lewis x (sLe^x) and related antigens allow cells to bind weakly to the epithelial cells, forcing the leukocytes to roll along the blood vessel, resulting in tighter bindings and eventually passing of the leukocyte across the epithelial barrier. The powerful leukocyte homing mechanism, offered by selectin interactions with their ligands, has been hijacked by different types of tumors during metastasis. This allows the circulating tumor cells to pass through the blood vessel wall. In addition to sLe^x, a related carbohydrate structure, Le^y, has also been implicated in tumor progression. Le^y was for a long period of time believed to be expressed only on tumor cells, but recently Le^y has been shown to be expressed in very high amounts in sperm and seminal fluids, where the most prominent hypothesis includes a role for Le^y in immune suppression. Tumor cell expression of Lewis antigens has been described in detail and antibodies against these antigens are under investigation at various pharmaceutical companies.

Virus-infected leukocytes, or cells that are detached by virus infection, may circulate in a manner similar to that described above for metastasizing tumor cells. In analogy, expression of selectin ligands, e.g. O-linked glycans, on cells would provide such viruses with an excellent mechanism for colonization of distal tissue. This appears as a particularly advantageous strategy for representatives of the families Retroviridae and Herpesviridae, owing to their long-lasting relationship with their hosts, including latent or persistent infection. Indeed, the significance of virus-induced sLe^x and its interactions with selectin has been proven for the pathogenesis of HTLV-1-induced lymphoma in humans. In spite of the potential relevance of selectin interactions for herpesvirus pathogenesis, not least in immunocompromised patients, we know little regarding to what extent herpesviruses express selectin ligands and essentially nothing regarding the strategies by which herpesviruses may induce such carbohydrates in the infected cells. It is however intriguing that essentially all human herpesviruses specify at least one glycoprotein that is tailor-made to present numerous O-linked glycans at the infected cell surface, including Lewis antigens, provided that the other requirements for synthesis of such glycotopes are fulfilled in the infected cell.

Aims of the present thesis

The overall aim of this thesis is to determine the ability of different herpesviruses to introduce Lewis antigens and related biologically active carbohydrate epitopes in infected cells and to identify the underlying mechanisms behind this phenomenon, with respect to viral and host factors involved. The specific aims are:

I. To demonstrate possible neoexpression of such carbohydrates and determine whether different herpesviruses induce different carbohydrate epitopes.

II. To examine the functionality of a putative signal sequence of a herpesvirus model glycoprotein regarding its ability to participate in the early steps in formation of such carbohydrates.

III. To develop a method for measuring the transcription rates of host genes, encoding the fucosyltransferases necessary for synthesis of relevant Lewis antigens.

IV. To characterize possible virus-induced changes in the transcription rates of these genes in herpesvirus-infected cells for identification of the host factors responsible for altered glycosylation.

V. To identify the viral factors responsible for altered glycosylation and to determine the viral mechanism behind the induction of specific Lewis antigens.

Herpesviridae

The herpesviruses, herpesviridae, are enveloped DNA viruses containing a large linear genome ranging between 100 and 240 kb in size. The viral DNA is enclosed in a 100nm large capsid, surrounded by a tegument consisting of viral and cellular proteins and RNAs important for viral gene transcription, see Fig 1 (73). All herpesviruses share a similar gene expression pattern with 40 open reading frames (ORFs), conserved among all the known herpesviruses (reviewed in (50)). When these genes are expressed, the herpesvirus infected cell enters a lytic phase and virions are released from the cell. Herpesviruses are also able to cause latent infection of various celltypes, where the circular viral DNA resides in the infected cell nucleus. Latency may involve expression of some viral genes, though progeny virions are not formed (73). Latency may, due to different factors, induce reactivation where the virus is able to enter the lytic phase of infection.

The herpesviridae are divided into three subgroups: α, β and γ. Herpesviruses in general cause subclinical infections, however, they may cause recurrent infections and are especially critical for immunocompromised individuals, resulting in life-threatening disease. α herpesviruses, herpes simplex type 1 and 2 (HSV-1 and -2) and varicella-zoster virus (VZV), latently infect neurons during primary infection. HSV-1 and HSV-2 may reactivate, causing oral and genital lesions respectively, but may also cause encephalitis or keratoconjunctivitis. VZV, unlike HSV, spreads through the respiratory route primarily infecting the mucous membrane of the lungs, where the virus is spread to regional lymph nodes infecting tonsillary memory T-cells. Infected T-cells transport the virus to the skin, finally causing skin lesion in the form of chickenpox in children or zoster during adult reactivation (39, 59). β herpes viruses, i.e. cytomegalovirus (CMV) infects white blood cells usually causing subclinical infection, although CMV causes severe infections in immunocompromised patients, i.e. transplantation patients and AIDS patients. γ herpes viruses, e.g. Epstein-Barr virus, inducing proliferation of B-lymphocytes causing mononucleosis.

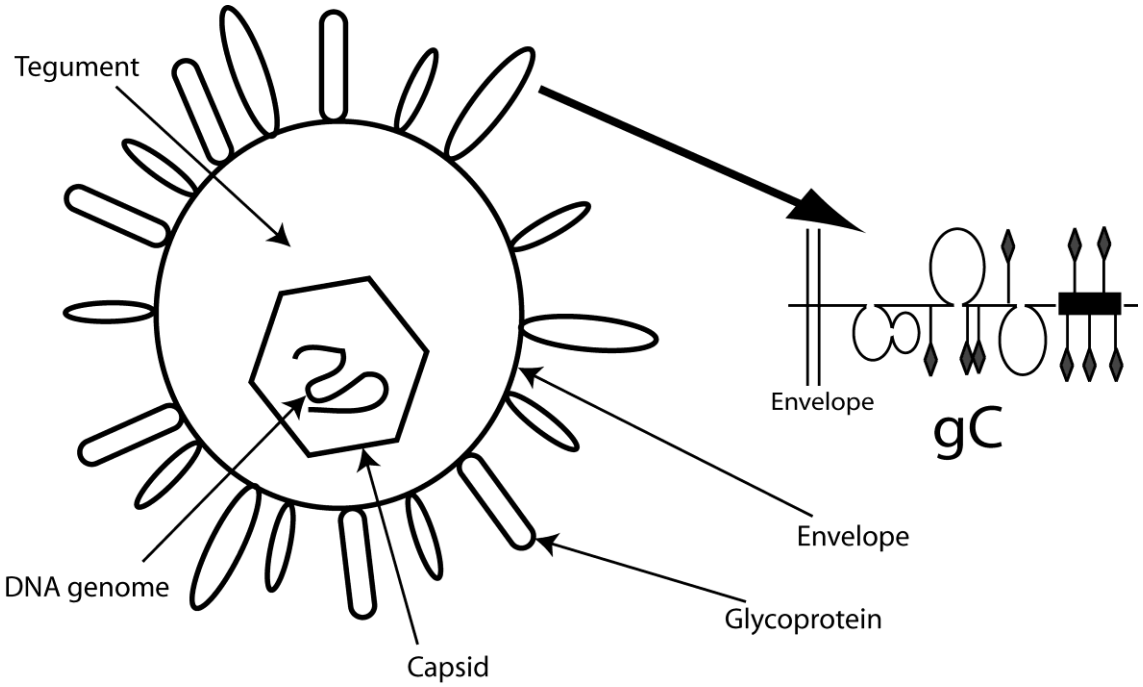

Fig 1. HSV animated structure including gC-1 detailed structure. Diamond shaped sticks represents N-linked glycans and the black box represents a peptide stretch, containing clustered O-linked glycans.

Viral Replication

A summarized description of the viral replication with emphasis on the steps relevant for this thesis is given below. For brevity, only HSV-1 is reviewed since all herpesviruses utilize the same replication strategy. The HSV-1 envelope contains as many as 11 different glycoproteins (g), of which gB, gC and gD are essential for the early steps in cellular infection. Initial binding of HSV-1 to the primary cellular receptors, glucosaminoglycans, via gC and gB is followed by fusion of the viral and cellular membranes. The viral capsid utilizes the nuclear pore complex to release the viral DNA and tegument proteins into the nucleus. The infected cell enters the lytic or latent pathway, depending on whether viral gene expression is engaged or not. During latent infection the viral genome is circularized and LAT (latency-associated transcripts) are the only transcripts detected in latently infected neurons (4). Latent infection can be reactivated, at which point the immediate early (IE) genes are transcribed. During the lytic infection, expression of IE genes induces early (E) and late (L) gene expression. Expression of the E genes allows replication of the viral DNA and the L, structural, genes. Virion particles are then formed and released from the cell (73).

Transcription of viral and cellular genes in the HSV-1-infected cell

The transcription program of the viral genome is initiated by the tegument protein VP16 along with cellular transcription factor Oct-1 and HCF, which together are responsible for induction of viral gene ICP0 (infected cell protein 0) by binding to the ICP0 promoter (15, 22). The VP16, Oct-1 and HCF complex also activates ICP4 transcription. ICP0 and ICP4 function together as crucial viral proteins for transcription of the other IE genes, ICP22, ICP27 and ICP47, and thereby initiation of transcription of all viral genes, though only ICP4 is essential for viral replication (15). ICP0 deletion mutants are able to replicate, however, low MOI results in a much slower lytic cycle (7). Several of the IE genes are also involved in regulation of cellular genes. ICP0 is not able to bind DNA, but activates many cellular genes, though no specific promoter has been described (Reviewed in (15)). IE protein ICP22 activates RNA polymerase II, essential for gene transcription (62), ICP27 also interacts with RNA polymerase II as well as promoting transportation of viral, single exon, RNA to the cytoplasm from the nucleus (11) and effects cellular RNA stability in association with tegument protein VHS (virion host shut-off) (56).

Viral transcription radically effects cellular gene transcription by altering mRNA processing. Cellular genes redundant to the herpesvirus are shut down by VHS degrading preexisting and newly transcribed cellular mRNA, and ICP27 blocks cellular pre-mRNA splicing, both playing a role in reducing cellular mRNA levels in the infected cell. Contrary to the paradigm that all cellular genes are shut-off, many studies indicate a much more fine-tuned regulation, where genes coding for transcription factors and/or genes involved in transcriptional regulation are up-regulated early in the infection. Genes involved in oncogenesis, stress-response and cell-cycle regulation have also been found to be up-regulated (70).

The IE genes induce transcription of the delayed early genes, DNA polymerase, DNA binding proteins, ORI binding protein and helicase/primase complex, all being essential for viral infection. Expression of the DE genes is followed by expression of the late and the true late genes, mainly coding for structural as well as viral DNA replication genes (74).

Glycoprotein C and glycosylation of viral glycoproteins

All human viruses rely on the host cell glycosylation machinery, implying that the resulting glycans are host specific (54). The specificity of a given oligosaccharide depends on the specific set-up of glycosyltransferases in a cell at a given time. Glycosyltransferases catalyze the addition of one monosaccharide to the growing oligosaccharide chain. The added monosaccharide is linked via its carbon atom to one of the free hydroxyl groups of an accepting monosaccharide. The structural information of the oligosaccharide is determined via the three levels of specificity of a

glycosyltransferase: Specificity for (i) the identity of the monosaccharide to be donated, (ii) the structure of the oligosaccharide acceptor and (iii) the glycosidic linkage to be generated (α or β anomery for the donated monosaccharide and up to four free hydroxyls available at the accepting monosaccharide) (23, 75, 79). This implies there is a huge number of different human glycosyltransferases (35) because although the number of the most frequent monosaccharides is less than ten, the number of possible acceptor glycan structures and possible linkage types is very large. The glycans of glycoproteins are classified according to the type of linkage between the innermost monosaccharide and the amino acid of the glycoprotein. Viral envelope glycoproteins contain two types of glycans, i.e. N-linked glycans where a GalNAc is attached to the amide nitrogen of Asn or the O-linked glycans that are linked to the hydroxyl group of Thr or Ser.

N-linked glycosylation is initiated by transfer of a pre-built, large, mannose-rich, glycan precursor from a lipid to Asn of the glycoprotein (Reviewed in(42)). The peptide signal essential for glycan acceptance is relatively well defined, N-X-S/T where X cannot be a proline. After addition of the mannose-rich precursor the N-linked glycans is processed further by glycosidases and glycosyltransferases to rather large and branched glycans. N-linked glycosylation will not be discussed in detail, as they are not studied in the present work.

O-linked glycosylation is sometimes referred to as mucin-like glycosylation if numerous O-linked glycans are tightly clustered on specific peptide stretches of the glycoprotein, which is the case for gC-1 and a number of other herpesvirus glycoproteins. Usually O-linked glycans consist of shorter chains than the N-linked glycans although there are exceptions from this rule. Another important difference is that O-linked glycans in contrast to N-linked glycans do not rely on a pre-created precursor glycan. Thus, each monosaccharide from the first one to be added to the polypeptide chain to the outermost are added individually. The innermost monosaccharide, always GalNAc, is linked to serine or threonine via one GalNAc transferase, belonging to a family of potentially as many as 24 isoenzymes (1, 71), encoded by the human genome, each with slightly different but sometimes overlapping specificities.

The signal for O-linked glycosylation is not as distinct as that mentioned above for N-linked glycosylation. Generally, the peptide stretches to be O-glycosylated contains many Ser or Thr units, to which the first GalNac is added, and several Pro residues. Charged residues are disfavored at positions -1 and +3 in regards to Ser/Thr (71), which is of relevance for the present work (see Paper I). These “rules” merely reflect tendencies, and are not sufficient for construction of a consensus amino acid sequence for O-linked glycosylation though much effort has been put into discovery of such a consensus sequence. The difficulties are not only due to the general complexity of the O-linked glycosylation for a given GalNAc transferase but also due to the fact that each of the many GalNAc transferases has its own sub-specificity (1).

The second sugar to be added to the GalNAc can be one of the three following: (i) α2,6-Sialic acid, resulting in the sialyl-Tn antigen, (ii) β1,3-Galactose, resulting in a Core 1 antigen, further addition of β1,6GlcNAc to the initial GalNAc produces the Core 2 antigen, which will be discussed further in the thesis. (iii) β1,3-GlcNAc for synthesis of the Core 3 antigen, further addition of another GlcNAc in the β1,6-position produces a Core 4 antigen. The Core 2 and Core 4 antigens may then be subject to addition of β1,3/4-Gal and β1,3-GlcNAc for production of longer polylactosamine arms where sialic acid and fucose are terminally added producing rather complex O-linked glycans (23, 75).

Glycosylation topology of gC-1

Glycoprotein C (gC) allows binding of the virion to glycosaminoglycans heparan sulfate (HS) and chondroitin sulfate (CS) on the cell surface (25, 44). gC is a class I transmembrane protein of 511 amino acid residues and is heavily glycosylated, expressing both N- and O-linked glycans. gC-1 is N-glycosylated at 8 of 9 possible glycosylation sites and has an O-glycan rich mucin domain between amino acids 55-104 consisting of many Ser, Thr and Pro (Fig 1). The structural outcome of O-linked glycosylation of gC is dependent on many factors. First, the amino acid sequence is crucial for which of the 20 GalNAc transferases is able to add the first GalNAc (See above). In addition, some GalNAc transferases contain a lectin-binding domain able to bind to various carbohydrate epitopes, thereby prolonging the time the glycoprotein is in contact with the glycosyltransferases of a given compartment (76). Finally, the variations in the expression pattern and intracellular location of glycosyltransferases are of greatest importance for the specificity of the glycotope detectable on gC.

Lewis and related antigens

Lewis antigens are a group of fucosylated glycans and the name refers to a family suffering from red blood cell incompatibility, leading to the discovery of Lewis a (Le^a) (79). Later a closely related compound, Lewis b, was discovered. Both Le^a and Le^b are expressed on the glycolipids of erythrocytes. The Lewis antigen family, consisting of Lewis a (Le^a), Lewis b (Le^b), Lewis x (Le^x), Lewis y (Le^y) and sialylated forms of Le^x and Le^a, is expressed on O-linked and N-linked glycans as well as on glycolipids. The Lewis antigens may be divided into two groups depending on their synthesis. Le^a, Le^b and sLe^a all are synthesized from a type 1 precursor (Galβ1-3GlcNAc) where a Fucose (Fuc) is added in a α1,2 or α1,4 position to synthesize the different antigens. Le^x, Le^y and sLe^x, on the other hand, are synthesized from a type 2 precursor (Galβ1-4GlcNAc) and Fuc is added to the same monosaccharide but in α1,3 position to create the type 2 Lewis antigens (Fig 2).

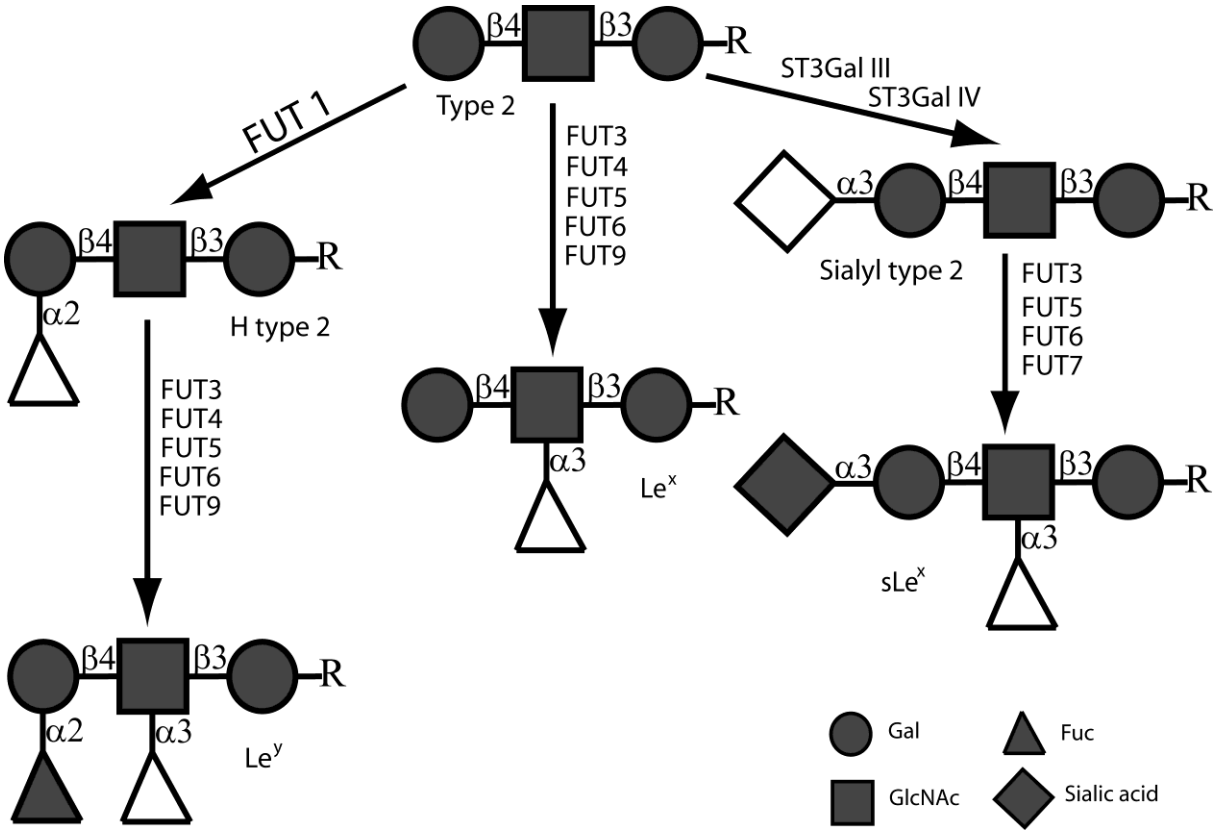

Fig 2. Schematic drawing of the synthesis of the Lewis antigens related to the present thesis, type 2 Lewis antigens.

Lewis antigens as selectin ligands

Lewis antigens are involved in several different biological processes. One of the most important roles of Lewis antigens *in vivo* are as ligands for selectins. Selectins are molecules expressing a lectin-like O-glycan binding domain linked in tandem to an epidermal growth factor-like domain, followed by varying numbers of short consensus repeats connected to transmembrane and cytoplasmic domains (40, 41, 66). There are three different types of selectins, E (endothelial) – P (platelet) - and L (leukocyte) - selectin. L selectins main ligand is sialyl 6-sulfo Le^x but L selectin is also able to bind sLe^x. P selectin and E selectin both bind sLe^x and sialyl 6-sulfo Le^x, though current data suggest that there are other undetermined ligands of E selectin (32, 48). The selectins bind sLe^x and sialyl 6-sulfo Le^x, but on different glycoproteins and in slightly different manners. P-selectin binds the P-selectin glycoprotein ligand-1 (PSGL-1) and CD24. L-selectin also binds PSGL-1, but is less dependent on the protein scaffold than P-selectin. L-selectin binds, other than PSGL-1, CD34, glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1) with a binding that is stronger than that of P-selectin binding (40). This binding to selectins is involved in the recruitment of leukocytes to sites of infection and injury, where the binding between sLe^x and selectins initiate rolling of the leukocytes, the first step in the process of leukocyte adhesion.

Lewis antigens in cancer

Malignant cells are known to exhibit abnormal glycosylation patterns. Generally, O-glycans of cancer cells are short and truncated compared to wild-type glycans (6). Monoclonal antibodies recognizing sLe^a, sLe^x and Le^y on tumor cells, providing Lewis antigens an important role in cancer biology (6). Detection of Lewis antigens on various types of cancer cells are used for serum diagnostics of cancer, and have been used for nearly two decades by clinicians (34).

There is a statistically significant correlation between the severity of the prognosis for the patient and the degree of sLe^x expression on many different types of cancer cells, also correlating with E selectin expression in the patient's serum (34). Immunohistochemical studies reveal an expression of E selectin in small blood vessels surrounding tumors (34). Metastatic cells expressing sLe^a and sLe^x, are able to bind to selectins in a similar fashion to homing leukocytes, allowing for extravasation of the tumor cells (33).

The role of Le^y expression on cancer cells has not yet been determined. Non-sialylated Lewis antigens, i.e. Le^y, are able to bind dendritic cells and are involved in the T-cell immunological response as discussed below. If Le^y on cancer cells are engaged in this immune response has not been investigated. Le^y antibodies are currently in trail studies for use in cancer treatment (60, 65).

Lewis antigens as viral receptors

Lewis antigens are also important as viral receptors, and human noroviruses are able to bind to Lewis antigens causing acute gastroenteritis. Many studies have shown that humans lacking a functional FUT2 gene are not susceptible to norovirus infection and hence do not express the norovirus receptor (47, 72). Recently, different norovirus strains have been found to have different specificity for histo blood-group antigens, resulting in a variable spreading pattern, dependent on the infectious viral strain (28, 29).

Fucosyltransferases

Fucose is a key monosaccharide in Lewis antigens and related glycotopes. The genetics and biology of the responsible fucosyltransferases is characterized by a high degree of complexity. There are nine different fucosyltransferases, Fuc-T, expressed in human cells. The fucosyltransferases add a fucose (Fuc) in α1,2- position to galactose (Gal) (Fuc-TI and Fuc-TII), add a Fuc at α1,3 or 4- position to GlcNAc (Fuc-TIII, IV, V, VI, VII and IX) or add Fuc α1,6 to GlcNAc (core fucosylation of N-glycans) (Fuc-TVIII). The different fucosyltransferase genes (FUTs) are differently expressed in a cell- and tissue-specific manner (Table 1), but still have some redundancy in function. FUT1 and FUT2 generally use different substrates for α1,2- fucosylation, FUT1 predominately uses a type 2 precursor (Galβ1,4GlcNAc) while FUT2 fucosylates type 1 precursors (Galβ1,3GlcNAc). FUT3 and FUT5 differ from the other α-1,3FUTs, being the only FUTs

able to fucosylate both type1 and type2 precursors and adds a Fuc in different positions depending on the precursor, α1,4- position (for type1) and a α1,3- (for type 2) (13, 49, 55).

Table 1. Properties of the human fucosyltransferases used in this thesis.

Gene (GenBank)	Enzyme abbreviation/ Linkage[a]	Synthesized Type 1 Structures[a]	Synthesized Type 2 Structures[a]	Cellular expression pattern[a]	HEL cell genotype[b]
FUT1 (H) (M35531)	Fuc-TI Fuc α(1,2)		H Type 2	Mesenchymal, erythroid	ND[c]
FUT2 (Se) (U17894)	Fuc-TII Fuc α(1,2)	H Type 1		Epithelial cells (savlia and mucous membranes)	$se^{428}se^{428}$
FUT3 (Le) (X53578)	Fuc-TIII Fuc α(1,3/4)	Le^a, Le^b, sLe^a	Le^x, Le^y, sLex	Epithelial cells in gastrointestinal tissue; kidneys	$Lele^{202.314}$
FUT4 (Le^x) (M58596)	Fuc-TIV Fuc α(1,3)		Le^x, Le^y	Myeloid cells; embryonic; ubiquitous	ND
FUT5 (M81485)	Fuc-TV Fuc α(1,3/4)	Le^a, Le^b, sLe^a	Le^x, Le^y, sLe^x	Low level tissue expression; spleen, gastrointestinal	ND
FUT6 (M98825)	Fuc-TVI Fuc α(1,3)		Le^x, Le^y, sLe^x	Gastrointestinal tissue; kidney; liver; plasma	$FUT6^{wt}FUT6^{370}$
FUT7 (X78031)	Fuc-TVII Fuc α(1,3)		sLe^x	Leukocytes; spleen	$FUT7^{wt}FUT7^{wt}$
FUT9 (AJ238701)	Fuc-TIX Fuc α(1,3)		Le^x, Le^y	Leukocytes; brain; stomach	ND

[a] (8, 13, 14, 36, 37, 64)

[b] Data from the present work (III)

[c] Not determined

Methodological considerations

Cell culture, herpesvirus strains, and procedures for viral infection

Throughout this thesis human embryonic diploid lung fibroblasts (HEL) cells (43) were used for herpes virus infectivity because this is a human, non-tramsformed, diploid cell line, permissive for HSV-1, CMV and VZV thus enabling comparison between the different herpesviruses. Low-passage cultures were used throughout the studies to avoid side effects that could occur in cells after many passages. For plaque titration assays, on the other hand, Green Monkey Kidney (GMK) cells were used for their ability to form easily detectable plaques using well characterized assays (9) (I). To investigate specific binding of HSV-1 to HS and CS murine fibroblasts L cells expressing both HS and CS, the CS expression variant gro2C and the HS expressing variant sog9 EXT-1 were used. Details for these cells are presented in (I). The wild-type viral strains used for herpesvirus infection are: Syn17+ (HSV-1), Towne (CMV) and patient isolate C822 (VZV), presented more in detail in (II, III, IV). Several mutant viruses were also analyzed: gC mutant HSV-$1_{114,117}$, originally designed for studies on HSV-1 receptor-binding (I), ICP0 deletion mutant *dl*1403 (IV), temperature sensitive ori binding protein mutant tsS (IV) and temperature sensitive ICP4 mutant tsK (IV). The parental strain of all HSV-1 mutants was Syn17+. Infection of different types of cells was performed as described (I, II, III and IV). Cycloheximide (CHX) was used for blocking viral and cellular RNA translation and MG-132, a drug which inhibits the proteasome, was used. Cells were pretreated with drugs prior to infection (IV).

In vitro glycosylation of gC-1 mutant

Immunoprecipitation and subsequent analysis of the relative molecular weight of gC and its precursor pgC, was used to characterize gC-1 mutants with expected altered O-linked glycosylation. One mutant gC-1 ($gC_{114,117}$) with suspected hyperglycosylation was further analysed in an in vitro system based on individual recombinant GalNAc-transferases and synthetic peptides representing a relevant part of the O-glycosylation signal of wild type (gC_{rescue}) and mutant gC-1 ($gC_{114,117}$) (I). Thus, the efficacy of O-glycosylation signals was investigated by the ability of GalNAc transferases T1, T2, T3 and T6 to add GalNAc from UDP-GalNAc to the peptide. The amount of GalNAc added to specific Ser and Thr residues of the target peptide were assayed by MALDI-TOF MS and the specific Ser and Thr residues were identified by their failure to produce an expected signal during Edman degradation. Together MALDI-TOF and Edman degradation provide very detailed information regarding the position of O-glycans on the peptides as well as the extent of glycosylation of each site.

Transcriptional analysis

Analysis of gene transcription through real-time PCR was extensively described in paper II. The transcription of relevant host and viral genes was studied through total RNA

isolation on automated systems. The RNA was then subjected to real-time RT PCR, where normalization of the investigated RNA against a highly expressed housekeeping gene proved difficult, as no mRNA transcript was found to be unaffected by HSV-1 infection. The remaining option was to investigate total RNA and use 18S rRNA as a housekeeping gene, where total expression of 18S rRNA proved to be constant in herpes virus-infected HEL cells. Calculations of the relative RNA concentrations were formulated according to the $2^{-\Delta CT}$ (II, III).

Lewis antigen expression

Immunofluorescence using a confocal microscope was utilized for visualization of the Lewis antigens. Mouse monoclonal antibodies against sLe^x, Le^x and Le^y, and human sera positive for HSV-1, CMV, and VZV were bound to infected cells on teflon-coated slides. Secondary FITC (green) conjugated antibodies directed against mouse antibodies and TRITC (red) conjugated anti-human antibodies were utilized. In the present experimental setting confocal microscopy provided a method superior to FACS or ELISA for information regarding expression pattern of the glycotopes mentioned above (III, IV).

Results

Glycosylation is important for the function of most membrane proteins, not least viral envelope proteins. Glycosylation influences a multitude of events from correct folding to specific binding of cells, bacteria or viruses. Mammalian viruses rarely encode glycosyltransferases, only two viral glycosyltransferases are known, encoded by bovine herpes virus type 4 (77) and myxoma virus (31), despite the fact that most enveloped viruses express glycoproteins. Glycosylation is a complicated process dependent on many factors, including the amino acid sequence to be glycosylated and cellular expression and localization of glycosyltransferases just to mention a few. One of the aims of the present study was to determine two of the parameters available for a herpesvirus to influence the O-linked glycosylation in the infected cells: By varying the peptide signal in viral glycoproteins for O-linked glycosylation and to influence expression patterns of host glycosyltransferases that determine the structural properties of O-linked glycans.

O-glycosylation signal features (I)

The well characterized HSV-1 glycoprotein gC, consisting of an O-glycan rich domain in the N-terminal portion of gC (Fig 3), appears to be an ideal experimental system for studies on the significance of the O-linked glycosylation signal of herpesvirus glycoproteins. The rationale behind the experiments reviewed in this section is that a mutant HSV-1, designated $HSV_{114,117}$, originally designed for studies on the significance of basic amino acids for HSV-1 attachment, encodes a gC-1 that became hyperglycosylated as determined by its electrophoretic mobility (I). In the mutated gC ($gC_{114,117}$), two basic aminoacids, Arg^{114} and Lys^{117}, of the gC-1 mucin domain are substituted for alanine. The introduced mutations surprisingly increased the molecular weight of gC, suggesting that the mutations caused a change in O-glycosylation rather than in N-linked glycosylation (I). Infection of a cell-line unable to extend O-glycosylation further than the first GalNAc residue of O-glycans (C1300) resulted in wild-type and mutant gC-1 with identical electrophoretic mobility, confirming that amino acids 114 and 117 influences the extent of O-linked glycosylation (Fig 4).

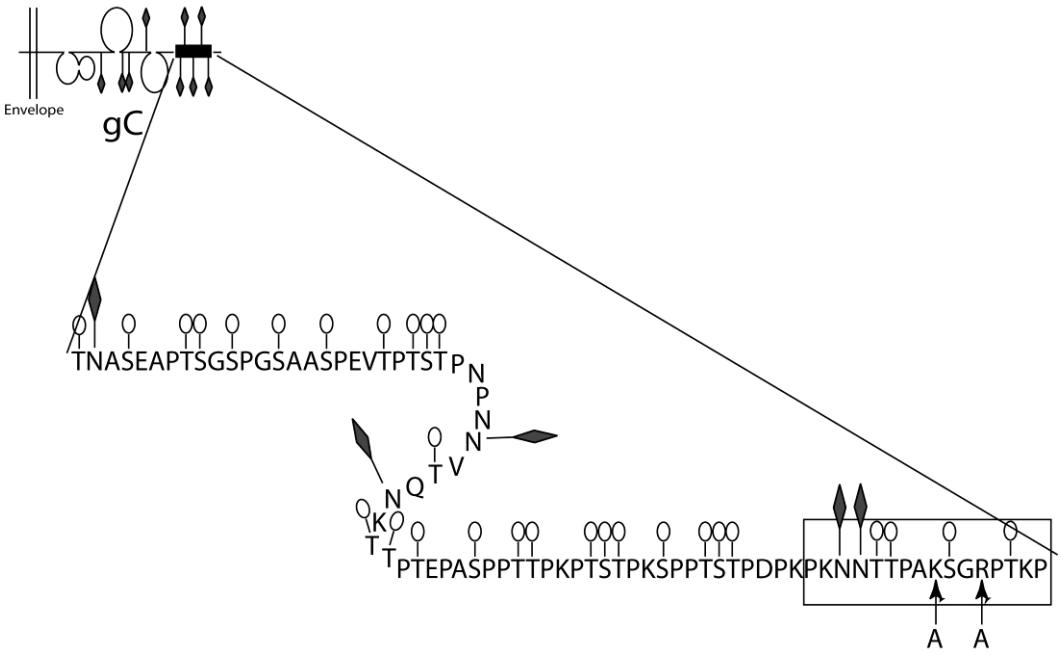

Fig 3. Schematic drawing of the mucin domain of gC-1. The N-glycans are symbolized by diamond shapes and the possible O-glycans are symbolized by circles. The peptide used in Paper I is the area enclosed by a box, with the mutated amino acids indicated by arrows.

One intriguing question was whether the increased O-linked glycosylation of $gC_{114,117}$ is due to an increased number of serines/threonines becoming O-glycosylated or an enlargement of existing O-glycans. This was investigated using an *in vitro* system with different recombinant GalNAc-transferases and a synthetic peptide, representing the relevant part of the gC-1 O-linked glycosylation signal (Fig 3), including Arg^{114} and Lys^{117}. *In vitro*, O-glycosylation assays, including a panel of purified GalNAc transferases, indicated that GalNAc T2 was most efficient in O-glycosylation of the peptide. The products were further assayed by MALDI-TOF and Edman degradation of the glycosylated peptide, where MALDI-TOF demonstrated a final GalNAc amount of 2 units on the gC_{rescue} peptide and 3 units on the $gC_{114,117}$ peptide (Fig 5). Edman degradation confirmed this result and also provided mapping data of the serine and threonine glycosylated residues. Thus, Ser^{115} and Thr^{119} were glycosylated on the gC_{rescue} and the additional site Thr^{111} was completely O-glycosylated on the $gC_{114,117}$ peptide (I). The addition of one extra O-glycan in $gC_{114,117}$ is not sufficient to account for the total difference in molecular weight between $gC_{114,117}$ and gC_{rescue}, and there was no increase in sialylation between $gC_{114,117}$ and gC_{rescue} (I). Hence, the increased O-glycosylation of $gC_{114,117}$ is due to an additional O-glycosylation site, addition of other O-glycosylation sites in the remaining portion of gC and/or an increased size of O-glycans in general, though not via addition of sialic acid.

The altered O-linked glycosylation of $gC_{114,117}$ was found to affect the ability of the viral attachment and social behavior of infected cells. Thus, infecting cells expressing only chondroitin sulfate (Gro2C), cells expressing only heparin sulfate (sog9 EXT-1) and cells

expressing both (L cells and GMK) with $HSV_{114,117}$ and HSV_{rescue}, showed that $HSV_{114,117}$ was less efficient in infecting cells via the CS receptor route (I). A decreased capacity to bind to CS also resulted in a decreased capacity for cell-to-cell spread, determined by plaque-size reduction assays (I).

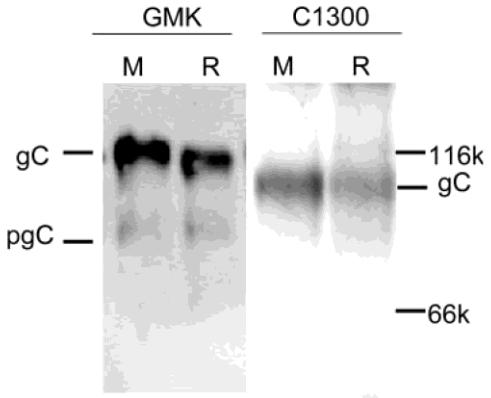

Fig 4. Positions of gC-1 and pgC-1 from $HSV_{114,117}$ (M) and HSV_{rescue} (R)-infected GMK and C1300 cells. Positions of molecular weight markers are indicated.

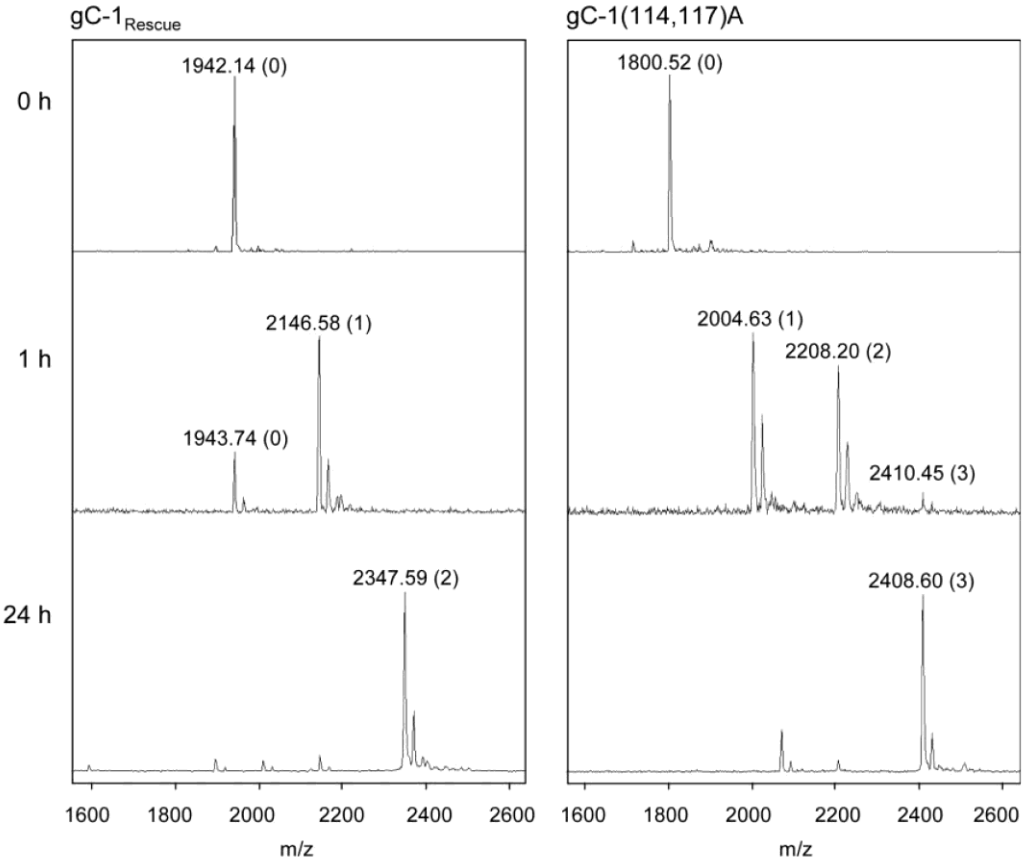

Fig 5. MALDI-TOF mass spectra of $gC_{114,117}$ and gC_{rescue} glycosylated *in vitro* by GalNAc-T2 for 0, 1 and 24h. The *m/z* values of differently glycosylated peptides are indicated. Calculated numbers of attached GalNAc residues are indicated within brackets.

Establishment of Real time RT-PCR for transcriptional analysis (II)

Investigating expression of cellular glycosyltransferases is an important part in determining possible changes in glycosylation in herpesvirus-infected cells. Real time reverse transcriptase (RT) PCR is a powerful tool for detailed studies of transcriptional regulation when used correctly. Factors such as sample to sample variation in cell count and RNA isolation must be corrected for, preferably by utilizing transcription of cellular house-keeping genes for standardization. Thus, identification of a highly expressed gene not effected by cell differentiation or viral infection is tedious but crucial. Herpesvirus infection alters cellular RNA levels through a number of mechanisms and "classical" house-keeping genes normally used for this purpose were detected at considerably lower levels in herpesvirus-infected cells compared to uninfected cells (II). The only RNA found to be unaltered during the current conditions was 18S rRNA. An incorrect choice of house-keeping gene alters results with several orders of magnitude, as shown by analysis of gC RNA expression in HSV-1 infected cells (II). The same method was used to determine transcription of a glycosyltransferase, fucosyltransferase 5 (FUT5), where HSV-1 infection induced gene transcription 80-fold compared to uninfected cells.

Lewis antigen expression in herpes virus infected cells (II, III and IV)

The aim here was to evaluate if herpesviruses were able to modify glycosylation by modulating the transcriptional rates of host enzymes necessary for, particularly, O-linked glycosylation. A relatively broad approach was applied including screening of GalNAc-transferases, responsible for adding the first monosaccharide to the peptide of the growing O-linked glycan, the so called Core 2 transferase (β1,6- GlcNAc transferase), sialyltransferases, and a family of fucosyltransferases. The only up-regulation detected was among the fucosyltransferase family, no induction was found in any of the other transferases investigated (Table 2). Two different expression patterns emerged, fucosyltransferases being the focus for herpes viral regulation, α herpesviruses HSV-1, HSV-2 and VZV induces transcription of FUT3, FUT5 and FUT6. This induction was also detected in β herpesvirus CMV infected-cells, where it was accompanied by a massive induction of FUT1.

The effects of increased FUT gene transcription were investigated by immunofluorescence of herpesvirus-infected HEL cells. Monoclonal mouse antibodies against Le^x, Le^y and sLe^x along with human sera directed against HSV, VZV or CMV were incubated with infected and uninfected HEL cells. Green fluorescent antibodies directed against mouse antibodies and red fluorescent antibodies directed against human antibodies were used for detection using a confocal microscope (Fig 6). HSV and VZV induced expression of sLe^x with Golgi-like staining, compatible with the induced expression pattern of FUT genes. sLe^x expression could also to some extent be detected

on the surface of VZV-infected cells. CMV, on the other hand, induced surface-expression of Le^y, in line with the additional extreme FUT1 transcription induction. No sLe^x or Le^y expression could be detected in uninfected cells.

Viral factors responsible for FUT5 induction

HSV-infected cells and FUT5 induction were used as a model system for closer studies of the FUT3, FUT5 and FUT6 sLe^x induction. HSV-1 binding to the cell was not sufficient for FUT5 transcription induction, and tegument proteins were themselves unable to induce FUT5 transcription (IV). Hence, viral transcription is required for FUT5 transcription. The first gene to be transcribed in lytic HSV-1 infected cells is ICP0, whose role was explored using an ICP0 deletion mutant (*dl*1403). Infection of cells with wild-type HSV-1 and *dl*1403 demonstrated the ICP0 gene to be involved in FUT5 transcriptional up-regulation (Fig 7). Cycloheximide treatment of HSV-1 infected cells inhibiting protein translation did not inhibit FUT5 transcription (Fig 8). FUT5 transcription is massively induced during CHX treatment as early as 2 hours p.i., though MG-132 treatment, inhibiting the proteasome, together with CHX was unable to induce FUT5 (preliminary data, IV). This may be a result of possible MG-132 induced inhibition of ICP0 function or effects of MG-132 on the transcriptional machinery (19, 61). All available data here support the latter explanation, but does not definitely rule out that MG-132 effects on ICP0 protein, demonstrated in Fig 8 of paper IV, are involved in FUT5 transcription. This option may be considered owing to the known function of ICP0 protein as an ubiquitinylating agent associated with proteasomal degradation of ND10 (21), but as MG-132 inhibited FUT5 transcription together with CHX at 2.5h p.i., time points when ICP0 is first detected in the infected cell (63) an effect of the ICP0 protein on FUT5 transcription this early is unlikely. In conclusion, the initiation of FUT5 transcription is, therefore an effect of ICP0 RNA.

Table 2. A summary of Real time RT-PCR of glycosyltransferase expression in HSV-1-, VZV- and CMV-infected HEL cells. Data from (II, III, IV) if not otherwise stated. → indicates no transcriptional induction; ↗ indicates slight transcriptional increase; ↑ represents a large transcription induction.

Transferase	HSV-1	VZV	CMV
FUT1	→	→	↑
FUT2	→	→	→
FUT3	↑	↑	↑
FUT4	→	→	→
FUT5	↑	↑	↑
FUT6	↑	↑	↑
FUT7	→	→	→
FUT9	↗	→	↗
ST3GalIII[a]	→	→	→
ST3GalIV[a]	→	→	→
GST2[b]	-	-	→
C2GnT[c]	→	-	→
GalNAc-T3[d]	-	-	→
GalNAc-T6[d]	-	-	→

[a] α2,3-sialyltransferase III and IV (preliminary data, III)
[b] 6-O sulfotransferase (preliminary data)
[c] β1,6-GlcNAc transferase, Core 2 transferase (preliminary data)
[d] GalNAc transferase T3 and T6 (preliminary data)

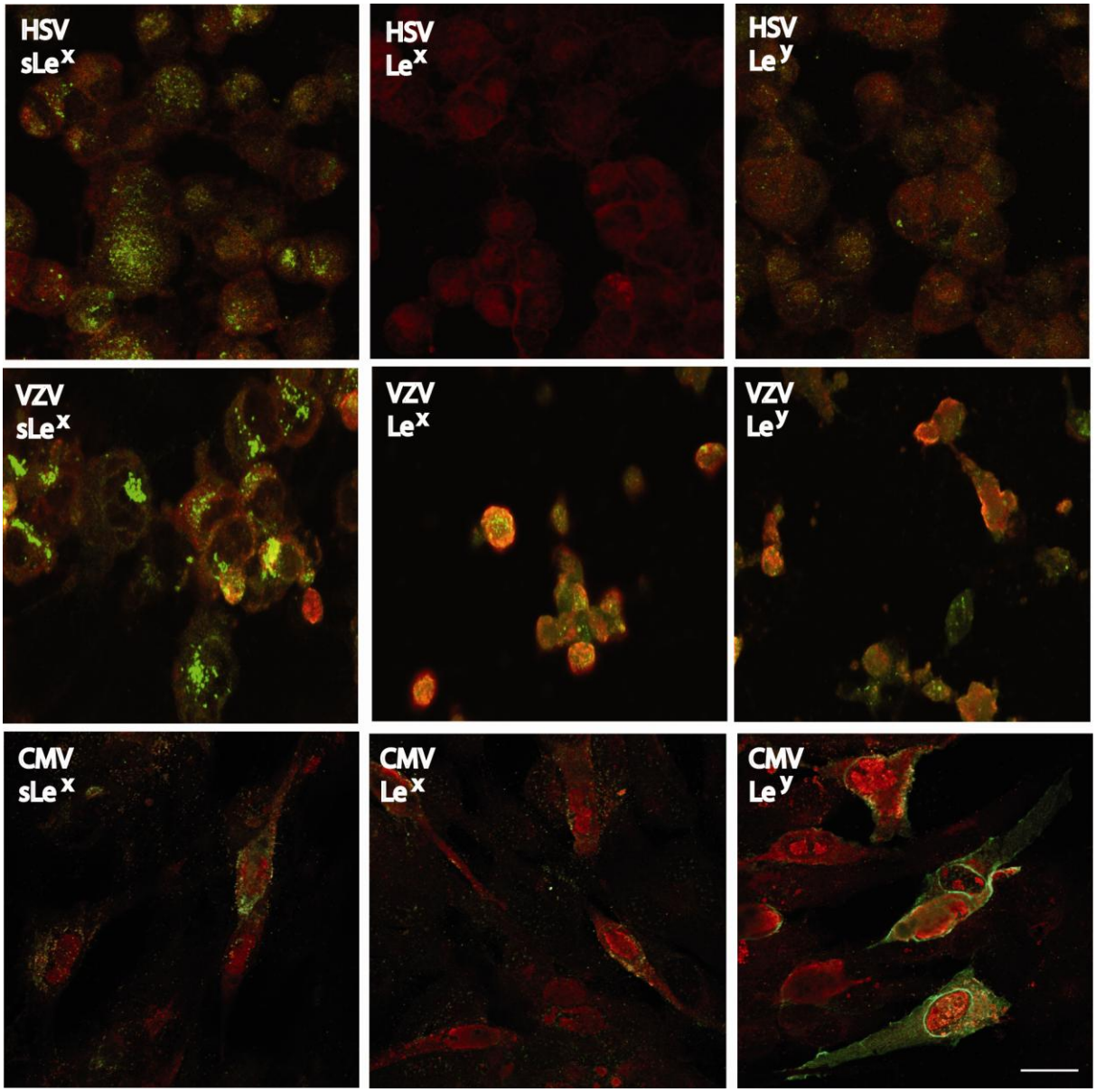

Fig 6. Immunofluorescence images from HSV-1-, VZV- and CMV-infected cells. Green secondary antibodies were used for detection of Lewis antigens and red secondary antibodies were used for detection of viral antigens. The white bar represents 20μm (III, IV).

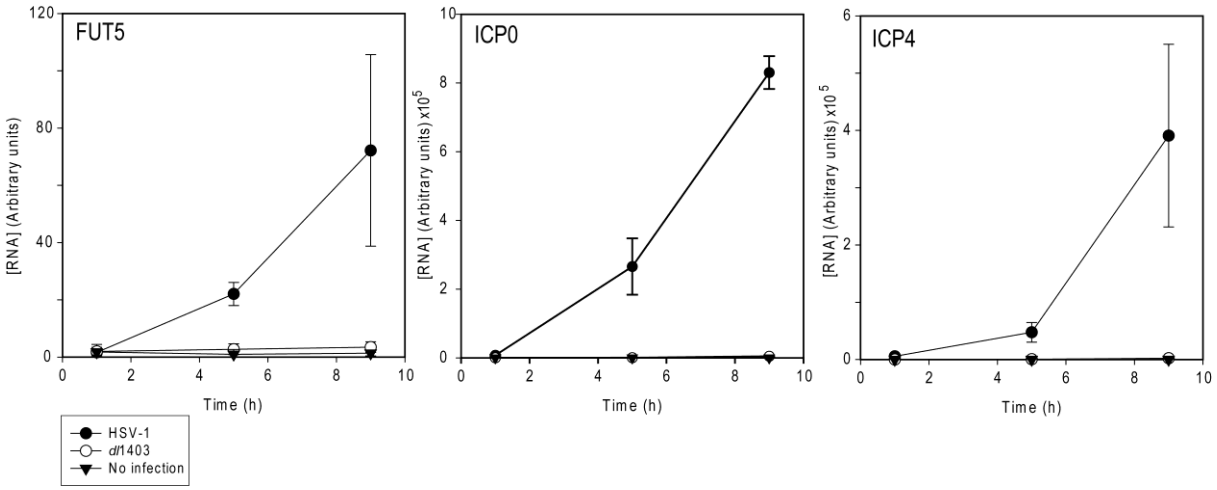

Fig 7. Expression of FUT5, ICP0 and ICP4 in cells infected with wild-type HSV-1, *dl*1403 and uninfected cells. The ICP0 PCR system was designed to detect the non-deleted portion of ICP0(IV).

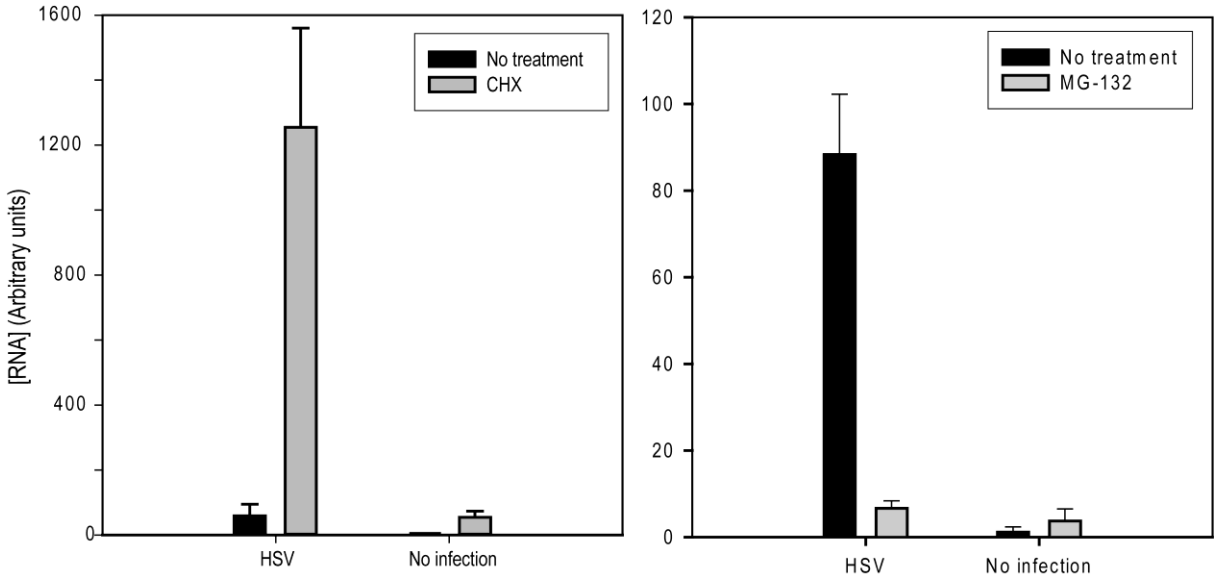

Fig 8. Detection of FUT5 RNA in cells infected with HSV-1 and uninfected cells. The cells were treated with CHX, MG-132 or ethanol. The cells were infected 9h (IV).

General Discussion

The present work describes the mechanism behind herpesvirus-induced expression of a family of biologically active carbohydrates on the cell surface. Most studies of glycosylation in virus-infected cells regards N-glycans on viral glycoproteins, where the glycans are crucial for general functions for example correct protein conformation (12, 38, 51, 53, 58, 69). Here, it is described how a virus actively can determine the structure of O-linked glycans, a relatively new field in virology. These glycans most likely have different roles in the infected cell compared to the N-linked glycans. One new viral strategy implies recruitment of specific O-linked glycans that actively interact with and modulate various immune- and non-immune cells in the infected host, data from (III, IV) (26, 27, 33). This is at variance with the immunologically more passive viral use of host-specific N-linked glycans for masking of viral epitopes from neutralizing antibodies (68).

As discussed in more detail below, herpesviruses are able to induce host factors necessary for expression of new O-linked glycans very early in the infection cycle, epitopes that do not appear in the uninfected cell (II, III, IV). A natural consequence of this is that early virus-induced alterations of glycosylation, prior to de novo viral protein synthesis, will target cellular proteins (or glycolipids), whereas later manipulation of glycosylation mainly effects viral glycoproteins. During HSV-1 infection, substantial amounts of viral glycoproteins are detected as early as 5 h p.i., whereas VZV and CMV glycoproteins are expressed later in the infection and for longer periods of time. The present results therefore suggest that the majority of the virus-induced glycans in HSV-1 infected cells relatively soon are exposed on viral glycoproteins whereas in particular CMV-induced carbohydrates may reside on host-specified glycoproteins for a longer period of time after infection (III, IV).

Herpesviruses are able to control glycosylation in at least two different manners explored in the present work: (i) As reviewed above, the signal requirement for O-linked glycosylation of mucin-like regions of glycoproteins are complex and to some extent heterogenous, owing to the large number of human GalNAc-transferases with capacity to initiate O-linked glycosylation. Thus, one viral strategy for controlling the extent of O-linked glycosylation could be via variations in the peptide signals of a mucin region of a viral glycoprotein (I). (ii) Viruses may also be able to control glycosylation by inducing transcription of critical host cell genes, resulting in expression of specific carbohydrate antigens (III, IV).

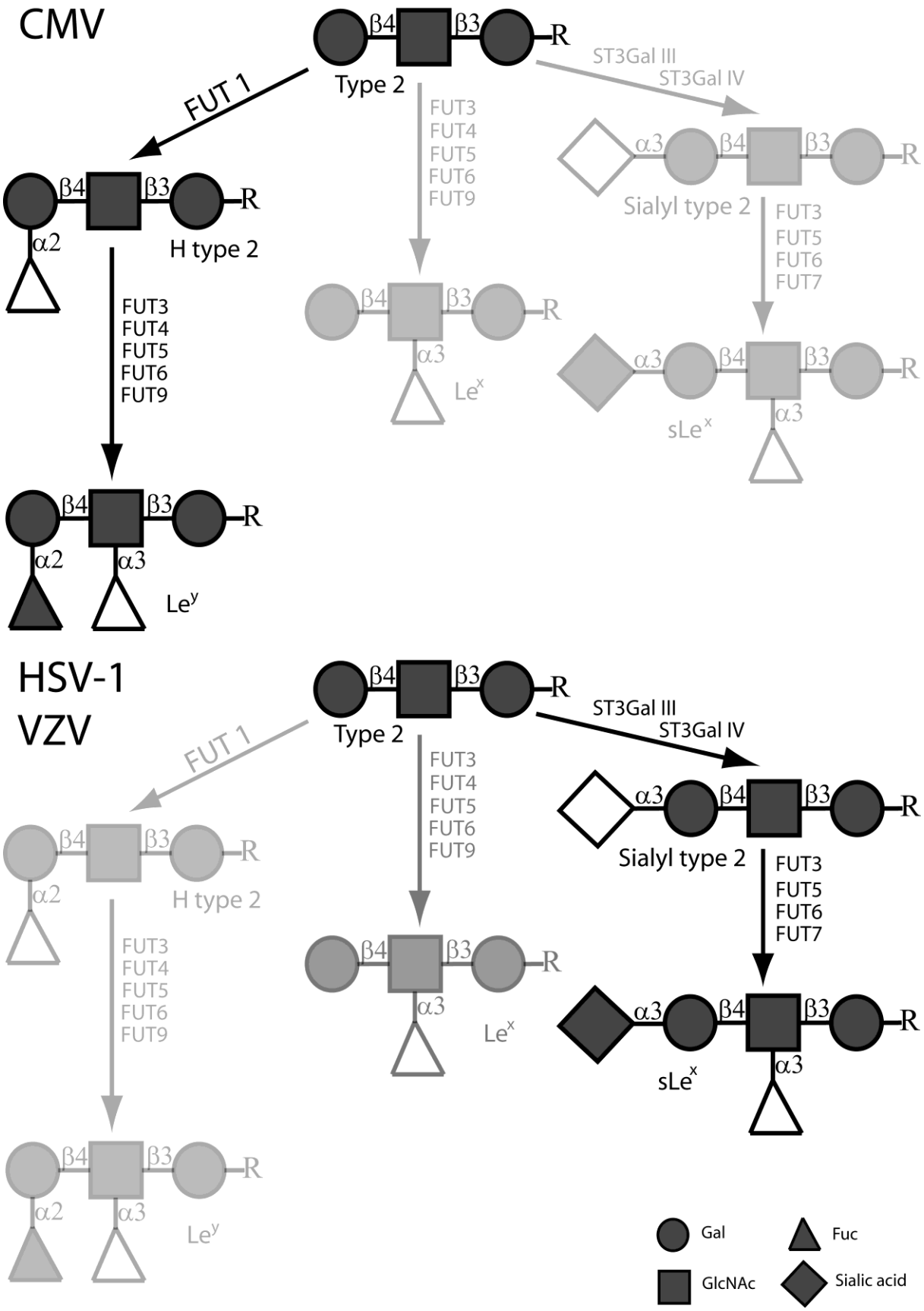

Fig 9. Summary of the fucosyltransferases involved in induction of Le^y and sLe^x in CMV- and HSV-1, VZV-infected cells respectively, represented in darker shades.

The present results suggest that the O-linked glycosylation signal in the mucin region of HSV-1 gC-1 is optimized for its interactions with the cell during the infectious cycle and that it is not a viral strategy to maximize the extent of O-linked glycosylation in the mucin region of gC-1. This is best illustrated by the finding that the consensus O-linked glycosylation signal of gC-1, for a number of sequenced HSV-1 isolates, contain two basic amino acids, whose presence is not compatible with utilization of all available Thr residues, especially the non-glycosylated Thr^{111}, in the mucin domain for O-linked glycosylation (I). This restriction was overcome by creating an HSV-1 mutant, where these basic amino acids were substituted by Ala, which results in hyperglycosylated gC-1 compared to its wild-type counterpart. The large difference in the extent of O-linked glycosylation, corresponding to 8,000 daltons, is only partly explained by the observation that non-glycosylated Thr^{111} in the wild type signal environment is switched to a 100% efficient glycosylation acceptor by the induced mutations (I). O-linked glycans of 8,000 daltons correspond to at least 20 monosaccharides, which have not been described for viral glycoproteins. It is therefore reasonable to assume that the mutations induced also increased the extent of glycosylation of several O-linked glycans in the mucin region of gC-1. Alternatively, additional available non-glycosylated Ser/Thr regions in the wild type signal have been recruited for glycosylation. The latter hypothesis is supported by findings that a few of the GalNAc-transferases, adding the innermost GalNAc of O-linked glycans, contain lectin domains that prolong the enzyme-glycoprotein interaction, thereby increasing the efficacy of O-linked glycosylation (24).

The extent of gC-1 O-glycosylation appears to be biologically relevant for the interactions between HSV-1 and the infected cell, where two phenomena are discernible: Hyperglycosylation of gC-1 (as introduced by changing the basic amino acids in the O-glycosylation signal) resulted in (i) impaired binding to an important receptor, chondroitin sulfate and (ii) altered cell-to-cell spread as illustrated by decreased plaque size (I). It is therefore tempting to speculate that the length and O-glycosylation density of gC-1 is adjusted for optimal performance regarding HSV-1 attachment to permissive cells and subsequent cell-to-cell spread of progeny virus.

It is evident from these studies that sequence variation of the O-glycosylation signal is a blunt tool for viral regulation of glycosylation that does not allow for a specific induction of defined carbohydrate epitopes such as Lewis or ABH antigens. A more appropriate strategy would be to induce synthesis of key glycosyltransferases that are essential for formation of the intended glycotopes. As previously mentioned, no glycosyltransferase encoded by a human virus has been identified, so the only remaining way for a human virus to induce specific glycotopes in the infected cell would be to activate the dormant host genes, encoding the relevant glycosyltransferases. The approach used here was to study transcriptional induction of glycosyltransferase genes, whose gene products could be implied in the induction of virus-specific changes in O-linked glycosylation. Initially, a

broad search was performed including genes encoding GalNAc-transferases (preliminary data) responsible for the addition of the innermost GalNAc, Core 2-transferases (preliminary data) necessary for formation of a specific branching of O-linked glycans, sialyltransferases (III) and fucosyltransferases (II, III, IV), both responsible for completing the terminal structure of an O-linked glycan (Table 2). Owing to their significance for expression of cell surface glycans, it was expected that the transcription of genes, representing several of these families should be subject to regulation by the infecting herpesvirus. Surprisingly, only one family of glycosyltransferases, the fucosyltransferase genes, was found to contain representatives whose transcription was induced by several orders of magnitude after infection with different herpesviruses. As discussed below, the different transcription pattern of fucosyltransferase genes was correlated to a compatible expression of different virus-induced glycotopes in the infected cells. This is in line with a notion that herpesviruses use regulation of host glycosyltransferase gene transcription for intended expression of specific carbohydrate neoantigens, in particular of the Lewis lineage, at the cell surface and that the fucosyltransferase genes represent a family of strategic importance for this purpose.

How is the herpesvirus-induced expression of different Lewis antigens accomplished? All of the herpes viruses investigated, HSV-1, VZV and CMV, induce transcription of dormant FUT3, FUT5 and FUT6 (II, III, IV). This pattern of fucosyltransferase induction results in a neo-expression of sLe^x in HSV-1 and VZV-infected cells (Fig 9, lower panel). From the present data it is however not possible to deduce if any of these three FUT genes is more important than the others for sLe^x induction. In addition to these three FUT genes, CMV but none of the other investigated herpesviruses also massively up-regulate FUT1 (III). FUT1 induction introduces a dramatic change in the glycosylation landscape, it prevents sialylation of type 2 precursor, i.e. the final substrate for FucT-III, V or VI directly resulting in sLe^x formation. Instead, the type 2 precursor is α1,2-fucosylated by FucT-I in CMV-infected cells following FucT-III, V or VI dependent α1,3-fucosylation resulting in Le^y (Fig 9, upper panel).

The herpesviral use of FucT-III, V or VI for virus-induced synthesis of Lewis antigens represents a different strategy than that used by HTLV-1, a human cell transforming virus, which also induces sLe^x. Thus, HTLV-1 up-regulates FUT7 transcription (26, 27) whose transcription remains more or less constant at a low level in herpesvirus-infected cells (III, IV). This allows for sLe^x synthesis, but does not permit a switch to Le^y, even during high FucT-I activities, because in contrast to FucT-III, V and VI, FucT-VII is strictly dependent on a sialylated precursor (5, 13). It is tempting to speculate that the induction of FUT3, 5 and 6 represents a common ancestrial feature among herpesviruses, and that the β herpesvirus-induced activation of FUT1 is a more recent event in order to recruit Le^y as an active glycotope of the infected cell.

One intriguing question pertains to how viruses increase the transcriptional rate of selected cellular genes despite the fact that many herpesviruses activate powerful mechanisms aiming at shutting off host cell macromolecular synthesis, including transcription of infected cell genes. This problem was addressed by exploring the nature of the viral factors responsible for HSV-1 induced transcription of host fucosyltransferase genes, using FUT5 as a model gene. From these studies it was clear that the most important difference, as to viral factors engaged in induction of sLe^x between HTLV-1 and HSV-1, is that the former utilizes a classical retroviral transactivator protein, Tax, which induces transcription via a CRE sequence in the human FUT7 promoter (26, 27, 33). This is in contrast to the somewhat surprising mechanism used by HSV-1 for activation of FUT5 demonstrated here (IV), requiring specific viral RNA and thus active in the absence of viral protein synthesis.

The conclusion that ICP0 RNA is a key actor is based on the finding that FUT5 transcription was induced during CHX treatment of infected cells and by the fact that FUT5 transcription was an immediate early process with kinetics congruent with ICP0 transcription (IV). It is important to note that binding of the virion to the cell surface or tegument proteins alone is not sufficient for FUT5 transcriptional induction, though tegument proteins are necessary for initiation of transcription of viral genes (IV). It is unlikely that other viral factors, e.g. RNA encoded by other HSV-1 genes, are involved in FUT5 induction since the ICP4 gene is not necessary for FUT5 induction (IV), and ICP0 RNA induction of other HSV-1 genes is not known.

The suggestion that ICP0 RNA is able to be processed to miRNAs (57) is intriguing and it could be worthwhile to address the possibility that ICP0-derived miRNAs are involved in the induction of FUT5. The notion that ICP0 RNA rather than the translated gene product is responsible for inducing cellular activities is not new. Thus, ICP0 RNA alone may induce apoptosis in HSV-1-infected cells (67), a process later reversed via expression of several different HSV-1 proteins (Reviewed by (20) and (52). This is, however, the first demonstration of a viral RNA, acting as a specific inducer of a dormant host glycosyltransferase gene.

The HSV-1 strategy to use a viral RNA, and in particular an RNA encoded by an IE gene, for the initial step of FUT5 induction offers advantages for the virus compared to the Tax-dependent HTLV-1 induced activation of FUT7. Firstly, this is a prerequisite for a very rapid activation of the host FUT5 gene as the concept of an RNA inducer eliminates delays caused by RNA transport to the cytoplasm, translation, and transport of the translated protein to the nucleus, inevitable and time-consuming processes associated with immediate early viral proteins as inducers of intranuclear activities. Our finding that 2-fold levels of FUT5 RNA is detected as early as one hour p.i. underscores that HSV-1 induction of FUT5 transcription indeed is a rapid process. Moreover, the RNA concept also permits that at least the first step of the virus-induced activation of FUT5 takes place without any presentation of viral B and T cell epitopes in the newly infected cells.

This latter aspect may be of relevance for the latent or persistent state of HSV-1 in the infected neuron, where it at least theoretically should be possible for the viral genome to influence the cell surface glycotopes of the latently infected neuron by a short pulse of ICP0 transcription. The possible significance of this aspect is illustrated by the multitude of surface glycotopes of relevance for the functional status of neuronal cells (30, 80).

The conclusions presented above concern only the very first step in the herpesvirus control of FUT gene expression. In other words, the ICP0 RNA-dependent activation of host fucosyltransferase genes, here exemplified by FUT5, is followed by several consecutive steps in the virus-induced regulation of sLe^x expression over time. For example, inhibition of viral DNA synthesis is associated with hyperexpression of FUT5 RNA (II, IV), compatible with the notion that viral late proteins are also engaged in attenuation of FUT5 transcription (II). It should also be kept in mind that the ICP0 protein, aside from being the first transcribed gene during lytic viral infection, degrades ND10 sites, releasing transcriptional factors in the nucleus that could be of relevance for the continued regulation of FUT5 transcription. This is noteworthy considering the analogies and differences between HSV-1 and HTLV-1 regarding virus-induced neoexpression of sLe^x, mentioned above. Thus, the FUT7-inducing HTLV-1 protein, Tax, acts on ND10 in a similar fashion to ICP0, by disruption of ND10, though via different mechanisms and thereby activating CREB pathways (3). The possibility that there exist similarities in at least the later parts of the transcriptional regulation behind sLe^x should therefore not be overlooked. It is therefore reasonable to assume that virus-induced regulation of sLe^x expression over time involves several points for viral control of which the ICP0 RNA-dependent FUT5 activation represents the first step.

The use of FUT5 as a model gene does not confirm that FUT3 and FUT6 are activated via the same mechanism as FUT5. But, there is at least a theoretical possibility for a regulatory element to participate in activation of FUT3, FUT5 and FUT6 transcription simultaneously because all three genes are situated on the same chromosome in close proximity of one another (10, 14). The likelyhood of this scenario may be evaluated in part by investigating FUT3 and FUT6 transcription using ICP0 and ICP4 HSV-1 mutants along with CHX treated cells. However, there are data discouraging the notion of co-induced transcription of the FUT3, FUT5 and FUT6 genes, because expression of these genes varies amongst different organs and cell types (8). On the other hand, the possibility cannot be excluded a priori that the thorough re-organization of the transcription program induced by herpesvirus infection, including manipulation of histone binding regions and enhancer proteins, may pave the way for a mutual up-regulation of all three FUTs.

How do herpesviruses benefit from the specific induction of Le^y and/or sLe^x at the surface of the infected cell? Several putative functions of these glycotopes in the viral context probably can be obtained from studying a far more explored system: the metastatic tumor cell. The glycotopes sLe^x is often exposed at the surface of circulating

tumor cells with a primary task to induce attachment to the endothelial cells and subsequent extravasation and colonization of peripheral tissue (16, 45, 46). During conventional viremia, the naked viral particles spread in the circulation are threatened by neutralizing antibodies and other immune factors. A circulating cell, infected with herpesviruses such as CMV or VZV, is relatively long-lived and could therefore serve as a Trojan horse for the genome of the infecting virus, rendering it inaccessible for immune effectors, not least circulating neutralizing antibodies. Virus-induced exposure at the infected cell surface of sLe^x on strategic occasions in the absence of antigenic viral proteins would provide a mechanism not only for adhesion of the infected cell to the endothelial wall but also for recruiting the selectin-dependent machinery for extravasation and access to the final tissue target. So far the only proven viral application of this strategy concerns HTLV-1 where the amount of virus-induced sLe^x at the surface of the virus-transformed cells is directly correlated to their ability to colonize skin cells. In analogy, the viremic spread of VZV to the skin is caused not primarily by circulating virus particles, but by circulating VZV-infected tonsillary memory T-cells. However, the possible role of VZV-induced sLe^x in this process remains to be elucidated.

The exact function of Le^y expression on tumor cells is enigmatic and not as well understood as that of sLe^x, but yet, its biological significance has been emphasized by inhibitor studies in animal models (Reviewed in (17)). One recently discovered function with possible relevance for interactions with microbial agents and the immune system is that Le^y is able to bind to dendritic cells (DC) via DC-SIGN (Dendritic cell-specific ICAM-3-grabbing nonintegrin), as are all non-sialylated Lewis antigens (2). Interestingly, several pathogens causing chronic infection, including *Mycobacterium tuberculosis, Schistosoma mansoni* and *Helicobacter pylori* have developed a Le^x-dependent strategy for causing a Th1 to a Th2 shift in the T-cell response via a DC-SIGN-dependent mechanism (2, 18, 78). CMV also causes chronic infection and virus-induced Le^y could interact with DC-SIGN in the same manner with the potential to shift the T-cell response to Th2, although it remains to be elucidated whether Le^y is fully compatible with Le^x also in DC-SIGN function. Thus, studies on the DC response to CMV infected cells regarding cytokine expression may be able to shed light on the significance of Le^y on CMV-infected HEL cells.

The profound significance of sLe^x and Le^y for tumor progression has stimulated the development of chemotherapeutic agents based on interference with these glycotopes. These strategies are based on development of humanized monoclonal antibodies to sLe^x or Le^y, of stable hapten glycotopes, and of specific inhibitors with capacity to interfere with biosynthesis of these agents. A few leads have been brought to clinical trials (65). Should a similar active role for Lewis antigens also be established for herpesvirus infection then a completely new arsenal of therapeutics of low toxicity may provide improved treatment of herpesvirus infections, not least in immunocompromised patients.

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Basic amino acids as modulators of an O-linked glycosylation signal of the herpes simplex virus type 1 glycoprotein gC: functional roles in viral infectivity

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The herpes simplex virus type 1 (HSV-1) glycoprotein gC-1 is engaged both in viral attachment and viral immune evasion mechanisms in the infected host. Besides several N-linked glycans, gC-1 contains numerous O-linked glycans, mainly localized in two pronase-resistant clusters in the N-terminal domain of gC-1. In the present study we construct and characterize one gC-1 mutant virus, in which two basic amino acids (114K and 117R) in a putative O-glycosylation sequon were changed to alanine. We found that this modification did not modify the N-linked glycosylation but increased the content of O-linked glycans considerably. Analysis of the O-glycosylation capacity of wild-type and mutant gC-1 was performed by *in vitro* glycosylation assays with synthetic peptides derived from the mutant region predicted to present new O-glycosylation sites. Thus the mutant peptide region served as a better substrate for polypeptide GalNAc-transferase 2 than the wild-type peptide, resulting in increased rate and number of O-glycan attachment sites. The predicted increase in O-linked glycosylation resulted in two modifications of the biological properties of mutant virus—that is, an impaired binding to cells expressing chondroitin sulfate but not heparan sulfate on the cell surface and a significantly reduced plaque size in cultured cells. The results suggested that basic amino acids present within O-glycosylation signals may down-regulate the amount of O-linked glycans attached to a protein and that substitution of such amino acid residues may have functional consequences for a viral glycoprotein involving virus attachment to permissive cells as well as viral cell-to-cell spread.

Key words: attachment/heparan sulfate/O-linked/plaque size

Introduction

The herpes simplex virus type 1 (HSV-1)-specific glycoprotein gC-1 is present in practically all clinical HSV-1 isolates and plays important roles during establishment of HSV infection in humans despite the fact that this glycoprotein is dispensable for viral replication in cell culture (Hidaka *et al*., 1990; Liljeqvist *et al*., 1999; Olofsson, 1992). Thus gC-1, together with another HSV-1 glycoprotein, gB-1, is responsible for viral binding to cell surface heparan sulfate, which is the primary viral receptor (Gerber *et al*., 1995; Herold *et al*., 1991; Herold and Spear, 1994). Moreover, gC-1 is exclusively responsible for viral binding to an alternative receptor of glycosaminoglycan nature, that is, chondroitin sulfate (Mårdberg *et al*., 2002). Finally, gC-1 constitutes a receptor for factor C3b of the complement system, thereby contributing to HSV-1 immune evasion mechanisms (Friedman *et al*., 1986, 1996).

Being a highly glycosylated protein, gC-1 contains nine N-linked glycosylation sites of which at least eight are utilized and equipped with complex type glycans exclusively, ranging from di- to tetraantennary structures (Olofsson *et al*., 1999; Rux *et al*., 1996). In addition, gC-1 contains numerous O-linked glycans, most of which are localized in two pronase-resistant clusters in the N-terminal part of gC-1 (Biller *et al*., 2000; Dall'Olio *et al*., 1985; Olofsson, 1992). One possible function of these O-linked glycans is to contribute to an extended shape of gC-1, thereby facilitating contacts between virion-associated gC-1 and its different ligands (Stannard *et al*., 1987). The clustered O-linked glycans of gC-1 may also have other functions, including direct glycoepitope signaling to effectors of the immune system. In this context it is intriguing that another herpes virus, for instance, bovine herpes virus type 4, encodes a virus-specific glycosyltransferase, β-1,6-N-acetylglucosaminyltransferase (core 2 transferase), which is engaged in formation of immunologically active selectin receptors of O-linked glycan nature (Markine-Goriaynoff *et al*., 2003; Vanderplasschen *et al*., 2000). Finally, the O-glycosylated stretches of gC-1 in the vicinity of, say, the heparan sulfate–binding domain (Mårdberg *et al*., 2001) may affect its binding specificity.

The biosynthesis of O-linked glycans is initiated by the addition of N-acetylgalactosamine (GalNAc) moieties to serine and threonine residues of a completely translated and folded polypeptide chain, a reaction catalyzed by 1 of at least 13 distinct cellular UDP-GalNAc:polypeptide GalNAc-transferases, which are localized throughout the Golgi apparatus (Hassan *et al*., 2000a). These GalNAc-transferase isoforms have different but partly overlapping peptide acceptor substrate specificities, and they are differentially expressed in cells and tissues (Gruenheid *et al*., 1993), and gC-1 appears to serve as substrate for multiple human GalNAc-transferases (Biller *et al*., 2000). This suggests that gC-1 will become O-glycosylated in any cell type included in the broad host cell range of HSV-1.

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Here we report that a minor modification of a glycosylation signal of gC-1, that is, substituting alanines for two basic amino acids, resulted in major changes in the extent of O-linked glycosylation. Moreover, this modification changed the biological properties of mutant virus in two different ways, for instance, altered pattern of adsorption to primary glycosaminoglycan receptors and altered plaque morphology, indicating differences in the complex process of viral cell-to-cell spread.

Results

Glycosylation status of gC-1 from HSV-1 with a modified O-glycosylation signal

During screening of HSV-1 mutants with modified gC-1, where alanine was substituted for basic amino acids, we found one mutant virus (HSV-$1_{gC\text{-}1(114K,117R)A}$) where gC-1 displayed a significantly lower electrophoretic mobility, indicating a significantly larger apparent molecular weight (~8–9 kDa) compared with gC-1 specified by corresponding wild-type virus (HSV-1_{Rescue}) (Figure 1). This difference could not be explained by the mere substitutions themselves because the theoretical peptide molecular weight of mutant gC-1 is in fact lower than wild-type gC-1. Because gC-1 is highly glycosylated it was initially considered conceivable that the mutation interfered with addition or processing of N-linked or O-linked glycans, although the mutations did not alter any of the putative N-glycosylation sites per se. To asses if N-glycan processing was affected mutant and rescue gC-1 were treated with N-glycanase (Figure 1, N-gly lanes). N-glycanase treatment resulted in increased electrophoretic mobilities of the rescue as well as the mutant gC-1, indicating that the enzyme released N-linked glycans from gC-1. However, the difference in electrophoretical mobility mutant and rescue gC-1 remained, demonstrating that differences in the number or composition of N-linked glycans were not responsible for the difference in electrophoretical mobility between gC-1_{Rescue} and gC-$1_{(114K,117R)A}$.

In contrast to the differences in mobility between completely glycosylated wild-type and mutant gC-1 another picture was observed for the precursor gC-1 (pgC-1) band (Figure 1), which has been demonstrated to contain immature high-mannose N-linked glycans but no O-linked glycans (Biller *et al.*, 2000; Olofsson *et al.*, 1983; Serafini-Cessi *et al.*, 1984; Sommer and Courtney, 1991). Thus mock-treated mutant and wild-type pgC-1 demonstrated identical eletrophoretic mobility, which was the case also for N-glycanase-treated (and hence more rapidly migrating) mutant and wild-type pgC-1 bands. The separation into two very close bands of N-glycanase-treated pgC-1_{Rescue} and pgC-$1_{(114K,117R)A}$, respectively, was occasionally seen and may correspond to pgC with or without the signal peptide sequence present. The results obtained for gC-1 ruled out that possible differences in complex type N-linked glycans were responsible for the high-molecular-weight phenotype of gC-$1_{(114K,117R)A}$, so the present results suggested that O-linked glycans rather than N-linked glycans accounted for the size difference between gC-1_{Rescue} and gC-$1_{(114K,117R)A}$.

This was further evaluated by comparing the electrophoretic mobilities of gC-1_{Rescue} and gC-$1_{(114K,117R)A}$ produced in C1300 cells, a mouse neuroblastoma cell line with a general deficiency in galactosyltransferases (Lundström *et al.*, 1987b). Pertinent here is that all O-linked glycans of gC-1 produced in C1300 cells constitute GalNAc monosaccharides, a few of which are sialylated, which should be compared with a variety of differently sized O-linked glycans up to at least tetrasaccharides that are associated with gC-1 produced in African green monkey kidney (GMK) cells (Lundström *et al.*, 1987a,b). There was no demonstrable difference in electrophoretical mobility between gC-1_{Rescue} and gC-$1_{(114K,117R)A}$ produced in C1300 cells (Figure 2), confirming that the difference in electrophoretic mobility indeed was caused by a higher content of O-linked glycans in gC-$1_{(114K,117R)A}$: In C1300 cells, the possible extra O-linked glycosylation of

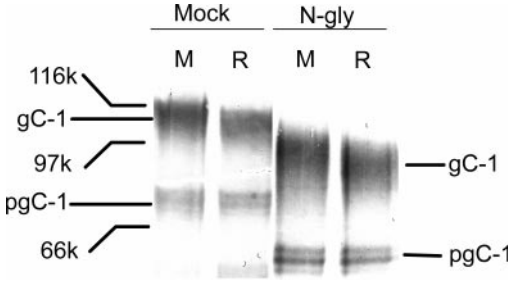

Fig. 1. Effects of N-deglycosylation of wild-type and mutant gC-1. Immunoprecipitates of gC-1 produced in HSV-$1_{gC\text{-}1(114K,117R)A}$- or HSV-$1_{Rescue}$-infected cells were subjected to N-glycanase (N-gly) or mock treatment. The solubilized glycoproteins were separated by SDS–PAGE in gradient gels and the protein bands were visualized by immunoblot, using a gC-1-specific monoclonal antibody (Olofsson *et al.*, 1983). Wild-type (R) and mutant (M) gC-1 were analyzed. Positions of molecular weight markers and gC-1 and pgC-1 are indicated.

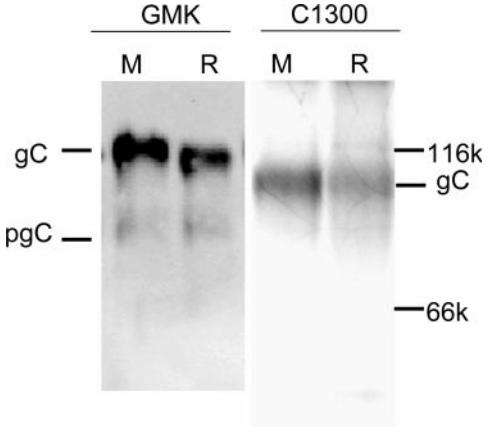

Fig. 2. Positions of gC-1 and pgC-1 from HSV-$1_{gC\text{-}1(114K,117R)A}$- (M) or HSV-$1_{Rescue}$-infected cells (W), respectively. The solubilized glycoproteins were separated by SDS–PAGE using homogenous gels, and the protein bands were visualized by immunoblot using a gC-1-specific monoclonal antibody. Glycoproteins from virus-infected GMK and C1300 cells are depicted. Positions of molecular weight markers and gC-1 and pgC-1 are indicated.

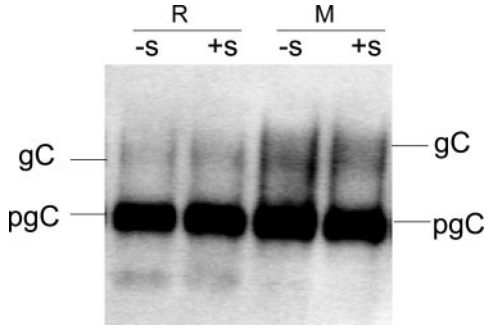

Fig. 3. Immunoblot of proteins of the gC-1 family, treated with N-glycanase (all) and sialidase (+s), and separated by homogenous SDS–PAGE. Lanes representing mock-treated glycoproteins are indicated (−s). Sialidase efficacy was confirmed by release of radiolabel as described in the legend to Figure 4.

gC-$1_{(114K,117R)A}$ was obviously insufficient to induce a corresponding shift for the gC-1 variants produced in C1300 cells. In addition, this result suggested that the difference in electrophoretic mobility between gC-1_{Rescue} and gC-$1_{(114K,117R)A}$, produced in GMK cells, reflected not only a larger number of but also physically larger O-linked glycans of mutant gC-1.

The influence of the gC-$1_{(114K,117R)A}$ mutation on the content of sialic acid in gC-1 was analyzed. Unlabeled glycoprotein extracts from HSV-infected GMK cells were treated with N-glycanase in the presence or absence of sialidase, separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and subjected to immunoblotting (Figure 3). Two distinct bands were detected for the extracts of each virus: One sharp strong band represented essential unglycosylated pgC-1, owing to N-glycanase treatment, and one broader band representing gC-1 containing O-linked glycans but not N-linked glycans. The strong appearance of the pgC-1 band reflected that the linear epitope used as a target for immunoblotting was partially hidden in completely glycosylated gC-1 (Sjöblom *et al*., 1992). The sialidase treatment did not result in any consistent and reproducible shift in electrophoretic mobility of gC-$1_{(114R,117K)A}$ or gC-1_{Rescue}.

To further explore possible differences in sialic acid content of gC-1 O-linked glycans, we determined the amount of radioactivity released by sialidase treatment of affinity-purified [^{3}H]-GlcN-labeled gC-1 produced in GMK and C1300 cells, respectively. We have previously shown that gC-1 from C1300 cells does not contain any N-glycan-associated sialic acid, but the materials originating from GMK cells were treated with N-glycanase prior to sialidase treatment to ensure that only sialic acid of O-linked glycans was assayed. The released radiolabeled sialic acid was separated from remaining glycoprotein by Sephadex G25 gel filtration (Figure 4). A small but reproducible difference in the relative content of sialic acid between gC-1_{Rescue} (7.5%–8.0%) and gC-$1_{(114R,117K)A}$ (10.0%–10.5%) was observed, whereas no such difference in the relative sialic acid content was noted for the corresponding gC-1 preparations from GMK cells.

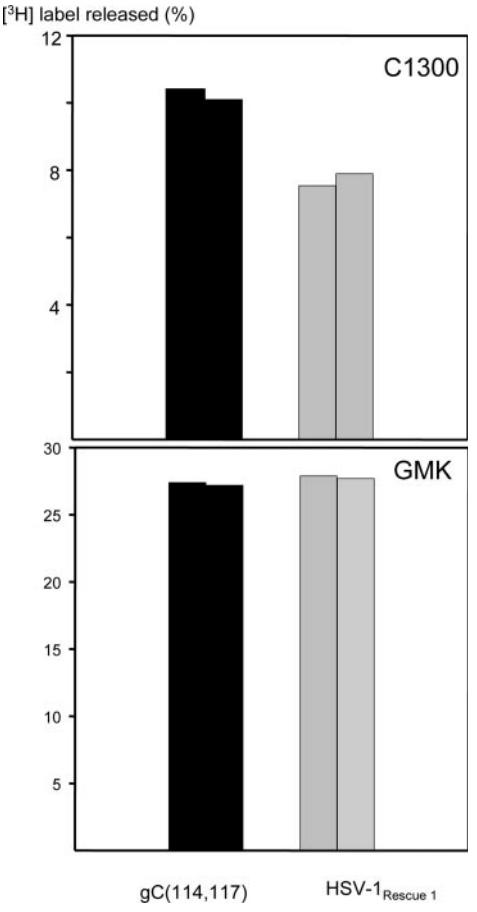

Fig. 4. Sialidase treatment of [^{3}H]-GlcN-labeled gC-1 purified from HSV-$1_{gC\text{-}1(114K,117R)A}$- or HSV-$1_{Rescue}$-infected C1300 cells and GMK cells, respectively. The gC-1 preparations from GMK cells were treated with N-glycanase prior to sialidase treatment. Released [^{3}H]-GlcN label was detected in the totally included volume of Sephadex G25 gel filtration and is expressed as percent of total gC-associated radiolabel.

Prediction of O-glycosylation sites in synthetic peptides by in vitro *analysis of the O-glycosylation capacity of multiple human GalNAc-transferase isoforms*

Synthetic peptides representing the O-linked glycosylation signal delimited by amino acids 106 and 124 were analyzed as substrates for individual, purified human GalNAc-transferases (Bennett *et al*., 1998; Biller *et al*., 2000). A wild-type peptide (representing rescue virus) as well as a peptide containing the gC-$1_{(114K,117R)A}$ mutations were analyzed with GalNAc-T1, -T2, -T3, or -T6, respectively. The wild-type and the mutant peptide were readily glycosylated by GalNAc-T2, however, the reaction velocity with the mutant peptide was considerably higher than with the wild-type peptide (Table I). The three other enzyme isoforms investigated demonstrated only low catalytic activities with both the wild-type and mutant gC-1 peptide substrates (data not shown).

The difference in velocity of the glycosylation reaction with GalNAc-T2 with the mutant peptide prompted us to assess the final product obtained with GalNAc- T2. Matrix-assisted laser desorption/ionization time-of-flight mass

spectrometry (MALDI-TOF MS) of the products revealed important differences. There are in total four potential O-glycan sites in the analyzed peptide substrates, and we found that GalNAc-T2 produced GalNAc-glycopeptides after prolonged incubation containing 2 moles of GalNAc with the rescue peptide and 3 moles of GalNAc with the mutant peptide (Figure 5 and Table II). Sites of O-glycan attachments were analyzed by Edman degradation. Initial analysis of number of moles GalNAc incorporated by MALDI-TOF showed GalNAc-T1 and -T2 to produce glycopeptides containing one to two (rescue peptide) and two to three (mutant peptide) GalNAc residues, respectively. The sequencing results, which are presented in Table II, indicated that T^{111}, S^{115}, and T^{119} of the mutant peptide and that S115 and T119 of the rescue peptide incorporated GalNAc residues. This conclusion is in line with the MS results (Figure 3). Evidence for glycosylation of T110 was found neither for the wild-type nor the mutant peptide.

Table I. *In vitro* O-glycosylation of gC-1 wild-type and mutant peptide substrates by human recombinant GalNAc-T2

	GalNAc-T2 activity[a]	
Peptide substrates	200 μM[b]	800 μM
$gC\text{-}1_{Rescue}$ $_{106}$PKNNTTPAKSGRPTKPPGP$_{124}$	184	558
$gC_{(114K,\ 117R)A}$ $_{106}$PKNNTTPAASGAPTKPPGP$_{124}$	1466	3402

[a]Expressed as pmol/min. Background values without exogenous substrate subtracted.
[b]Peptide substrate concentration; 0.25 mU of enzyme per reaction was used and the reaction time was 1.0 h.

Modified O-linked glycosylation signal and HSV-1 replication in cell culture

We also determined whether the altered glycosylation influenced HSV-$1_{gC\text{-}1(114K,117R)A}$ replication in cell culture. This was explored on two levels, that is, influence of the mutation on (1) interactions between HSV-1 and the primary receptor, using an *in vitro* assay for attachment to permissive

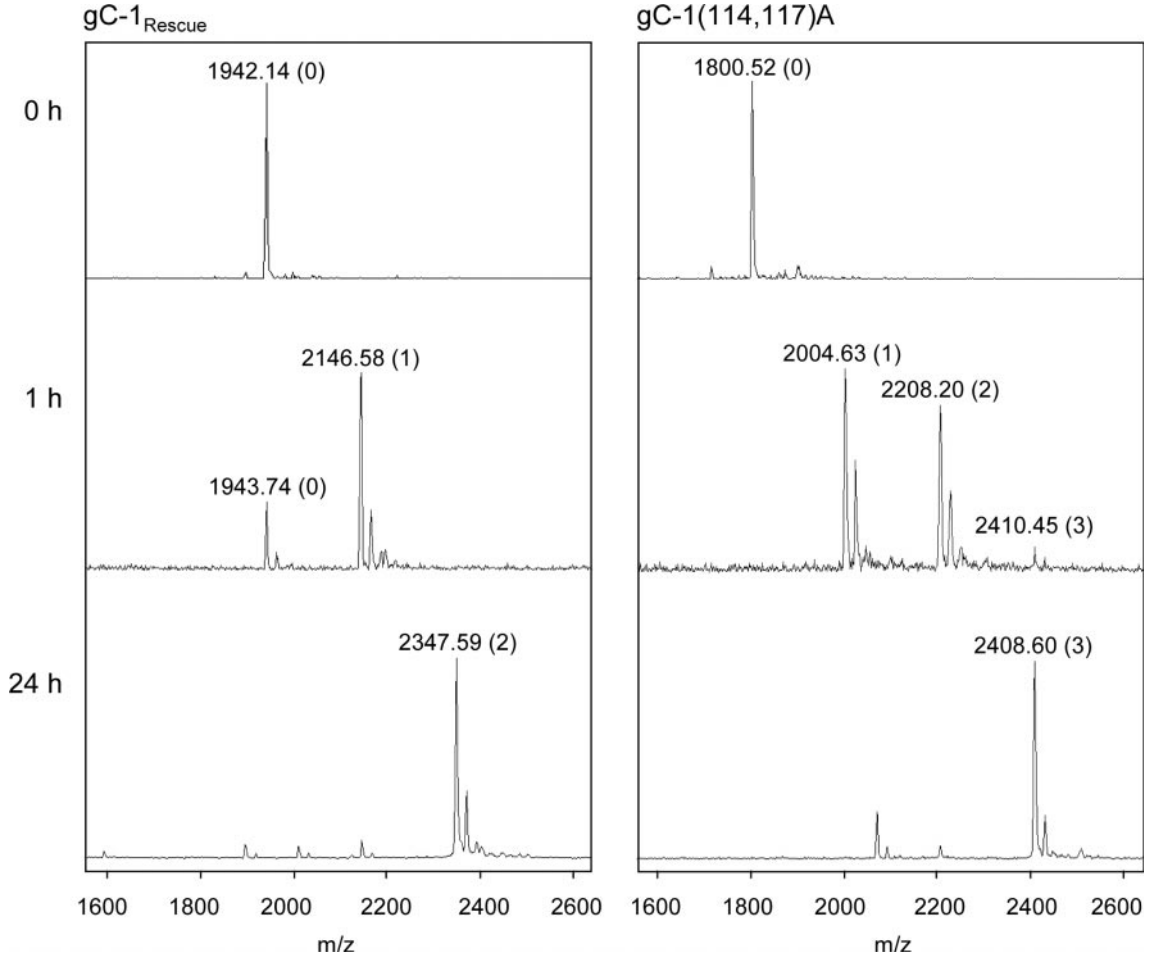

Fig. 5. MALDI-TOF mass spectra of synthetic wild-type and mutant gC-1 peptide glycosylated *in vitro* by GalNAcT-2 for 0 h, 1 h, and 24 h. The *m/z* values of peaks of differently glycosylated peptides are indicated. Calculated numbers of attached GalNAc residues are indicated within brackets.

Table II. Analysis of number of GalNAc residues incorporated and sites of occupancy with GalNAc-T2

		GalNAc-T2	
Acceptor peptide	Amino acid sequence	GalNAc incorporated[a]	Acceptor sites[b]
$gC\text{-}1_{Rescue}$	$_{106}$PKNNTTPAKSGRPTKPPGP$_{124}$	1/2	S^{115},T^{119}
$gC_{(114K,\ 117R)A}$	$_{106}$PKNNTTPAASGAPTKPPGP$_{124}$	2/3	T^{111},S^{115},T^{119}

[a]Total number of moles of GalNAc incorporated after 1 h (figure before slash) or after 24 h (terminal reactions; figure after slash) as described under *Materials and methods*.
[b]Acceptor sites identified for human GalNAc-T2 in exhaustive/terminal reactions, with numbers indicating positions in the corresponding acceptor peptide. Identification of the acceptor sites were performed with Edman degradation.

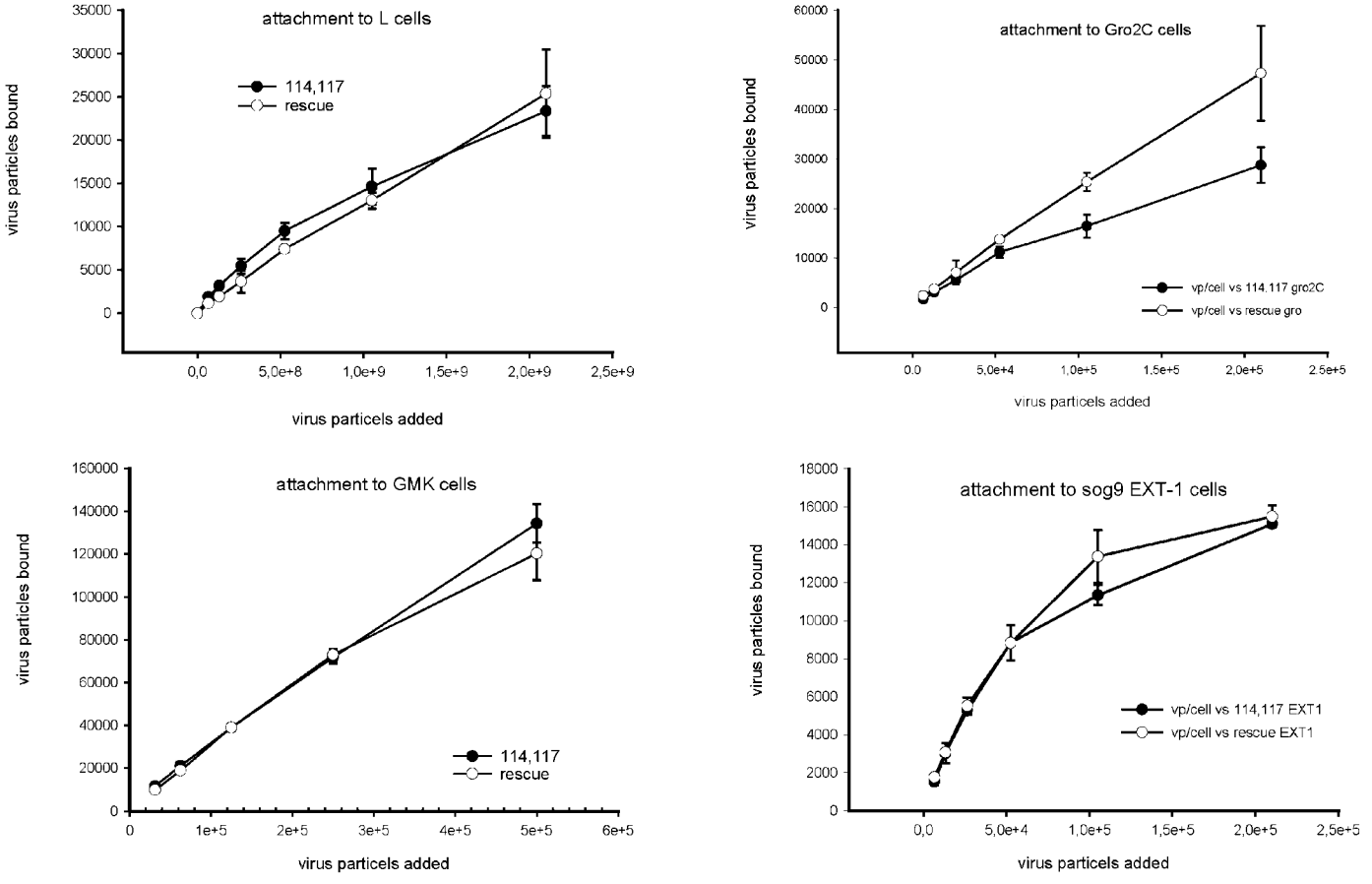

Fig. 6. Binding of HSV-$1_{gC\text{-}1(114K,117R)A}$ or HSV-1_{Rescue} to different types of cells. The attachment assay was performed by adding the indicated amounts of radiolabeled virus to monolayers of GMK AHl, Gro2C cells, sog9 EXT-1 cells, and L cells, respectively. All virus preparations used for experimentation were derived from HSV-$1_{gC\text{-}1(114K,117R)A}$- or HSV-$1_{Rescue}$-infected GMK cells. Mean values and SEMs ($n = 3$) are indicated.

cells; and (2) cell-to-cell spread of newly produced virus as manifested in differences in plaque morphology. This latter phenomenon involves mainly other glycoprotein-dependent processes than binding of free virus to cells, which is outlined more in detail later. Owing to the role of gC-1 in HSV-1 binding to cell surface heparan sulfate and chondroitin sulfate, an analysis of the attachment kinetics of mutant and rescue virus was performed (Figure 6). The target cells were GMK cells, mouse L cells, and two mutant cells of L cell origin, that is, cells synthesizing chondroitin sulfate but not heparan sulfate (Gro2C cells), and cells synthesizing heparan sulfate but not chondroitin sulfate (sog9 EXT-1 cells) (Gruenheid *et al.*, 1993; McCormick *et al.*, 1998).

We found that the adsorption graphs of HSV-$1_{gC\text{-}1(114K,117R)A}$ and HSV-1_{Rescue} were overlapping over a wide range of virus concentrations for all cells analyzed except for Gro2C cells, where HSV-$1_{gC\text{-}1(114K,117R)A}$ demonstrated a significantly decreased adsorption ability as compared with

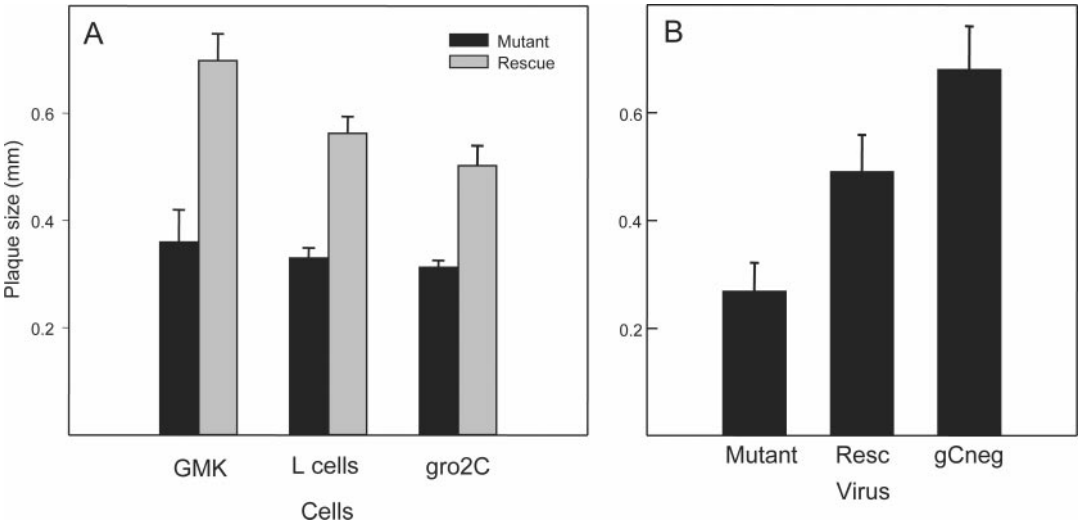

Fig. 7. (**A**) Plaque size of HSV-$1_{gC\text{-}1(114K,117R)A}$ and HSV-1_{Rescue} in cultures of GMK and L and GMK cells. Virus preparations used for inoculation were derived from GMK cells. The plaque size was read 4 days postinfection. (**B**) A separate comparison of the plaque sizes in GMK cells of mutant, rescue and parental gC-null HSV-1 virus strain (gC-39). The plaque size was read 3 days postinfection. Mean values and SEMs ($n = 20$) are indicated.

HSV-1_{Rescue} for the two highest virus concentrations. These results, which were reproducible in three complete, consecutive triplicate experiments ($p < 0.005$, $n = 9$), would indicate that the two basic amino acids at positions 114 and 117 are necessary for full binding capacity of HSV-1 to chondroitin sulfate but not to heparan sulfate. Attempts to use peptide attachment blocking experiments failed to assign any direct role of K^{114} and R^{117} in binding to cell surface glycosaminoglycans; only small inhibitory effects (20%) were achieved using a blocking peptide concentration of 50 μM and in fact the mutant peptide was a better inhibitor of wild type as well as mutant virus than the wild-type peptide (data not shown).

We also investigated whether the mutations induced affected the plaque morphology of infected GMK, L cells, and Gro2C cells (Figure 7), because this is a phenomenon largely related to late functions of HSV-1 glycoproteins in the infectious cycle (reviewed by Johnson and Huber, 2002; Rajcani and Vojvodova, 1998). We found that the plaques generated by HSV-$1_{gC\text{-}1(114K,117R)A}$ were almost 50% smaller than those formed by HSV-1_{Rescue} ($p < 0.001$). The difference in plaque size between HSV-$1_{gC\text{-}1(114K,117R)A}$ and HSV-1_{Rescue} was observed for GMK and L cells as well as Gro2C cells. This similarity between the three cell types was in contrast to the finding that the different attachment rate between mutant and wild-type virus was evident for only one of the investigated cells, Gro2C. This supports the notion that the changed plaque morphology on one hand and attachment rate to Gro2C on the other hand each are caused by different and independent consequences of the altered O-linked glycosylation signal. It is important that although the parental gC-1 null virus strain, designated gC-39, produced syncytial plaques (syncytial and nonsyncytial morphology depicted in Figure 8), we found that neither the plaques induced by HSV-$1_{gC\text{-}1(114K,117R)A}$ nor by HSV-1_{Rescue} were syncytial, suggesting that no alterations in the membrane fusion capacity of HSV-1 glycoprotein were involved in the plaque size transition between these two latter virus strains. As an extra control, we analyzed confluent plates with more than 200 plaques each of HSV-$1_{gC\text{-}1(114K,117R)A}$ and HSV-1_{Rescue} using a gC-specific monoclonal antibody. Interestingly, we found only a few nonstained gC-1-negative revertant plaques from plates infected with mutant as well as rescue virus, but as expected these plaques were of the same syncytial, large phenotype as the parent gC-1 negative virus strain. Altogether, the results indicated that the O-glycosylation peptide signal also affected viral spread in cell culture even in the absence of cell surface heparan sulfate.

Discussion

The findings of this article are of significance at two levels. First we have identified what seems to constitute labile elements in an O-linked glycosylation signal of the highly O-glycosylated protein gC-1. Thus, by changing two basic amino acids of the glycosylation signal that were not targets themselves for O-linked glycosylation, it was possible to generate a mutant gC-1 equipped with a considerably higher degree of O-linked glycosylation. In fact, the SDS–PAGE mobility shift suggested a difference of more than 8000 Da between mutant and wild-type gC-1, implying that the extra content of O-linked glycans of mutant gC-1 could be as much as 40 monosaccharide units. Although care should be taken when calculating absolute masses for the carbohydrate complement of a glycoprotein from electrophoretic data (Olofsson and Bolmstedt, 1998), our reported increase in glycosylation is based on SDS–PAGE results from continuous as well as gradient polyacrylamide gels, representing two different principles, sieving and stacking, respectively, for separation. Moreover, these systems have demonstrated a good correlation between the calculated carbohydrate content and electrophoretic mobility for

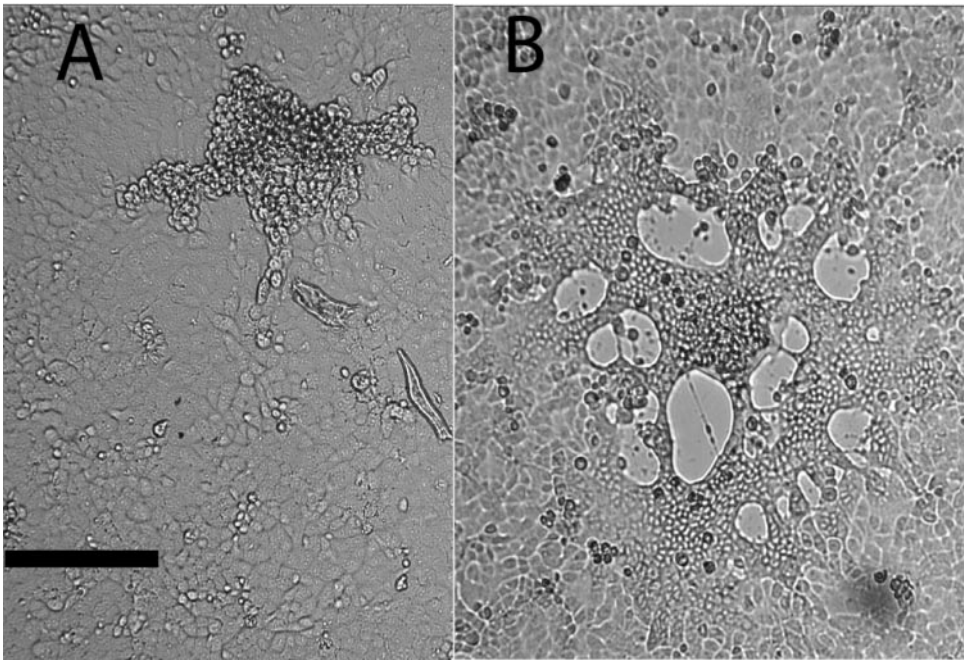

Fig. 8. Morphology of nonsyncytial HSV-1_{Rescue} (**A**) and syncytial HSV-1 strain gC-39 plaques. Bar corresponds to 200 μ. To improve visualization of plaque fine structure, no immunostaining was applied.

mutant viral glycoproteins, lacking different numbers and combinations of N-glycosylation sites (Bolmstedt *et al*., 1991; Olofsson *et al*., 1999). Second, the peptide glycosylation signal harboring these two amino acids seems to influence two different processes in the HSV-1 life cycle: viral binding to cell surface chondroitin sulfate and viral cell to cell spread, at least as it is manifested in plaque morphology. Thus the present wild-type gC-1 O-linked glycosylation signal may be suboptimum for maximal O-linked glycosylation but optimum for maximal biological functionality.

The *in vitro* studies of O-linked glycosylation with individual GalNAc-transferases, using synthetic peptides as substrates, revealed some of the mechanistic details behind the increased O-linked glycosylation of $gC_{(114K,117R)A}$. We choose GalNAc-T1, -T2, -T3, and -T6 because these are enzymes that function with naked peptide substrates and do not require prior GalNAc-glycosylation by other isoforms. They also represent the best characterized isoforms both in terms of specificity and expression patterns. GalNAc-T1 and -T2 further represent the most universally expressed isoforms (for a review, see Hassan *et al*., 2000a). Of the investigated GalNAc-transferases, only GalNAc-T2 demonstrated efficient activity with the wild-type and mutant peptide substrates, but the mutant peptide sequence was a significantly better substrate than the wild type. Furthermore, amino acid sequencing and MALDI-TOF MS showed that the mutant peptide was glycosylated with three moles of GalNAc (T^{111}, S^{113}, and T^{119}), whereas the wild-type peptide was only glycosylated with two moles of GalNAc (T^{119} and S^{113}). Thus the presence of positively charged amino acids among serine and threonine residues in the wild-type gC-1 peptide is likely to direct a lower density of O-glycosylation in this region, although the net difference was only one additional GalNAc residue attached with the mutant peptide.

It is clear that the total number of extra monosaccharides of O-linked glycans of $gC\text{-}1_{(114K,117R)A}$ compared with wild-type glycoprotein is considerably higher than what could be harbored in one extra O-linked glycan of $gC\text{-}1_{(114K,117R)A}$. Considering that most of the O-linked glycans of gC-1, produced in GMK cells, constitute structures up to tetra- or possibly pentasaccharides (Lundström *et al*., 1987a), it appears unlikely that the contribution one or possibly two new O-linked glycans of $gC\text{-}1_{(114K,117R)A}$, as suggested from the peptide model study, is sufficiently large to accommodate that many monosaccharides. Hence our results suggest that the induced mutations also affected the O-linked glycans of regions outside the peptide range (residues 106 and 124), most likely the domain delimited by amino acids 40 and 100, where numerous clustered O-linked glycans are harbored (Biller *et al*., 2000; Olofsson, 1992). This notion is in several ways in accordance with our current view of the temporal regulation of O-linked glycosylation. First, some GalNAc-transferase isoforms are not able to add GalNAc to a serine or threonine unless an adjacent serine or threonine residue is preglycosylated (Bennett *et al*., 1998). Thus it is possible that the facilitated addition of the first few GalNAc residues in the mutated gC-1 may pave the way for subsequently acting GalNAc-transferases, resulting in further increase in O-glycan density of gC-1 outside the peptide substrate sequence studied in this report. GalNAc-transferases further contain lectin domains with specificity for GalNAc-glycopeptides (Hassan *et al*., 2000b), and these are involved in enhancing the O-glycosylation density and possibly have other roles in directing O-glycosylation. Thus a seemingly marginal improvement of the O-glycosylation

signal could have profound effects on the total content of O-linked glycans in a glycoprotein, such as gC-1. The notion that gC-$1_{(114K,117R)A}$ produced in cells with an intact O-glycosylation machinery contains larger O-linked glycans reflects a general increase in the size of each O-linked glycan rather than an increased number of O-linked glycans is supported by the comparison by the studies of gC-1_{Rescue} and gC-$1_{(114K,117R)A}$, produced in C1300 cells, containing only O-linked monosaccharides and to some extent sialylated GalNAc (STn) (Lundström *et al.*, 1987b).

The induced modification of the O-glycosylation changed the biological properties of HSV-1 in two ways. First, the decrease in the ability of HSV-1 to bind to the surface of chondroitin sulfate–expressing cells, which represents the initial step in a series of interactions between HSV-1 and different cellular receptors (Campadelli-Fiume *et al.*, 2000; Mårdberg *et al.*, 2002). Second, the reduced size of the mutant virus plaques, which represents altered viral cell-to-cell spread, a more complex process as outlined in detail later. The chondroitin sulfate– and heparan sulfate–binding sites of gC-1 are largely overlapping, and several arginine residues and a few hydrophobic amino acid residues in the peptide stretch aa 129–160 are important determinants for binding to both types of glycosaminoglycan (Mårdberg *et al.*, 2001, 2002; Trybala *et al.*, 1994). Thus the increased content of O-linked glycans in gC-$1_{(114K,117R)A}$ may interfere either sterically or electrostatically with the interactions between gC-1 and chondroitin sulfate. Therefore one function of K^{114} and R^{117} could be to maintain an optimum net charge of a domain of significance for chondroitin sulfate binding, achieved by negative modulation of O-glycan sialylation, further supported by their cationic nature. Still, it is possible that identical domains of gC-1 are involved in chondroitin and heparan sulfate binding; the interference by O-linked glycans may affect the access for each type of glycosaminoglycan differently. Hence the dynamic responsiveness of this O-glycosylation signal may be a viral means to moderate the level of gC–chondroitin sulfate interactions.

Regarding the other biological effects of the modified O-linked glycosylation sequence, there is so far no specific role for gC-1 defined in the processes determining plaque size and viral cell-to-cell spread. The interplay between different HSV-1 gene products in promoting cell-to-cell spread is complex, involving several HSV-1 glycoproteins. Thus gD-1 seems to be a key factor for cell-to-cell spread of wild type HSV-1 strains in cultured cells (Cocchi *et al.*, 2000), whereas cell-to-cell spread in keratinocytes may be mediated by gI/gE without any involvement of gD-1(Huber *et al.*, 2001; Johnson and Huber, 2002). Moreover, syncytial mutants of HSV-1 may spread among cells owing to the activity of gK-1, possibly supported by gC-1 (Pertel and Spear, 1996). If the function of gC-1 is regulatory, it may be difficult to assign a role to gC-1 in this multitude of interactions by use of deletion mutants. In spite of this, the present data suggest that the O-linked glycosylation signal of gC-1 constitutes one of several factors affecting the plaque size of HSV-1, probably by one or more of at least three conceivable ways. First, gC-1 interaction with cell surface chondroitin but not heparan sulfate is essential for the large plaque size phenotype of HSV-1. This explanation seems unlikely, because of the small decrease in chondroitin sulfate binding as observed for HSV-$1_{gC\text{-}1(114K,117R)A}$ compared with wild-type virus (Figure 4). Second, the O-linked glycans of gC-1 may modulate the cell-to-cell spread-promoting of other HSV-1 glycoproteins, including, gE/gI, gK, or gD. Third, the O-linked glycosylated peptide signal with its O-glycan array may itself be engaged in cell-to-cell spread or cell morphology activities affecting plaque size. This latter hypothesis is in line with both findings that (1) the degree of *Ebola* virus–induced cell detachment is proportional to the length of a used O-linked glycosylation signal in the *Ebola* virus glycoprotein (Simmons *et al.*, 2002), and (2) the finding that such signals are engaged also on nonviral cell detachment mechanisms (Chervenak and Illsley, 2000).

Materials and methods

Cells and viruses

GMK-AHI cells were grown in Eagle's minimum essential medium (EMEM) supplemented with 4% heat-inactivated fetal bovine serum and 0.05% Primaton RL substance. The murine fibroblast cell line LMtk-, clone ID, and its HSV-resistant variants gro2C, expressing chondroitin sulfate only (Gruenheid *et al.*, 1993), and sog9 EXT-1 cells, expressing heparan sulfate but no other glycosaminoglycan marker (McCormick *et al.* 1998), were grown in Dulbecco modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum in 5% CO_2 atmosphere. The mouse neuroblastoma cell line was propagated as previously described (Lundström *et al.*, 1987b).

The mutated HSV strain HSV-$1_{gC\text{-}1(114K,117R)A}$ has two amino residue substitutions, specifically lysine and arginine residues at positions 114 and 117 replaced with alanine residues. The HSV-1 virus designated HSV-1_{Rescue} was used as a wild-type control, and this virus expresses an unmodified gC gene introduced into the HSV-1 gC null strain gC-39 (Holland *et al.*, 1984). HSV-$1_{gC\text{-}1(114K,117R)A}$ and HSV-1_{Rescue} were originally constructed for studies on the heparin sulfate–binding site of gC-1, and both viruses have been characterized in detail previously (Mårdberg *et al.*, 2001). To avoid possible differences in other genes than gC-1, the wild-type and the mutated gC-1 genes were inserted into gC-39 using identical protocols for homologous recombination to produce infectious virus clones. Three plaques each of mutant and wild-type virus were selected for further experimentation and the entire gene for gC-1 was control sequenced (Mårdberg *et al.*, 2001). The gC-1-null strain gC-39 produced essentially syncytial plaques, whereas the plaques of HSV-$1_{gC\text{-}1(114K,117R)A}$ and HSV-1_{Rescue} were nonsyncytial. The number of physical viral particles was calculated as previously described (Mårdberg *et al.*, 2001). The virus particle/PFU ratio varied between 500–1000, with no significant difference between mutant and rescue virus. The term $gC_{(K114,\ R117)A}$ is used for designation of the mutant gC-1 protein.

In vitro *assessment of O-glycosylation capacity*

Polypeptide GalNAc-transferase assays were carried out essentially as described using soluble, secreted, purified

recombinant human GalNAc-T1, -T2, -T3, and -T6, expressed in insect cells (Bennett *et al*., 1996; Wandall *et al*., 1997). Acceptor peptides, representing rescue gC-1, $_{106}$PKNNTTPAKSGRPTKPPGP$_{124}$, and gC(K114, R117)A, $_{106}$PKNNTTPAASGAPTKPPGP$_{124}$, were synthesized by Chiron Mimotopes (Victoria, Australia), and purified by the manufacturer to 99% purity. Briefly, the assays were performed in 25 µl total reaction mixture containing 25 mM cacodylate (pH 7.4), 10 mM $MnCl_2$, 0.25% Triton X-100, 200 µM UDP- [^{14}C]-GalNAc (3,700 cpm/nmol) (Amersham Pharmacia Biotech AB, Uppsala, Sweden) or 2000 µM UDP-GalNAc, 15 µg acceptor peptide for assays with GalNAc-T1, -T2 and -T3 and 0.78 to 25 µg acceptor peptide for assays with GalNAc-T6, and 0.25 mU of each purified GalNAc-transferase, respectively. Soluble, secreted forms of the human GalNAc-transferases analyzed were expressed in High Five cells, grown in cell-free media, and purified to near homogeneity with specific activities of 0.6 U/mg for GalNAc-T1, 0.5 U/mg for GalNAc-T2, 0.5 U/mg for GalNAc-T3, and 2.35 U/mg for GalNAc-T6 measured using peptides derived from MUC2, MUC1, and MUC7 human mucin tandem repeats as described previously (Wandall *et al*., 1997). Products were quantified by liquid scintillation counting after chromatography on Dowex-1, octadecyl silica cartridges (Mallinkrodt Baker Inc, Philipsburgh, NJ), or HPLC (PC3.2/3 or mRPC C2/C18 SC2.1/10 Pharmacia, Smart System; Amersham Pharmacia).

Evaluation of GalNAc incorporation into peptides was performed by MALDI-TOF mass spectrometry (Voyager DE; AME Bioscience, Toroed, Norway) as described previously (Schwientek *et al*., 2002). Sites of incorporation was analysed by Edman degradation on an Applied Biosystems Procise HT 494 sequencer using glass fiber filters precycled with BioBrene Plus (Applied Biosystems, Foster City, CA) as immobilizing support. (N-terminal sequence analysis was carried out at the Protein Analysis Center, Kardinska Institute, Stockholm, Sweden.) The peptide samples were aliquots of HPLC fractions isolated in a mixture of acetonitrile, 0.1% trifluoroacetic acid and water. The samples were spotted onto the BioBrene Plus treated and precycled glass fiber filters. The PTH derivatives were separated on-line using a Brownlee Spheri-5PTH, 5 micron, 220 × 2.1 mm C18 column at 0.325 ml/min. N-terminal sequence analysis was carried out using a Procise HT instrument (Applied Biosystems) after application of samples in solution to precycled Biobrene-treated glass fiber filters (Applied Biosystems).

Metabolic labeling and immunoprecipitation of HSV-1 glycoproteins

Confluent layers of GMK cells in 6-well culture plates were washed two times with serum-free EMEM and infected with HSV-1_{Rescue} or HSV-$1_{gC\text{-}1(114K,117R)A}$ at a m.o.i. of 3. The virus was allowed to adsorb to the cells for 1 h at 37°C, followed by addition of EMEM, containing antibiotics. After incubation in CO_2-incubator for 4 hours 50 µCi/ml D-[6-^{3}H]-glucosamine hydrochloride ([^{3}H]-GlcN; Amersham Pharmacia, 30 Ci/mmol) was added. The cells were radiolabelled for 20 hours until harvest. The labeled culture supernatant was removed from cells and supplemented with 1% (v/v) NP40 and 1 mM AEBSF (4-(2-aminoethyl)-benzenesulfonyl, HCL:*p*-aminoethylbenzenesulfonyl fluoride, HCl; Calbiochem/Novabiochem, San Diego CA). Cells were detached using rubber policemen, washed twice with TBS (tris-buffered saline; 150 mM NaCl, 50 mM tris HCL, pH 7.5) and resuspended in 1 ml TBS containing, 1 % (v/v) NP40 and 1 mM AEBSF. Cell suspensions were sonicated on ice followed by centrifugation to remove cell debris.

Radioimmunoprecipitation was carried out essentially as previously described (Lundström *et al*., 1987a). Heat-inactivated formalin-fixed *S. aureus*, coated with anti-mouse antibodies (Dakopatts, Glostrup, Denmark), were mixed with anti-HSV-1gC-specific mouse monoclonal antibodies B1C1 or C4H11B for 2 hours at 4°C (Olofsson *et al*., 1983; Sjöblom *et al*., 1992). The antibody-staphylococcus complex was then washed three times in TTB [(TBS, containing 1% Triton X-100 and 0.1% bovine serum albumin (BSA)] and mixed with labeled culture supernatants or labeled cell lysates overnight at 4°C. The samples were washed twice in TTB and twice in TBS prior to analysis by homogenous SDS-PAGE (9.25% polyacrylamide gels) or gradient gel SDS-PAGE (NuPAGE 4-12%; Invitrogen; Carlsbad, CA) and fluorography (Amersham Pharmacia). In some experiments gC-1 was detected by immunoblot, using a monoclonal antibody specific for a linear epitope (Sjöblom *et al*. 1992).

In some experiments radiolabeled gC-1 was affinity-purified as previously described and subjected to sialidase (*V. cholerae*, Behringwerke, Marburg, Germany; 100 U/ml) treatment at pH 4.5 for 2 h at 37°C. The glycoprotein and the released sialic acid were separated by gel filtration on short disposable Sephadex G-25 columns (Amersham Pharmacia).

N-Glycosidase (N-glycanase) digestion of immunoprecipitated glycoproteins

The enzymatic elimination of N-linked glycans was performed essentially as previously described (Olofsson *et al*., 1999). Briefly, [^{3}H]-GlcN-labeled culture supernatants were immunoprecipitated as described above but after final wash the samples were resuspended in 10 µl 20 mM sodium phosphate buffer pH 7.5, containing 10 mM EDTA, 0,005% sodium azide, 1% (v/v) β-mercaptoethanol, 1% (w/v) sodium dodecyl sulphonate (SDS) and incubated for 20 minutes at 37°C. The glycoprotein was denatured and eluted from the Staphylococci by boiling for 5 minutes followed by centrifugation. The samples were then diluted 10 times in 20 mM sodium phosphate buffer pH 7.5 containing, 10 mM EDTA, 10 mM sodium azide, 1% (v/v) β-mercaptoethanol, 2% (v/v) NP40 to avoid SDS denaturation of PnGase. PnGase (6 µl of 200IU/ml; Roche Applied Sciences, Stockholm) was added to samples prior to incubation at 37°C for 20 hours. After acetone precipitation samples were finally analyzed on SDS-PAGE and autoradiography (Amersham Pharmacia).

Purification of extracellular virus

The purification of radiolabeled HSV-1 rescue and mutant strains were performed as previously described (Mårdberg *et al*., 2001). In brief, roller bottle cultures of GMK-AH1

cells were infected with either virus strain. Following virus adsorption the cells were washed, and 45 ml fresh EMEM supplemented with 40 μC/ml of [methyl-^{3}H]- thymidine (25 Ci per mmol, Amersham) was added. The cells were incubated for further 48 h, and thereafter the virus was pelleted from culture medium and purified using a tree-step discontinuous gradient of sucrose as described (Karger *et al.*, 1995). Unless otherwise stated, purified virus was resuspended in PBS containing 0.1% BSA and stored at −70°C. The numbers of virus particles in the purified preparations were calculated based on the determination of the DNA content (Karger *et al.*, 1995).

Binding of radiolabeled virus to cells

Monolayers of GMK AHl, Gro2C cells, sog9 EXT-1 cells and L cells in 96-well plates were precooled for 30 min at 4°C, then washed twice with cold PBS and blocked for 1 h at 4°C with PBS-BSA. Different virus strains, adjusted to contain the same initial number of viral particles, were serially diluted in PBS-BSA, as indicated in the figure legends. Equal 50-μl portions of each virus dilution were added in triplicate, and the plates were left for virus adsorption for 5 h at 4°C under continuous agitation. Subsequently, the cells were washed three times in cold PBS to eliminate unadsorbed virions and lysed in 5% SDS. The radioactivity was determined by scintillation counting, and the results were expressed as the percentage of attached virions relative the number of virus particles originally added to the cells.

Virus plaque size assay on glycosaminoglycan-deficient cells

Monolayers of GMK-AHl and L cells in 6 well plates were infected with about 200 pfu per strain of the mutated HSV, $gC_{(114K,117R)A}$, as well as the rescue strain ($HSV\text{-}1_{Rescue}$). The monolayers were washed in complete medium, DMEM high glucose (L cells) and EMEM (GMK AHl) supplemented with penicillin and streptomycin prior to virus addition. The virions were allowed to adsorb at 37°C for 2 h, and thereafter the cells were washed three times in complete medium, and 1% methylcellulose solution was added. The cells were incubated for 3 or 4 days, as indicated in figure legends, and nonconfluent plaques were detected by black plaque assay with the gC-1-specific MAB B1C1 (Nilheden *et al.*, 1983; Olofsson *et al.*, 1983). The plaque size was determined essentially as described by Baigent *et al.* (2001). In brief, the plates were photographed together with a ruler, using a computer-assisted CCD camera, and the largest diameter of each plaque was determined at 3× magnification, using the ruler scale for standardization. A mean value was calculated for each strain and cell line, and the statistical significance of the measurements was determined by using Student *t*-test.

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Abbreviations

DMEM, Dulbecco's modified Eagle's essential medium; EMEM, Eagle's minimal essential medium; GMK, green monkey kidney; HSV-1 herpes simplex virus type 1; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBS, Tris-buffered saline.

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Real time PCR for monitoring regulation of host gene expression in herpes simplex virus type 1-infected human diploid cells

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Abstract

Herpes simplex virus type 1 (HSV-1) induces prominent shifts in the rates of transcription of host cellular genes of relevance for the outcome of the viral infection. The quantitative analysis of transcription may be obscured by virus-induced alterations in the levels of RNA encoded by cellular housekeeping genes that are used commonly for normalisation of real time reverse transcription PCR (RT-qPCR). In the present study, we analysed β-actin, GAPDH and 18S rRNA for their usefulness in normalisation of RT-qPCR analysis of the transcription of the HSV-1 gamma gB-1 gene and *FUT5*, a cellular gene induced by viral infection. The transcription of these genes was monitored in a TaqMan-based real time RT-PCR system over a 24 h interval of virus infection of human embryonic lung fibroblasts. The levels of gB-1 and *FUT5* RNA were normalised via difference in the threshold cycle (ΔC_t) values relative to each and one of the housekeeping genes or calculated in relation to the number of infected cells without any further normalisation. The levels of RNA encoded by β-actin or GAPDH were found to vary by several orders of magnitude during HSV-1 infection, introducing large errors in the estimation of the gB-1 and *FUT5* RNA levels. In contrast, the variation of C_t values for 18S rRNA was less than one cycle during 24 h period of HSV-1 infection. The *FUT5* and gB-1 RNA figures obtained by ΔC_t normalisation relative 18S rRNA were identical to those calculated in relation to the number of infected cells. These data recommend 18S rRNA for normalisation in HSV-1-infected human cells but discourage the use of β-actin and GAPDH RNA for this purpose. By applying these procedures, it was shown that the transcription of *FUT5* was increased by 50-fold 5–24 h after HSV-1 infection and 200-fold by the inhibition of viral DNA replication in HSV-infected cells.

Keywords: TaqMan; Transcription; Virus; Glycosyltransferase; *FUT5*; gB-1

1. Introduction

The infection of a cell by the herpes simplex virus type 1 (HSV-1) and other members of the herpesvirus family is often associated with prominent changes in the execution of the host cellular transcription program. During the lytic phase, HSV-1 infection such changes were considered to reflect an almost general virus-induced shut-down of the host cell-specific macromolecular synthesis (Spencer et al., 1997), but recent data demonstrate maintained or even increased rates in transcription of several cellular genes after HSV-1 infection (Taddeo et al., 2002). The virus-induced changes in the cellular transcription programme may also be more subtle and probably constitute parts of the herpesvirus strategy of establishing lifelong persistent infections in humans, with periods of latency and occasional episodes of recurrencies. In some cases, cellular genes whose transcription is a target for viral modulation are involved in adaptive and acquired immune mechanisms, and recently it was shown that virus-induced transcriptional changes in host cell glycosylation could be involved in such strategies. Thus, cytomegalovirus affects the transcriptional program of three glycosyl transferase genes involved in the formation of sialyl Lewis X, a carbohydrate determinant serving as a receptor for selectins, which are active in the homing of neutrophiles (Cebulla et al., 2000; Vestweber and Blanks, 1999).

In this context, the gene product encoded by *FUT5* is relevant (Weston et al., 1992) and might be important to the HSV-1 infection. This enzyme constitutes an α3/4-fucosyltransferase, engaged in formation of cell surface

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complex carbohydrates, but whose regulation of expression in different tissues or biological importance is not known (Cameron et al., 1995). However, pilot experiments suggested that HSV-1 infection could awake expression of *FUT5* during the infectious cycle, and therefore, *FUT5* was chosen as a model for cellular genes that are inducible by the virus infection.

Studies on how the virus regulates the expression of host cellular genes may reveal important features of the interplay between a herpesvirus and its host. The advent of real time PCR (qPCR) has facilitated such studies because older, cumbersome, and at most, semiquantitative techniques such as Northern blot have been replaced by accurate, sensitive and rapid real time RT-PCR-based procedures (RT-qPCR). RT-qPCR has a capacity to quantitate the transcription rates with a dynamic range of several orders of magnitude. However, the success of this approach is dependent on the use of housekeeping genes as internal standards for defining a background rate of transcription for normalisation of the analysis of a particular target gene. Examples of such housekeeping genes with presumed constant rates of transcription, independent of differences in cellular metabolic activity are β-actin, GAPDH, and 18S rRNA. Not surprisingly, there are types of experimental situations where manipulation of the cellular status considerably may affect the rate of transcription also of housekeeping genes (Weisinger et al., 1999). Hence, it can probably not be anticipated that the profound change in the cellular metabolism posed by a virus infection is compatible with maintenance of a constant transcription rate of all housekeeping genes, used for normalising transcriptional analysis, by RT-qPCR. In other words, if variations in transcription rates of classical housekeeping genes vary during normal cell development they may be expected to be much worse during virus replication of an infected cell.

The expression of some frequently used housekeeping genes was compared during herpes simplex virus type 1 infection of diploid cells, and it was determined whether they could be used for normalisation in analysis of transcriptional regulation of viral genes as well as of "non-housekeeping" genes in the infected cells. The results indicate that a careful choice of a suitable housekeeping gene, based on practical experimentation (Thellin et al., 1999), is necessary for a reliable transcriptional analysis of a virus-infected cells, using real time PCR. However, provided that such measures have been taken it is possible to find suitable housekeeping genes for transcriptional studies even in cells infected with herpes simplex virus, which has been considered to shut-off most cellular genes.

2. Materials and methods

2.1. Cells and virus infection procedures

Human diploid embryonic lung fibroblasts (HEL cells) of low passage number were used throughout the study (2% fetal calf serum during cell growth). Cell cultures in 6-well tissue culture plates (750,000 cells per well; serum depletion) were infected with 12 plaque forming units (PFU) per cell of the laboratory prototype HSV-1 strain Syn 17+. The virus was allowed to bind for 1 h and cultures infected in parallel were harvested at 40 min, 1, 3, 5, 7, 9, and 24 h, respectively. In some experiments indicated in the figure legends, HSV-1 DNA replication was inhibited, using 80 μM acyclovir (GlaxoSmithKline, Stockholm). Virus-infected and parallel non-infected cells were harvested by trypsination (0.4% trypsin in phosphate-buffered saline) and centrifuged at 260 × *g* for 6 min. The cells were washed before lysis (RNA isolation kit II, Roche Diagnostics Scandinavia AB). The lysed cells were processed for total RNA extraction as described below.

Green monkey kidney AH-1 (GMK) cells (Günalp, 1965) were used for plaque titration.

2.2. RNA extraction

The MagNA Pure LC Isolation station (Roche Diagnostics Scandinavia AB, Stockholm) was used for the isolation of total RNA from the lysed cells. Briefly, 200 μl of the lysed cells were isolated in duplicate using the RNA isolation kit II (for cultured cells) (Roche Diagnostics Scandinavia AB) exactly as described by the manufacturer. Total RNA was eluted in a final volume of 100 μl. The purity of the RNA preparations was checked by the 260 nm/280 nm ratio (range 1.8 ± 0.2).

2.3. Total nucleic acid extraction for HSV-1 DNA analysis

For quantitation of progeny HSV-1 DNA the MagNA Pure LC Isolation station was used as described by the manufacturer, applying the total nucleic acid isolation kit (Roche Diagnostics Scandinavia AB, Stockholm). Control experiments revealed that there was no remaining RT activity of the Taq that could result in disturbing amplification of possible gB-1 RNA contamination in the samples used for HSV-1 quantification (see Section 2.4).

2.4. Real time PCR

Primers and probes (sequences of the primers and probes are listed in Table 1) were designed according to the guidelines of Applied Biosystems with help of the Primer Express 2.0 software (Applied Biosystems). Primers were custom synthesised by Thermo Electron Corporation (Ulm, Germany). The RNA analysis was undertaken using the TaqMan One-Step RT-PCR Master Mix reagents kit (Applied Biosystems) and 40 ng of total RNA. The reverse transcription reaction was run at 48 °C for 30 min and the following PCR according to the manufacturer's instructions, for 40 cycles (95 °C 15 s, 60 °C 60 s) using 2× AmpliTaq Gold DNA Polymerase mix, 40× RT enzyme mix, forward and reverse primers (0.5 μM) and probe (0.3 μM). All one-step PCR

Table 1
TaqMan real time PCR systems used in the present study

Gene	Ass number	Forward primer	Reverse primer	Probe[a]	Comments
18S rRNA[b]					RT-PCR.
β-Actin	NM_001101	CCCAGCCATGTACGTTGCTA	TCACCGGAGTCCATCACGAT	ACGCCTCTGGCCGTACCACTGG	RT-PCR method: the present paper.
GAPDH	NM_002046	GAGAAGGCTGGGGCTCAT	TGCTGATGATCTTGAGGCTG	CTCTGCTGATGCCCCCATGTTCGT	RT-PCR method: the present paper.
gB-1	AF259899	TTTGTGTACATGTCCCCGTTTTAC	AGAAGCCGTCGACCTGCTT	ACACCGAACACACCAGCTACGCCG	RT-PCR method: the present paper.
FUT5	NM_002034	CGCTGGATCTGGTTCAGC AT	CAGCCGTAGGGCGTGAAG	*CCCCCAGCAACTGCCGGC*	RT-PCR method: the present paper.
gB-1	AF259899	GCAGTTTACGTACAACCACATACAGC	AGCTTGCGGGCCTCGTT	CGGCCCAACATATCGTTGACATGGC	Direct PCR; for the estimation of HSV-1 genome copy number. Method: Lindh et al. (manuscript in preparation).

[a] All probes, except for the commerical 18S rRNA system, were fluorescently labelled with FAM in 5′ and TAMRA in 3′ position. The molarities of the constituents of the reaction mixtures are given in Section 2.

[b] Commercial pre-developed TaqMan Assay™ (Applied Biosystems). primer and probe sequences not public.

reactions were performed in 30 μl reaction volumes, and carried out in a 96-well plates, which was centrifuged for 1 min at 1000 × *g* at room temperature in a swing-out rotor (Rotina 48R; Hettich, Tuttlingen, Germany) to remove small air bubbles in the reaction vessels. The amplification and detection was carried out with an ABI Prism 7000 sequence detection system (Applied Biosystems). The C_t value was defined as the number of PCR cycles required for the fluorescence signal to exceed the threshold value (defined as 10 times the standard deviation of the baseline variation). ΔC_t values were defined as the absolute value of the difference between the C_i of the target RNA (gB-1 or *FUT5*) and the housekeeping gene RNA C_t value for each sample (Hamalainen et al., 2001), and approved only when the slope of the standard curve for the target gene was parallel with the standard curve for the housekeeping gene RNA (slope difference less than 0.1). The standards used (except for 18S rRNA) were plasmids created with Topo TA (Invitrogen AB, Stockholm) according to the manufacturer's instructions. The plasmids were used in 10-fold dilutions in the real time PCR in question and the reaction was carried out as described above. To facilitate comparisons, the relative concentration, C_R, of gB-1 or *FUT5* RNA were calculated using the formula $C_R = 2^{C-\Delta C_t}$, where C is the ΔC_t value for the lowest RNA concentration detectable for each target gene.

The HSV-1 gB-1 DNA qPCR was carried out in the same manner as the real time RT-PCR reaction but instead using 2× TaqMan Universal Master Mix (containing uracil *N*-glycolase; Applied Biosystems), forward primer, reverse primer (0.9 μM of each) TaqMan probe (0.2 μM), and 20 μl of isolated DNA. Cycling temperatures were the same as those in the one-step RT-PCR.

2.5. HSV-1 plaque titration

Monolayers GMK cells in 6-well plates were infected with 10-fold dilutions of media from infected cells at 5 h or 24 h post-infection, respectively. The virions were allowed to adsorb at 37 °C for 1 h, and thereafter, 1% methylcellulose solution was added. The cells were incubated for 3 days and plaques were detected after ethanol fixation and staining with Alcian Blue.

3. Results

3.1. Expression of the HSV-1 γ-gene gB-1

The HSV-1 gene encoding glycoprotein gB-1, which is transcribed a little before but abundantly after viral DNA replication (Roizman and Knipe, 2001) was chosen as a probe for monitoring virus-specific transcription. To determine the influence of DNA replication on the gB-1 expression virus infected cells were examined both in the presence or absence of inhibiting concentrations of acyclovir. First, the acyclovir effect on viral replication was analysed by assaying: (i) the reduction of progeny viral genome copies by qPCR in the DNA format and (ii) the reduction of viable progeny virus by plaque titration (Fig. 1). Prominent HSV-1 DNA replication could be detected as early as 5 h p.i. in the absence of acyclovir and the number of HSV-1 genome copies increased continuously during the incubation period. In the presence of acyclovir the DNA replication was less than 1% of that recorded from untreated, infected cells, and accordingly, acyclovir treatment decreased the virus infectivity by more than two orders of magnitude (Fig. 1). These

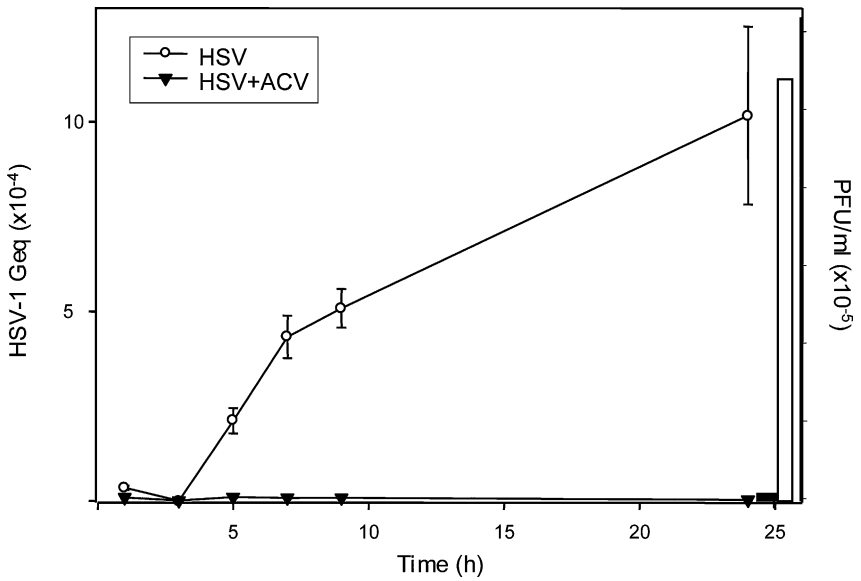

Fig. 1. Time course of HSV-1 production in infected cells. The line graphs show the number of HSV-1 genome equivalents (measured with gB-1 as the target gene) per 20 μl cell extract. Symbols and bars denote mean and standard deviations ($n = 3$), respectively. The open and the filled bars show number of PFU/ml at 24 h post-infection for non-treated and acyclovir-treated cells, respectively.

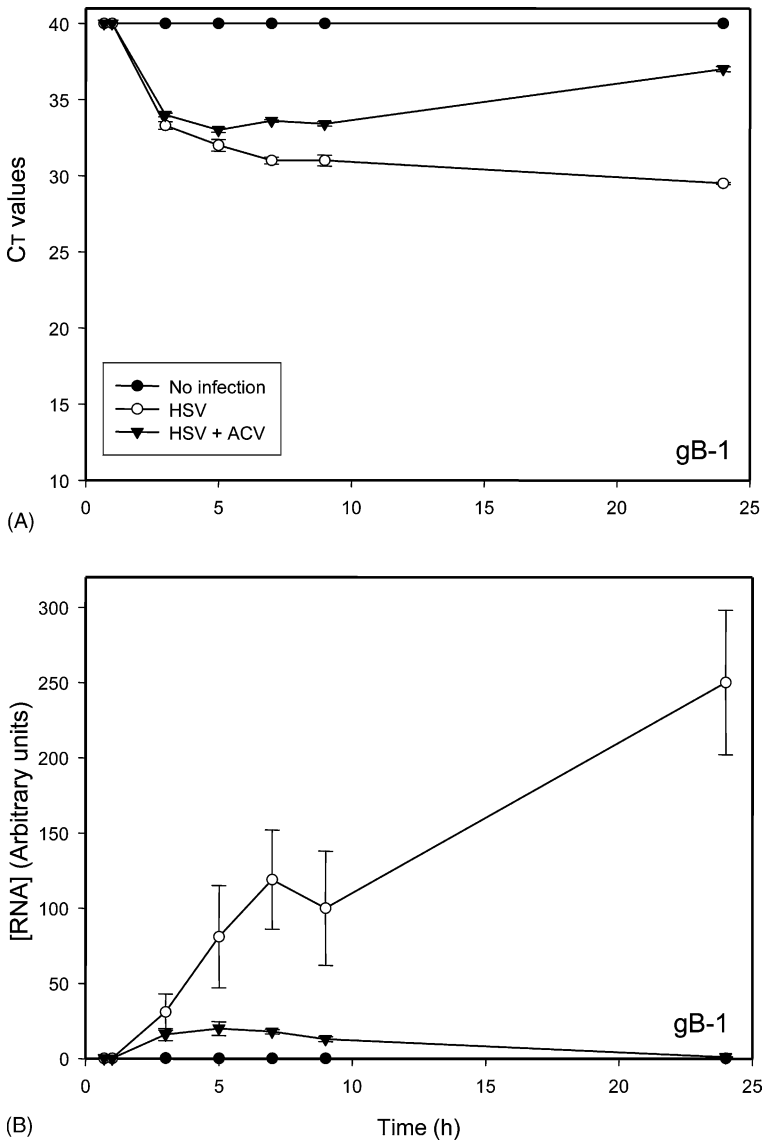

Fig. 2. Determination of gB-1 RNA expression in HSV-1-infected cells, using RT-real time PCR. Panel A shows the C_t values and panel B shows the relative concentration (C_R) of RNA calculated from the formula: $C_R = 2^{C-\Delta C_t}$, where C is a constant, which is assigned a value of near 1, for the lowest RNA concentration detected for the results of each panel, respectively.

data indicate that the acyclovir treatment abrogated DNA replication during the current experimental conditions.

The first sign of gB-1 transcription, reproducible although small and hardly discernable in the graph (Fig. 2), was obtained at 1 h post-infection and the kinetic graphs were similar for virus-infected cells propagated in the absence or presence of acyclovir at least up to 3 h of post-infection. To enable more accurate comparisons of the transcription rate the C_t values, representing the RNA concentrations in a logarithmic manner, were transformed into a linear format (Fig. 2, panel B). This procedure revealed prominent, but expected differences in the transcription rate of the gB-1 gene between acyclovir-treated and untreated cells. Thus, from 5 h post-infection and onwards, the RNA levels remained relatively constant for the acyclovir-treated cells, although a decline was detected at 24 h post-infection. In contrast, in the absence of acyclovir the level of gB-1-specific RNA from the infected cells was increased by several 100-fold, compared with the 1 h post-infection value. In spite of a small decrease at 9 h, a high gB-1 RNA concentration was also recorded at 24 h post-infection. The dramatic increase in gB-1 gene transcription coincided with the occurrence of more massive viral progeny DNA replication at 5 h post-infection. Most likely, the vast majority of gB-1 RNA originated from transcription of progeny HSV-1 DNA in the cells propagated without acyclovir. In acyclovir-induced absence of DNA replication the gB-1 transcription declined over time with only low levels detectable at 24 h. Altogether the results confirmed that the transcriptional machinery of HSV-1-infected cells remained intact at least up to 24 h

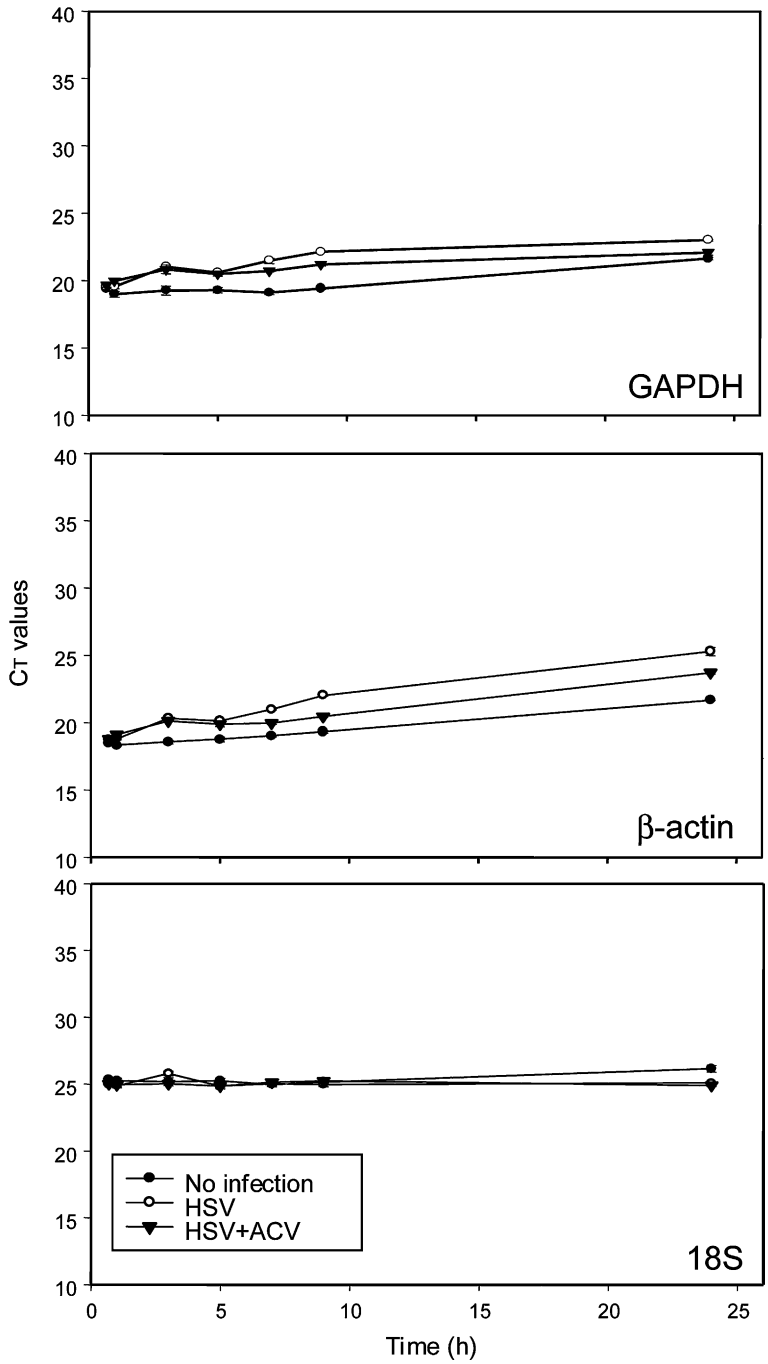

Fig. 3. Determination of housekeeping gene RNA expression in HSV-1-infected cells, using RT-real time PCR. The identity of the target genes is indicated in the lower right corner of each panel.

post-infection in spite of the cytopathic effects induced by the high virus inoculum used for infection.

3.2. Expression of classical housekeeping genes in HSV-1-infected cells

The same RNA extracts were also used to explore the feasibility of three housekeeping genes used commonly as internal standards in transcriptional analysis of herpes-infected cells. The recorded C_t values for expression of the human genes encoding GAPDH, β-actin and 18S rRNA are shown in Fig. 3. RNA originating from all three genes were readily detected in HSV-infected as well as non-infected cells with C_t-values ranging from 18 to 26, indicating the assay sensitivity did not pose a hurdle for use of any of these genes. However, the C_t values for GAPDH and β-actin varied to some extent not only dependent on the infection status of the cells but also dependent on length of the observation

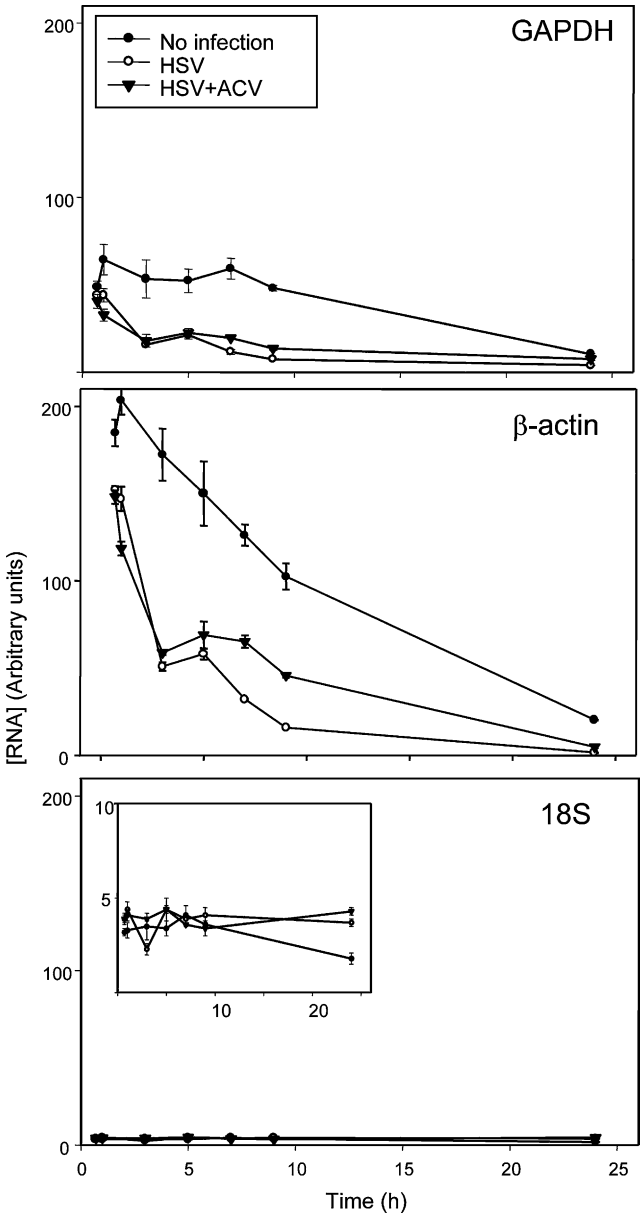

Fig. 4. Relative concentrations of housekeeping gene RNA in HSV-infected cells. The relative concentrations were calculated with the same formula as in Fig. 2. The ordinate scale is identical for the three panes in order to illustrate the differences in specific RNA variation between the three target genes. Inset in the 18S rRNA pane shows identical data but using an expanded *Y* scale.

period. To explore further, this phenomenon the C_t data were transformed into RNA concentration linear representations (Fig. 4). The results were also normalised to facilitate comparison of the concentrations between non-infected cells and HSV-infected cells in the presence or absence of acyclovir, respectively, for each of the housekeeping genes. However, only results within the same panel are comparable in terms of concentrations. These results demonstrated that GAPDH RNA concentrations varied more than one order of magnitude dependent on whether the cells were infected or not or dependent on the length of the incubation time. Even worse, the β-actin RNA concentrations varied more than two orders of magnitude. In contrast, the transcription rates of 18S rRNA were very similar for infected and non-infected cells over the time interval observed. The inset at the bottom panel of Fig. 4 shows the 18S rRNA variation at a higher scale, demonstrating no significant difference in the rate of transcription except for the decline for non-infected cells at 24 h post-infection.

3.3. Feasibility of classical housekeeping genes for normalisation of transcription in HSV-1-infected cells

The ΔC_t values for gB-1 RNA expression relative each of the three housekeeping genes from corresponding gB-1 RNA C_t value were calculated (Fig. 5). There were several similarities between the three panels although the span between the largest and the smallest gB-1 ΔC_t value was more prominent for the β-actin and GAPDH normalisation than corresponding span for the 18S rRNA normalisation. The great impact of these relative small differences in terms of logarithmic values became evident after transforming the ΔC_t values to linear relative concentration representations (Fig. 6). Thus, almost absurd overestimates of the rate of gB-1 transcription were found in the linear perspective, using β-actin (more than a 100,000-fold increase) and GAPDH (a 20,000-fold) for normalisation, reflecting the effects of the prominent decline in β-actin and GAPDH expression over time in HSV-infected cells. In contrast, the use of 18S rRNA for normalisation resulted in gB-1 RNA expression graphs congruent with those obtained in Fig. 2B, where the relative RNA concentration determinations were calculated versus a fixed concentration of total RNA. In conclusion, the 18S rRNA gene but neither the β-actin nor the GAPDH gene appears relevant for normalisation in transcriptional studies on HSV-1-infected human fibroblasts.

3.4. Application of the 18S rRNA gene for normalisation of FUT5 gene expression HSV-1-infected cells

Preliminary experiments showed that transcription of cellular *FUT5* was increased by HSV-1 infection of permissive cells, and *FUT5* was therefore chosen as a model for cellular genes that are inducible by the virus infection. In the next series of experiments, we evaluated whether it was possible to use 18S rRNA for normalisation of results obtained from RT-qPCR analysis of *FUT5* transcription. Thus, the levels of *FUT5* RNA were determined, using RNA extracts from the same HSV-1-infected cells as in the experiments described above. The relative concentration data, calculated from the actual C_t values are presented in Fig. 7.

It was found that the expression of *FUT5* RNA was low and constant in non-infected cells over time, whereas HSV-1 infection induced an almost 100-fold increase in expression of *FUT5* RNA. Acyclovir treatment of infected cells resulted in a further pronounced enhancement of the of *FUT5* RNA

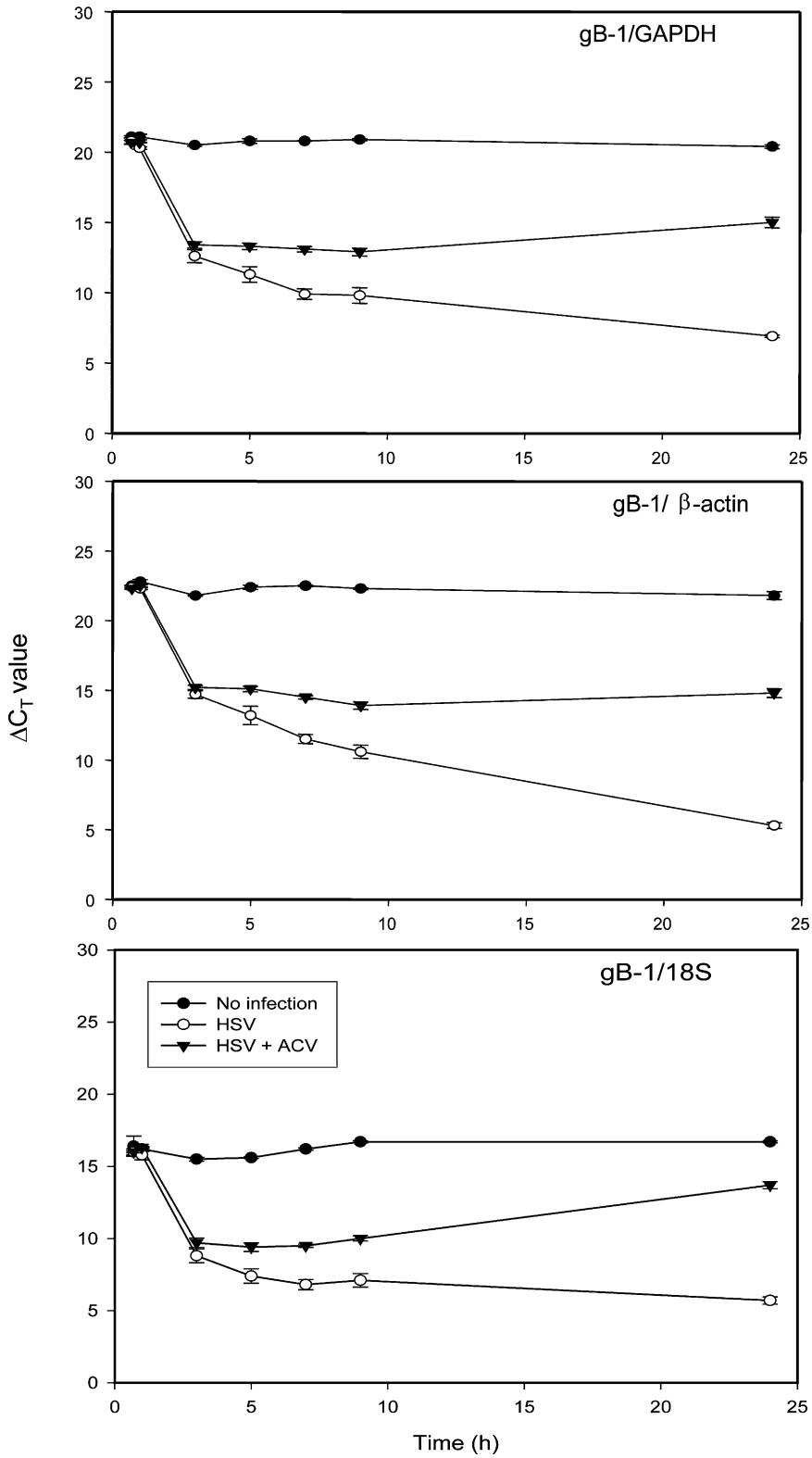

Fig. 5. Expression of the gB-1 RNA concentrations in terms of ΔC_t values relative to RNA concentration of each of the three housekeeping genes.

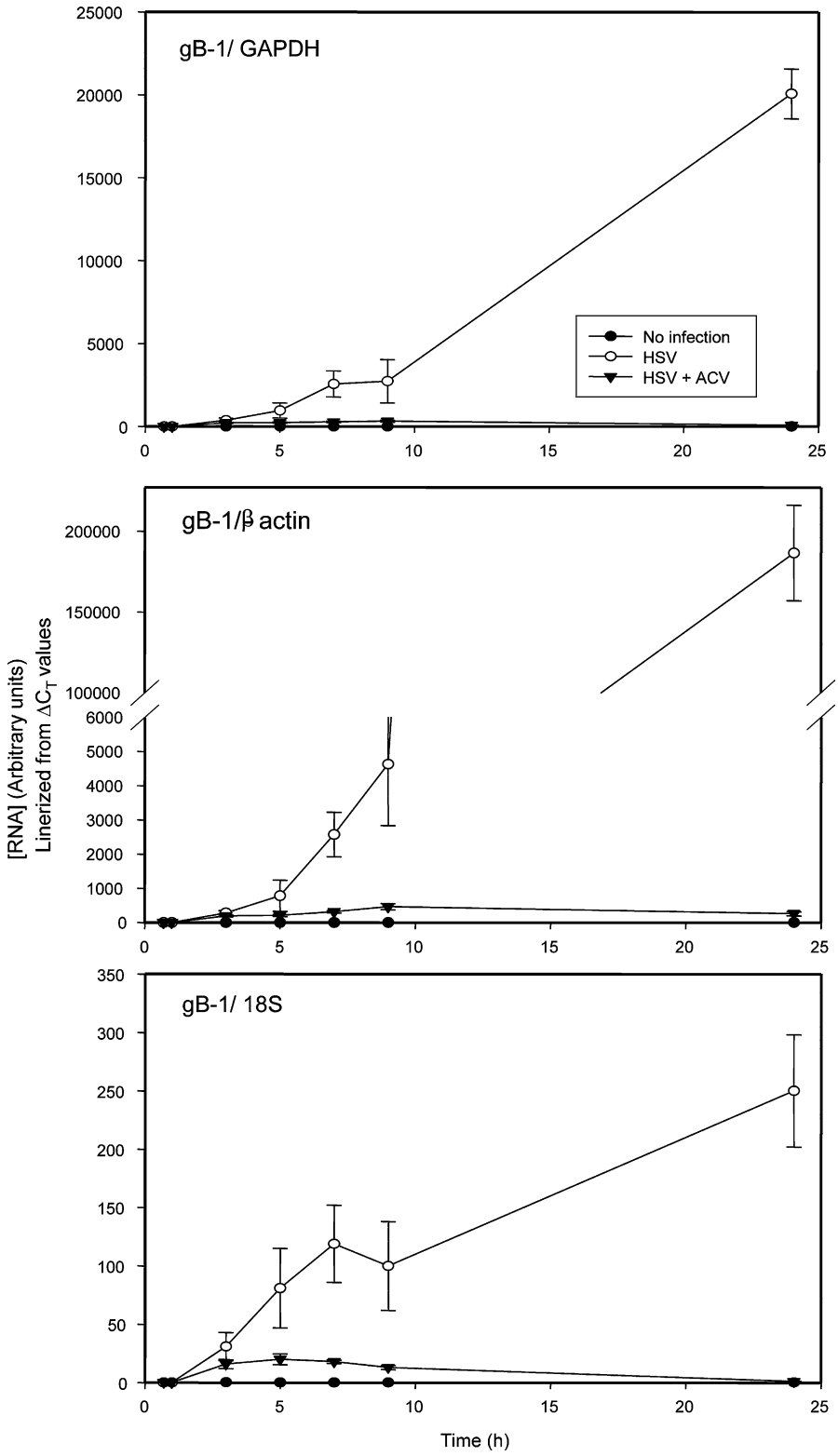

Fig. 6. Linearized gB-1 RNA concentrations calculated from the ΔC_t values of Fig. 5, using the formula indicated in Fig. 2.

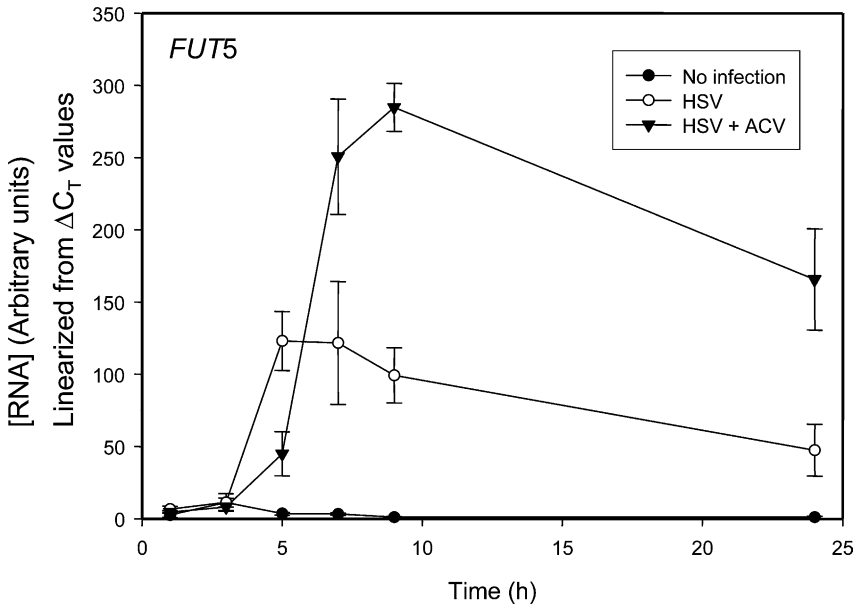

Fig. 7. Linearized *FUT5* RNA concentrations in HSV-1-infected cells expressed per 100,000 infected cells, normalised relative 18S rRNA, using the formula indicated in Fig. 2.

expression in HSV-1-infected cells, i.e. 250-fold compared with the expression in non-infected cells. The slightly earlier appearance of *FUT5* RNA in HSV-1-infected cells in the absence of acyclovir was reproducible although different batches of cells were used in different experiments.

Finally, control experiments were undertaken to ensure that the FUT5/18S rRNA ΔC_t values recorded were consistent and not variable due to differences in the number of cells used for extraction of RNA (Fig. 8). Non-infected cells (low levels of FUT5 RNA expected) and HSV-1-infected cells, harvested at 7 h post-infection (high levels of FUT5 RNA expected), were chosen for these experiments, and RNA, purified from cells of each density were assayed for the 18S rRNA and FUT5 C_t values, respectively. It was found that the ΔC_t values calculated for FUT5 from HSV-1-infected cells were approximately 3 with no significant variation between the three RNA concentrations analysed. Corresponding values for non-infected cells were approximately 8, but

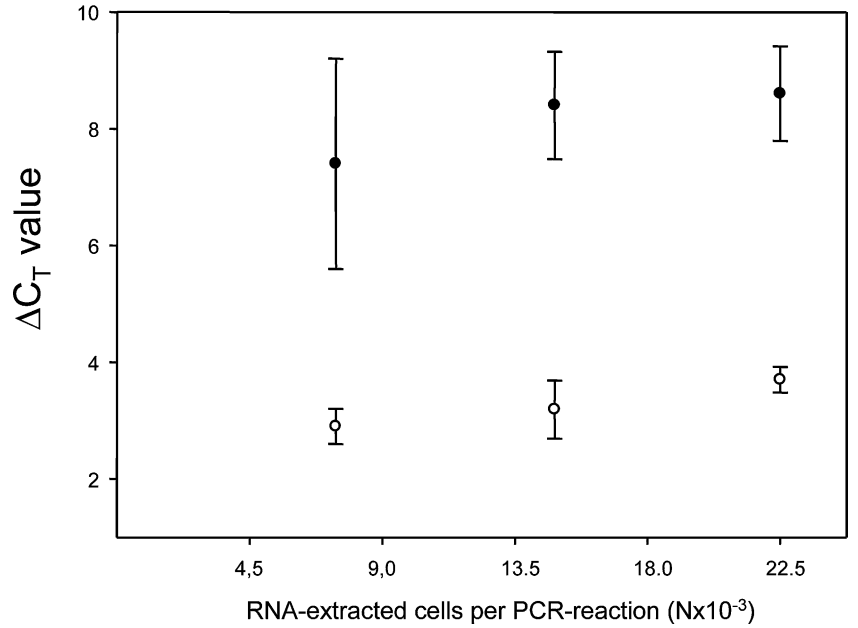

Fig. 8. Cell concentration dependence of FUT5/18S rRNA ΔC_t values. RNA preparations from different numbers of non-infected cells (filled circles) and HSV-1-infected cells, harvested at 7 h post-infection (open circles), were analysed for FUT5 RNA and 18S rRNA in RT-qPCR. The ΔC_t values were calculated as described in Section 2. The lowest cell density analysed in the graph corresponded to that used in Figs. 2–7 above.

the S.D. recorded for lowest cell concentration was high, reflecting the fact that the FUT5 C_t value was close to 40, and hence very variable due to the Poisson distribution of single target molecules. However, the constancy of the ΔC_t values independent of cell concentration and the consistent ΔC_t value difference of 5 (corresponding to a 32-fold difference in linear terms) between the HSV-1-infected cells and non-infected cells indicated that the system used for RNA purification and subsequent RT-qPCR was stable and sufficient for the conclusions drawn. Altogether, the results indicated that the 18S rRNA normalisation was reliable and could also be used in situations where the exact number of virus-infected cells is not known.

4. Discussion

The present investigation confirms a previous report that HSV-1 infection induces profound changes in the transcription of cellular genes (Taddeo et al., 2002). Transcriptional up-regulation as well as down-regulation were demonstrated for four investigated cellular genes, also involving normally constitutively expressed, so called housekeeping genes. This poses a major problem for interpretation of real time PCR-based techniques for determining of transcription rates, since the transcription of such housekeeping genes have been used for normalisation anticipating that such genes are transcribed relatively constantly over time. The results of the present study show the transcription rate of frequently used housekeeping genes, such as GAPDH and β-actin may vary by orders of magnitude during the course of HSV-1 infection, thereby introducing intolerable deviations from correct results.

In general, studies on virus influence on cellular functions are carried out on infected confluent monolayer cell cultures under serum-free conditions. An unexpected result, from the study, was that transcription of GAPDH and β-actin was strongly affected also in non-infected cells under such conditions. This was in particular true for β-actin where a significant decline in transcription took place already after 3 h of serum depletion in the medium to culminate with a reduction of transcription by almost two orders of magnitude after 24 h (Fig. 4). Therefore, β-actin, and for the same reasons also GAPDH, should be strictly avoided for normalisation of TaqMan real time RT-PCR results in human cultured diploid fibroblast irrespective if they are virus-infected or not. The transcription of 18S rRNA, on the other hand, remained constant over the period observed with fluctuations practically within the statistical variation of the assay (cf. Fig. 4), with exception for the 24 h value of non-infected cells.

When considering the ΔC_t values, which are logarithmic in nature, it is easy to underestimate the errors introduced by use of variable RNA references for normalisation. Thus, the ΔC_t graphs for gB-1 versus GAPDH or β-actin may at a first glance look similar to that of gB-1 versus 18S rRNA (cf. Fig. 5). However, the transformation of these data to linear relative concentration values revealed important differences, suggesting several thousands higher concentrations of gB-1 RNA in untreated HSV-1-infected cells compared with acyclovir-treated HSV-1-infected cells (Fig. 6). It is evident that such values are absurd and incongruous, and totally dependent on the large fluctuations in transcription of the two reference housekeeping genes during virus infection. In contrast, the almost perfect congruency between the linear concentration graph for gB-1/18S rRNA (Fig. 6, lower panel) and the corresponding concentration versus microgram curve of Fig. 2B emphasises that transcription of 18S rRNA is a stable and endurable standard for normalisation in determination of the transcriptional activity in virus-infected cells. 18S rRNA was used for standardisation in transcriptional analysis of host cell genes in bovine herpes virus-infected MDBK cells and the results obtained were reasonable in relation to the changes in gene products observed (Muller-Doblies et al., 2002).

In the present experimental setup the use of 18S rRNA for normalisation may appear as unnecessary owing to the great similarities between the two graphs described above. However, this reflects the standardised conditions of the present investigation with identical cell numbers for both infected and non-infected cells applied for RNA extraction with no systematic difference between virus-infected or non-infected cells in the amount of total RNA extracted (data not shown), explaining the similarity between these two graphs. However, given other experimental situations where the actual cell numbers are not known or where only more heterogeneous cell materials are available there are no other ways for standardisation than normalisation with a reliable housekeeping gene such as 18S rRNA. It must, however, be pointed out that one important drawback using 18S rRNA is that this is not a mRNA, which makes this type of normalisation valid only for total RNA protocols and not for mRNA protocols. Moreover, the 18S rRNA is usually expressed at several orders of magnitude higher rates than most mRNA species which might affect the accuracy of normalisation.

The data showed that the 18S rRNA protocol for normalisation was also applicable for analysis of a prototype silent cellular gene, whose transcription was induced by HSV-1 infection, i.e. *FUT5*, encoding a fucosyl transferase engaged in synthesis of complex carbohydrate antigens. The great enhancement of HSV-1-induced *FUT5* transcription by addition of acyclovir is an effect opposite to that observed for gB-1 transcription. One probable explanation for this phenomenon is that the induction of *FUT5* transcription is caused by early α or β HSV-1 proteins, or even earlier by the mere binding of HSV-1 particles to the cell surface. The acyclovir-induced enhancement of *FUT5* transcription may then reflect that one or more γ proteins could be engaged in moderation of the *FUT5* transcription and that this effect is abolished owing to the low synthesis rate of gB-1 and

other γ proteins during acyclovir-dependent HSV-1 DNA replication. Further studies on the mechanisms behind this finding are underway.

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III

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Virus-induced transcriptional activation of host *FUT* genes associated with neo-expression of Le^y in cytomegalovirus-infected and sialyl-Le^x in varicella-zoster virus-infected diploid human cells

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Cell surface carbohydrate structures including sialyl-Lewis X (sLe^x) and Lewis Y (Le^y) are important ligands in normal and malignant tissues. The aim here was to determine the possible influence on the expression of such antigens by two viruses varicella-zoster virus (VZV) and cytomegalovirus (CMV) involved in persistent infections of humans. We found that infection of human diploid fibroblasts with both viruses resulted in transcriptional activation of several fucosyltransferase (FUT) genes that were either dormant or expressed at low levels in uninfected cells. Both viruses induced *FUT3*, *FUT5*, and *FUT6*, encoding α1,3- and/or α1,4-specific fucosyltransferases. CMV, but not VZV, induced transcription of *FUT1* (encoding an α1,2-specific fucosyltransferase), *FUT7*, and *FUT9*. The changes in transcription of FUT genes were expectedly associated with expression of Le^y in CMV-infected cells and sLe^x in the VZV-infected fibroblasts although no expression of these antigens was observed in uninfected cells. One major explanation for this difference between CMV- and VZV-infected cells was that CMV, but not VZV, induced expression of *FUT1*, necessary for Le^y expression. The induced carbohydrate antigens in CMV- and VZV-infected cells could be of significance for virus spread and possible escape from immune responses.

Key words: cytomegalovirus/fucosyltransferase/Lewis Y/sialyl-Lewis X/varicella-zoster virus

Introduction

Several fucosylated histo-blood group antigens, including sialyl-Lewis X (sLe^x) and Lewis Y (Le^y, Figure 1) are deeply involved in cancer dissemination and metastasis (Irimura et al. 1993; Thurin and Kieber-Emmons 2002; Klinger et al. 2004; Magnani 2004), most likely by decoying the functions of these structures in normal cells. Thus, sLe^x or its sulfated derivatives participate in specific binding to E- and P-selectins, thereby promoting activation and homing of various white blood cells active in innate as well as acquired immune responses (Muramatsu 2000; Kannagi and Hakomori 2001; Hiraiwa et al. 2003; Uchimura et al. 2005). Le^y is found at the surface of activated granulocytes, possibly engaged in appropriate adhesion of circulating granulocytes although this process is less characterized (Dettke et al. 2000). Owing to biological significance of sLe^x and Le^y, the critical genes for their synthesis are only active on the appropriate occasions. The changes in expression of carbohydrate antigens in the tumor cells are in most cases achieved by tumor-induced changes in the transcription rates of one or more quiescent glycosyltransferase (FUT) genes, whose gene products synthesize sLe^x or Le^y (Taniguchi et al. 2000). For human T-cell leukemia virus type 1 (HTLV-1)-dependent leukemia, induction of sLe^x is accomplished by transactivation via HTLV-1 encoded Tax of the human fucosyltransferase VII (Fuc-T VII) gene (*FUT7*) (Kannagi and Hakomori 2001; Hiraiwa et al. 2003). This finding raised the question whether viral control of host cell fucosylation as a pathogenic factor could also be used by other viruses, where latency and/or persistent infection are characteristic for the virus–host relationship.

The genetics behind the synthesis of fucosylated carbohydrate antigens is however complex, involving a family of eight active human fucosyltransferase human genes, designated *FUT1–FUT7* and *FUT9*, encoding key enzymes in the formation of sLe^x and Le^y and related histo-blood group antigens of the ABH and the Lewis systems [Reviewed in de Vries et al. (2001) and Becker and Lowe (2003)]. The eight FUT genes mentioned belong to two groups with respect to catalytic activity, where the gene products of *FUT1* and *FUT2* add fucose in an α1,2 linkage, whereas the others may add fucose in an α1,3 and/or α1,4 linkage (de Vries et al. 2001). In spite of these similarities between the members of each of the two groups, there are important differences between all enzymes with respect to a number of parameters including acceptor specificity and tissue localization (Table I).

There are reasons to believe that also nontransforming viruses may significantly influence the structures of the carbohydrate antigens of viruses and the infected cell surface by controlling the regulation of the fine-tuned transcriptional program of these glycosyltransferase genes (Cebulla et al. 2000; Nystrom et al. 2004). The aim of the present study was therefore to explore the interactions of varicella-zoster virus (VZV) and human cytomegalovirus (CMV) with the host cell glycosylation machinery, focusing on the

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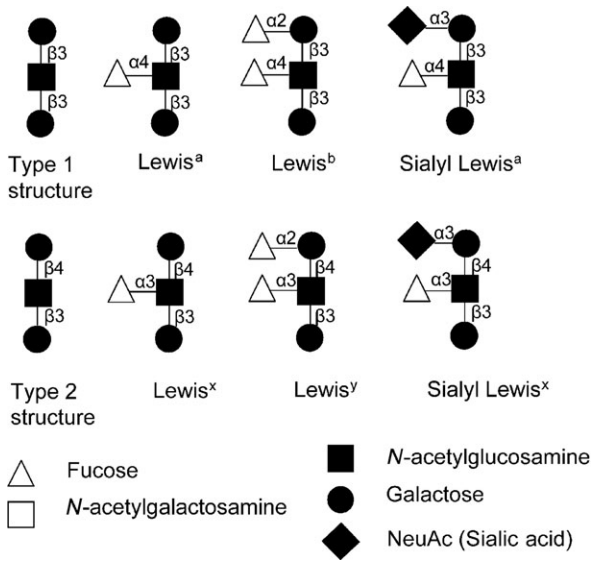

Fig. 1. Structural representation of glycoepitopes.

eight fucosyltransferases (*FUT1–FUT7* and *FUT9*). VZV, a representative for α-herpesviruses, causes chickenpox, and thereafter life-long latency in sensory neurons, from which reactivations may present as herpes zoster or, rarely, as encephalitis. This virus was chosen because of its tight interactions with T memory cells during viremia (Ku et al. 2005). CMV, a representative for β-herpesviruses, may cause fevrile illness including mononucleosis and is thought to remain life-long in monocytes and lymphocytes. Understanding the mechanisms of CMV infection has become increasingly important, because it may cause life-threatening infections in transplanted and other immunocompromised subjects. CMV was chosen because of its close interactions with the endothelium and various white blood cells (Mocarski and Tan Courcelle 2001). As a target cell an embryonic human diploid fibroblast cell line was chosen, maintained in our laboratory at low passages and characterized in previous studies (Lundström et al. 1987; Nystrom et al. 2004), being permissive for both CMV and VZV. We report that infection with both CMV and VZV viruses resulted in increased transcription rates by several orders of magnitudes for at least three of the *FUT* genes, but there were also differences between CMV and VZV, the most important being that only CMV induced *FUT1*, encoding a Fuc α1,2-transferase. This resulted in prominent differences between VZV and CMV with respect to the neo-carbohydrate antigens in the infected cells.

Results

Cell histo-blood group genotyping

As target cells for the present studies, we chose a diploid cell line of human embryonic fibroblasts (HEL), available at low passage numbers and equally permissive for both CMV and VZV. This enabled studies on the influence of the two viruses on expression of glycosyltransferase genes in one and the same cell type. Moreover, as several of the *FUT* genes studied are not vital for growth in cell culture, it was deemed advantageous to avoid transformed, aneuploid cells with possible asymmetric distribution of the actual *FUT* genes among individual cells. The cells were genotyped,

Table I. Properties of the human fucosyltransferases of the present study[a]

Gene (GenBank)	Fuc-T enzyme specificity		Tissue distributiona	Characteristics of HEL cells	
	Linkage	Substrate		Genotype[b]	Possible phenotype
FUT1(H) (M35531)	Fuc α(1,2)	Type 2[c]	Mesenchymal, erythroid	*ND*[d]	H type 2 (Bombay phenotype extremely rare)
FUT2 (Se) (U17894)	Fuc α(1,2)	Type 1	Epithelial cells (saliva and mucous membranes)	$se^{428}se^{428}$	No H type 1, no Le^b (Secretor negative)
FUT3 (Le) (X53578)	Fuc α(1,3/4)	Type 1 and 2; sialylated Type 1 and 2	Epithelial cells in gastrointestinal tissue; kidneys	$Lele^{202,\ 314}$	Le^a, sLe^a, Le^x, sLe^x (Lewis positive)
FUT4 (Lex) (M58596)	Fuc α(1,3)	Type 2	Myeloid cells; embryonic; ubiquitous	*ND*[d]	Le^x, Le^y
FUT5 (M81485)	Fuc α(1,3/4)	Type 1 and 2; sialylated type 1 and 2	Low level tissue expression; spleen, gastrointestinal	*ND*[d]	Le^a, sLe^a, Le^x, sLe^x, Le^y
FUT6 (M98825)	Fuc α(1,3)	Type 2 and sialylated type 2	Gastrointestinal tissue; kidneys; liver, plasma	$FUT6^{wt}FUT6^{370}$	Le^x, sLe^x, Le^y
FUT7 (X78031)	Fuc α(1,3)	Sialylated type 2	Leukocytes; spleen	$FUT7^{wt}FUT7^{wt}$	sLe^x
FUT9 (AJ238701)	Fuc α(1,3)	Type 2	Leukocytes; brain; stomach	*ND*	Le^x, Le^y
ABO (J05175)	Gal/GalNAc α(1,3)	H	Ubiquitous	*BO*[a]	H and B type 2

[a]Based on the references: Cameron et al. (1995), Clarke and Watkins (1996), Clarke and Watkins (1997), Kaneko et al. (1999), Natsuka et al. (1994), Sasaki et al. (1994).
[b]Results from present study.
[c]Type 1 and 2 imply the Type 1 structure and Type 2 structure, respectively as depicted in Figure 1.
[d]ND, not determined.

using polymerase chain reaction-sequence specific primers (PCR-SSP) and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFPL) (Larson et al. 1996; Procter et al. 1997; Elmgren et al. 2000; Bengtson et al. 2001), with respect to those glycosyltransferases previously shown to be subject to allovariation (de Vries et al. 2001) and which are synthesized histo-blood group antigens of relevance for the present study. The results are presented in Table I. The most important finding was that the cells were homozygously defective in *FUT2* (stop codon), implying that they are neither able to express Le^b, nor type 1 ABO antigens, despite the existence of one functional B allele. Furthermore, the cells contained at least one functional *FUT3*, *FUT6*, and *FUT7* allele of each gene.

Time course of CMV and VZV infection and FUT transcription in virus-infected cells

Replicate cell cultures were infected with CMV or VZV (see *Materials and methods*), and virus-infected cells were extracted for DNA and assayed via real-time PCR for viral progeny DNA (Figure 2). Typical cytopathic effects (CPE) were observed for both CMV and VZV at 24 h postinfection (p.i). Production of progeny VZV DNA was detected at 48 h p.i. and a plateau phase was reached at 72 h p.i., which is in accordance with the previously published data (Arvin 2001; Pass 2001). Production of progeny CMV DNA was detected at 24 h p.i. and maximal levels were reached at 72 h p.i.

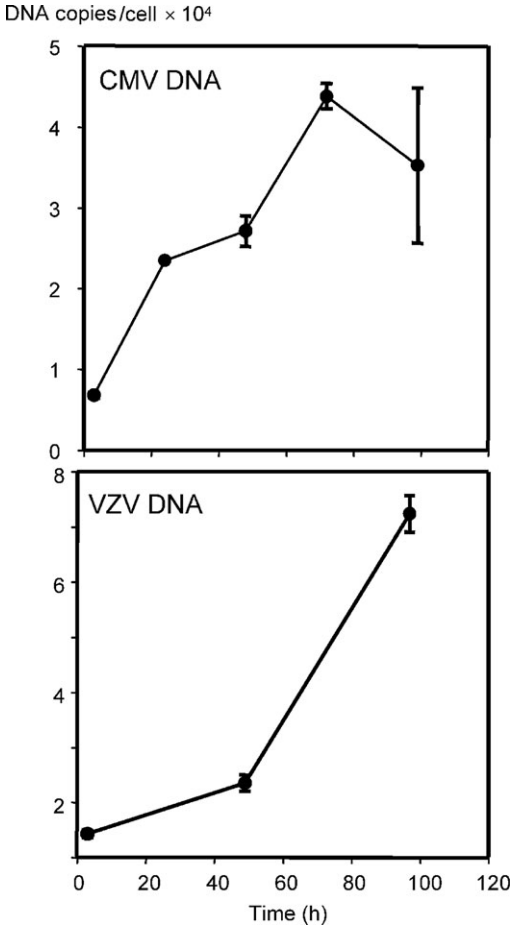

Fig. 2. Time course of CMV and VZV infections. CMV- and VZV-infected cells were lysed and DNA was extracted. Viral DNA was analyzed using real-time PCR.

Total RNA from VZV- and CMV-infected cells was extracted and analyzed by Taqman real-time PCR using the PCR systems described in Table II. Linear levels of different *FUT* transcripts were calculated from ΔC_T values (see *Materials and methods*). The arbitrary RNA concentration values were normalized against 18S RNA enabling direct comparisons of differences in the transcript abundances of each and one of the analyzed *FUT* genes. The *FUT* genes studied could be divided into three different groups with respect to their transcriptional patterns after infection with CMV or VZV: (i) *FUT* genes induced by CMV and VZV (Figure 3), (ii) *FUT* genes only induced by CMV (Figure 4), and (iii) *FUT* genes whose transcription remained high and relatively constant in virus-infected cells as well as uninfected cells during the time interval studied (Figure 5). The transcription rates in mock-infected cells of the *FUT* genes of groups (i) and (ii) were low or undetectable (Figures 3 and 4).

Three *FUT* genes encoding α1,3/α1,4- (*FUT3* and *FUT5*), or α1,3- (*FUT6*) fucosyltransferases belonged to the first group i.e. their transcription rates were strongly induced in VZV- as well as in CMV-infected cells (Figure 3). The maximal transcription rates of these *FUT* genes in virus-infected cells were 100–1600 times higher than the baseline levels obtained for mock-infected cells.

The second group of *FUT* genes represents exclusively CMV-induced genes, containing one α1,2-fucosyltransferase (*FUT1*) whose transcription was induced to 20 000 times over the baseline level by CMV, but not altered by VZV (Figure 4). The maximal levels during the observation interval were reached about 70 h p.i.. Also, *FUT7* and *FUT9* transcription was induced at lower levels and different kinetics (*FUT7*) in CMV-infected cells, but not altered in VZV-infected cells.

The third group comprised *FUT* genes that remained relatively unaffected by virus infection compared with the other two groups (Figure 5). The *FUT4* RNA concentration was high and in the same range (approximately 20 000 arbitrary units) as the maximal value observed for *FUT1* through CMV induction. In contrast to all other differences between virus-infected and mock-infected cells, the extraordinary high value recorded for CMV-infected cells at 24 h p.i. was not consistently observed from one set of infected cells to another. The transcription of the inactive (see Table I) *FUT2* gene was relatively constant in the CMV-, VZV-, and mock-infected cells.

Expression of carbohydrate neo-antigens in CMV- and VZV- infected cells

Next, we explored whether the induced changes in *FUT* transformation influenced the expression pattern of fucosylated carbohydrate antigens in the virus-infected cells. Confluent HEL cell cultures were cultured in teflon-coated glass wells where 30–60% of the cells were infected with either CMV or VZV during conditions in which cell-to-cell spread of virus was permitted as described in *Materials and methods*, *Immunofluorescence*. The cells were examined in immunofluorescence using antibodies to viral antigens and/or specific monoclonal antibodies to defined histo-blood group antigens. CMV-infected cells were identified by a typical granular

Table II. TaqMan real-time PCR primers systems used in the present study

Gene	Ass. number (mRNA)	Forward primer	Reverse primer	Probe
FUT1[b]	NM_000148	AGGTATAAACACACCCTCTGTGCTT	GAGTTCAGGGACAGACAGTGGTT	AAACTGGCAGGTACCGTGCTCATTGC
FUT2[b]	NM_000511	CTCGCTACAGCTCCCTCATCTT	CGTGGGAGGTGTCAATGTTCT	TGGTCACCAGTAATGGCATGGCCTG
FUT3[b]	NM_000149	GGGATCCCTTTTCGTCACACT	CGAACTGGTCTAAGCCTTGCA	AGGTGACCTACAGGCTCCGCTCGA
FUT4[b]	NM_002033	AATTGGGCTCCTGCACAC	CCAGGTGCTGCGAGTTCT	TGGCCCGCTACAAGTTCTACCTGG
FUT5[b,c]	NM_002034	CGCTGGATCTGGTTCAGCAT	CAGCCGTAGGGCGTGAG	CCCCCAGCAACTCCGGC
FUT6[b]	NM_000150	GCATCCAGACGGGATCCA	ACTGCTGCGTCTTGACACCTT	CCAGGTCCCCGATCCCTCTAGCAT
FUT7[a]	NM_004479	CCGCTTCTACCTGTCGTTTGA	GCGTTGCGCCAGAATTTCT	CGCGACTACATTACG
FUT9[d]	NM_006581	CAAGGATTACATCACGGAAAAGC	TGGTCCCAGAACAACAGGTACA	TACAATGCTTTTCTGGCTGGCT
ST3Gal-III[d]	NM_006279,NM_174963-72	AGGAAATCGTAAATCATGTGAAGATG	TTCCACGCAGAATAATACAAAAATC	CAATCTGCTGCTAGCCCTCTGCCTCTTT
ST3Gal-IV[d]	NM_006278	GGGACCAAGGGGAGTGAGG	CACCTGAGGCTCTGGATGTTC	TCTGCTCCTCCGGGTGCTAGCCA
CMV[e]	AY436380	TGATCACTGTTCTCAGCCACAAT	TCCTCTGATTCTCTGGTGTCACC	CCCGCGACTATCCCTCTGTCCTCA
VZV[e]	AY013747	TGCAGGGCATGGCTCAGT	CCCAAGAACCACATGTCCAAC	CGCGGTCCCAAGTCCCTGGA

[a]MGB (Major Grove Binding Probe), Applied Biosystems.
[b]Thermo Electron, Waltham, MA.
[c]Nystrom et al. (2004).
[d]Eurogentec, Seraing, Belgium.
[e]MWG-Biotech AG, Ebersberg, Germany.

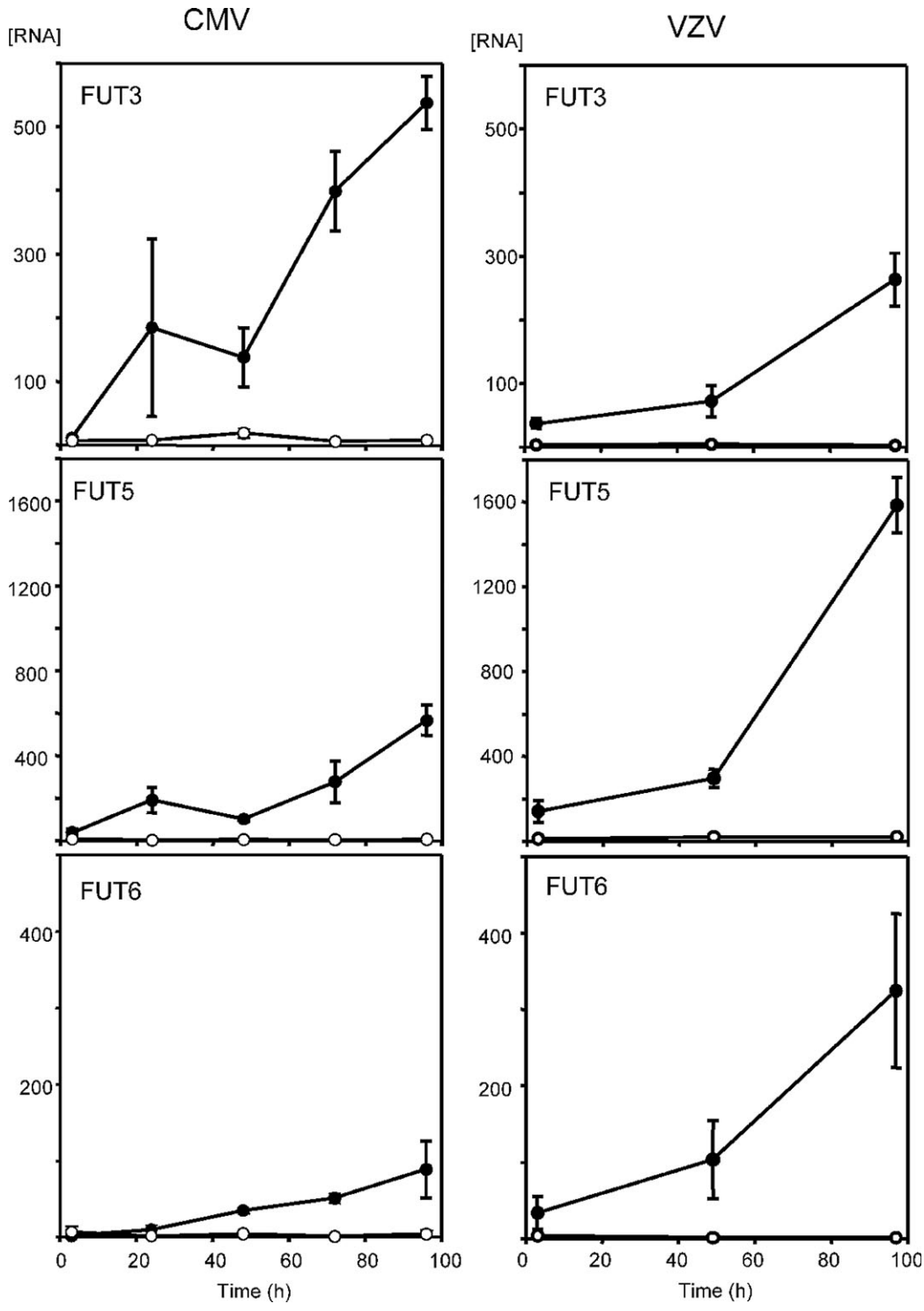

Fig. 3. RNA expression of *FUT* genes induced by both CMV and VZV. Total RNA from VZV- or CMV-infected cells (–•–) and mock-infected cells (–○–) was analyzed using real-time PCR. *FUT* gene assignations are indicated in the figure. The RNA concentrations indicate units are comparable between all graphs in Figures 3, 4, 5, and 7.

intranuclear stain with a CMV-specific antibody, whereas the VZV antibody resulted in a cytoplasmic granular staining (Figure 6). Uninfected cells, used for control purposes, expressed little if any detectable fluorescence with any of the antibodies used (K. Nyström unpublished data). In contrast, 30–40% of the CMV-infected cells demonstrated a prominent cytoplasmic and surface staining for Le^y. In addition, 5% of the cells were positive for sLe^x, preferentially in the cytoplasm, although the intensity of the fluorescence was less pronounced than that observed for the Le^y antibody. In contrast, the VZV-infected cells demonstrated no Le^y signal, but a clear, mainly Golgi-like sLe^x fluorescence, considerably

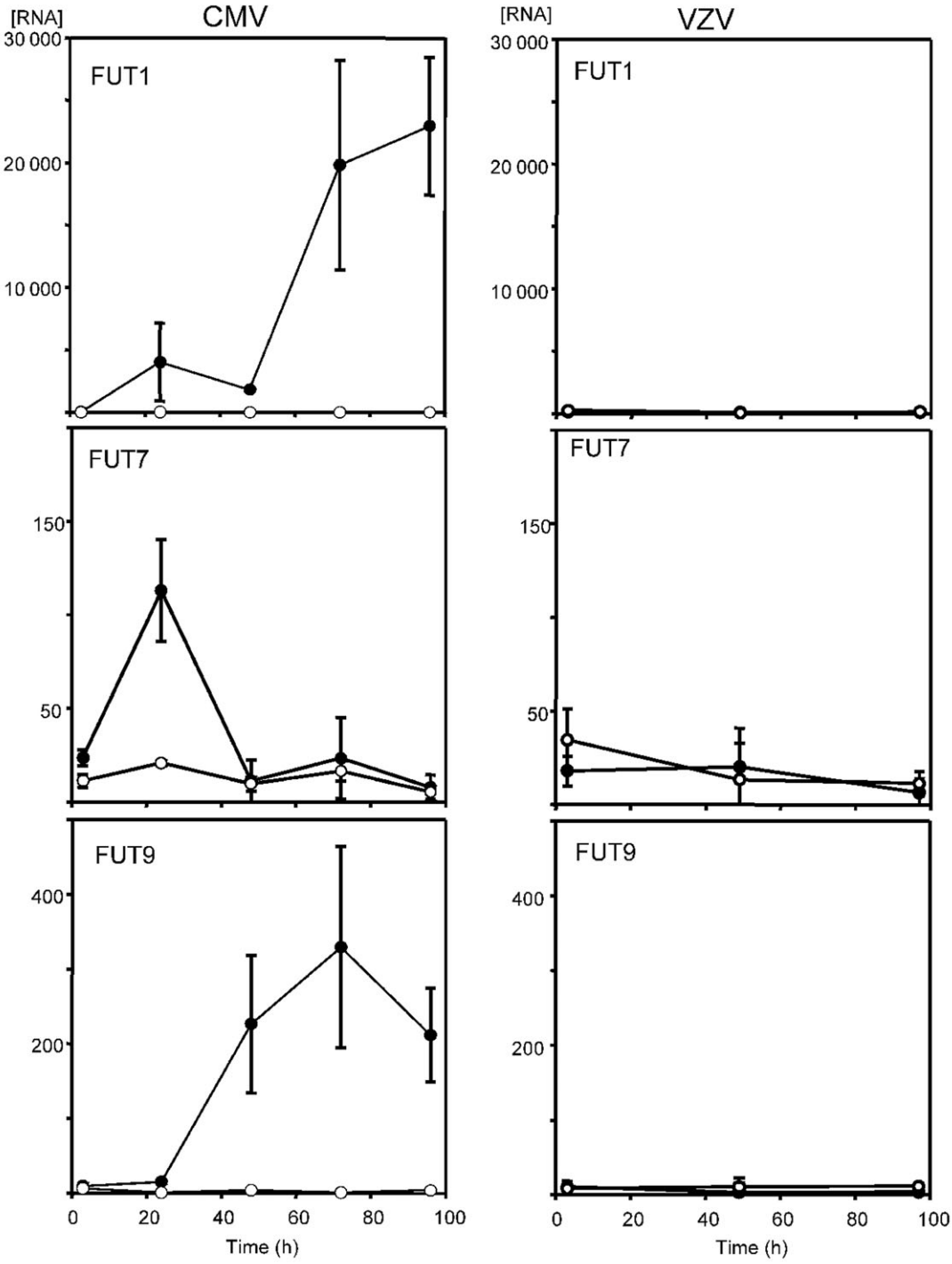

Fig. 4. RNA expression of *FUT* genes induced by CMV. Total RNA from CMV- and VZV-infected cells (–•–) and mock-infected cells (–○–) was analyzed using real-time PCR. *FUT* gene assignations are indicated in the figure.

more intense than in the CMV-infected cells. A weak but yet consistent surface-associated sLe^x surface staining, that was dimmed by the VZV-specific Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)-fluorescence in the double-fluorescence setting, became readily visible in Fluorescein Iso-Thiocyanate (FITC) single fluorescence (Figure 6, inset in the right middle panel). About 50% of the VZV-infected cells were positive for sLe^x. In some experiments, a weak Le^x fluorescence was detected in the CMV- or VZV-infected cells, but this phenomenon was occasional and not reproducible. No fluorescence was detected with antibodies towards Le^a, Le^b, or sLe^a (K. Nyström, unpublished data).

To further explore the role of sialyltransferase gene expression, we analyzed the levels of α(2,3)-sialyltransferase

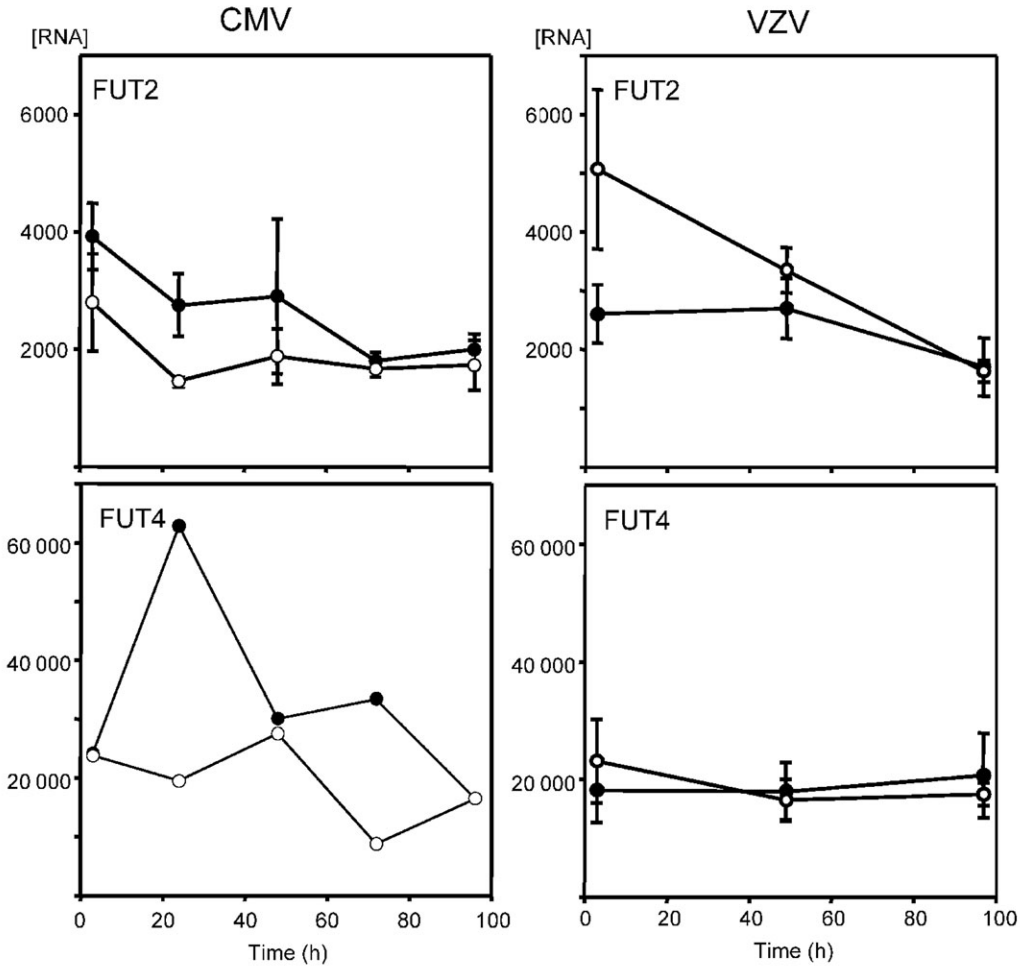

Fig. 5. RNA levels of constitutively expressed *FUT* genes. Total RNA from CMV- and VZV-infected cells (–•–) and mock-infected cells (–○–) was analyzed using real-time PCR. *FUT* gene assignations are indicated in the figure. The *FUT4* assay did not distinguish between RNA encoding the two catalytically active products of this gene (Taniguchi et al. 2000).

III (ST3Gal-III) and α(2,3)sialyltransferase IV (ST3Gal-IV) transcription in VZV- and CMV-infected cells (Figure 7). VZV- as well as CMV-infected cells expressed a high constitutive transcription of these human sialyltransferase genes with levels of 2000 arbitrary units for ST3Gal-III and 5000 arbitrary units for ST3Gal-IV using an identical arbitrary unit as in Figures 3, 4, and 5. This is in line with previous results regarding the sialylation capacity of the HEL cells (Lundström et al. 1987).

The relationship among the *FUT* genes studied and the possible carbohydrate structures generated by the gene products are depicted in Figure 8. The presence of Le^y in CMV-infected cells and absence in VZV-infected cells is well in line with the absence of *FUT1* transcription in the infected cells, as formation of H Type 2 is a rate limiting step in Le^y synthesis. The observation that sLe^x is detectable in VZV- as well as CMV-infected in the absence of Le^x expression is most likely explained by a high ratio between sialylated Type 2 structure and Type 2 structure, which is in accordance with the data above and previous reports regarding expression of highly sialylated glycoproteins in HEL cells (Lundström et al. 1987). The absence of detectable sLe^x in spite of high levels of *FUT4* expression in mock-infected cells may be explained by a reported restricted capacity of Fuc-T IV to fucosylated sialylated Type 2 structure (Satoh et al. 2005). The absence of Le^a and sLe^a is most likely reflected low levels of Type 1 structures in the virus- and mock-infected HEL cells.

Discussion

Here, we report that infection of diploid human fibroblasts with VZV and CMV resulted in expression of novel carbohydrate epitopes in a manner that is specific for each of the viruses. Moreover, we showed that this was correlated with virus-induced changes in the transcription rates of fucosyltransferases engaged in the synthesis of histo-blood group antigens (Figure 8). The particular carbohydrate antigens induced sLe^x and Le^y are involved in processes by which tumor cells spread and colonize distal tissues. One intriguing question is whether this principle is also used by viruses for spread of infected cells.

Regarding the molecular background to the differences in carbohydrate antigen expression between Le^y expressing CMV-infected cells and sLe^x expressing VZV-infected cells, the most prominent factor is the dramatic induction of *FUT1* in CMV-infected cells compared with no induction in

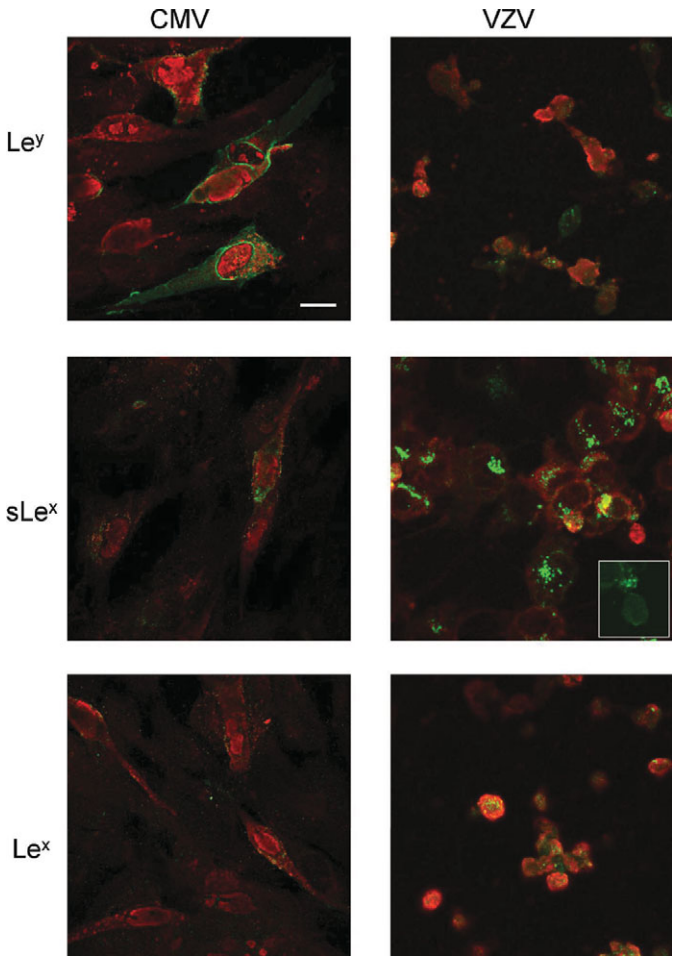

Fig. 6. Immunofluorescence images from CMV- and VZV-infected cells. Monoclonal human antibodies directed toward CMV or VZV and monoclonal mouse IgM antibodies against Le^y, Le^x, and sLe^x were allowed to bind to the cells. TRITC-conjugated (red) secondary antibodies were used for the detection of viral antigens and FITC-conjugated (green) antibodies were used for the detection of the glycoepitopes. Insert in right middle panel shows the single FITC fluorescence (double enlargement). The white bar represents 20 μm.

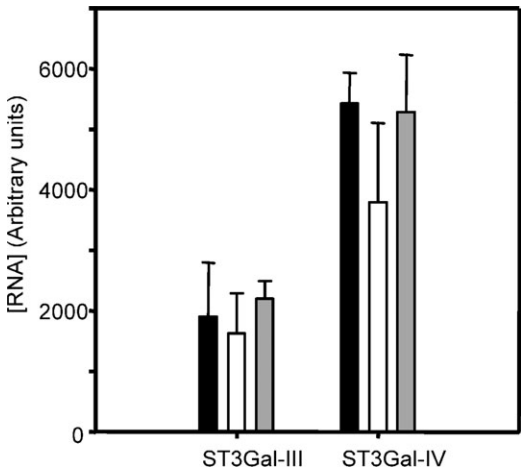

Fig. 7. RNA expression of sialyltransferase genes ST3Gal-III and ST3Gal-IV. Total RNA from CMV-infected cells (black bar), VZV-infected cells (white bar), and mock-infected cells (gray bar) was analyzed using real-rime PCR.

VZV-infected cells. A fucosyltransferase I(Fuc-T I) enzyme driven switch from sLe^x to Le^y has been analyzed in other experimental systems (Mathieu et al. 2004). Thus, transgenic expression of *FUT1* in a number of cell types initiates an efficient formation of the H Type 2 structure that efficiently competes with the synthesis of the sialylated Type 2 structure, i.e. the precursor to sLe^x (see Figure 8). Accordingly, in CMV-infected cells, the possibilities for Fuc-T III, IV, V, VII, and IX to form Le^x or sLe^x must be restricted at high Fuc-T I activities. On the other hand, in VZV-infected cells, the absence of Fuc-T I is favorable for sLe^x synthesis, but not for Le^x synthesis, owing to scarcity of nonsialylated structures in HEL cells (Lundström et al. 1987). The potential of the *FUT1* gene product to intercept the sLe^x synthesis is further underscored by the fact that this enzyme is localized to the medial Golgi region in contrast to its competitor for the Type 2 structure, the trans-Golgi-localized α2,3-sialyltransferase (Zerfaoui et al. 2002). Our results regarding CMV-infected cells is somewhat at variance with those published by Cebulla et al. (2000), reporting induction of sLe^x and Le^x, but not Le^y, in human endothelial cells. The reasons for this discrepancy could be that other cells were used in that study, no assays for Le^y were reported, and the cells were examined only after long-term culture.

One interesting feature is that only 30–40% of the CMV-infected cells expressed Le^y and about 50% of the VZV-infected cells expressed sLe^x. These seemingly low figures probably reflected that the cells in the object glass cultures for immunofluorescence were infected at low multiplicity of infection during longer periods of time to allow cell-to-cell spread of virus to ensure the existence of cells at different stages of infection. Carbohydrate signaling is usually transient in nature (Tsuboi and Fukuda 2001) and this is probably also true for the present sLe^x and Le^y appearance in the virus-infected cells. The *FUT* transcription data depicted in Figures 3–5 represent the sum of *FUT* transcription in all the cells, and it is reasonable that some of the cells are more active in *FUT* transcription and others are less active or even inactive, resulting in the heterogeneous Le^y and sLe^x expression pattern observed in immunofluorescence. As expected we found surface-associated sLe^x in the VZV-infected cells, but much of the sLe^x fluorescence was associated with Golgi-like structures in VZV-infected cells. This could reflect the fact that VZV glycoproteins generally remain Golgi-associated for a relatively long time resulting in a comparatively low surface exposition, compared with its Golgi appearance (Wang et al. 1998).

One key function of sLe^x is to act as a receptor for primarily E- and P-selectins during leukocyte trafficking and homing via the high-endothelial venules (Kannagi 2002; Lowe 2002; Schottelius et al. 2003). In this context, it is tempting to compare the early appearance of sLe^x in VZV-infected cells with the parallel phenomenon in adult T leukemia cells, where sLe^x is strongly upregulated due to transcriptional induction of the human *FUT7* gene by the human T-cell leukemia virus type 1 (HTLV-1) Tax protein (Hiraiwa et al. 2003). The degree of skin infiltration of leukemia in patients is directly proportional to the fraction of cells displaying HTLV-1-induced sLe^x (Kannagi and Hakomori 2001). It is now established that VZV viremia in primary virus infection

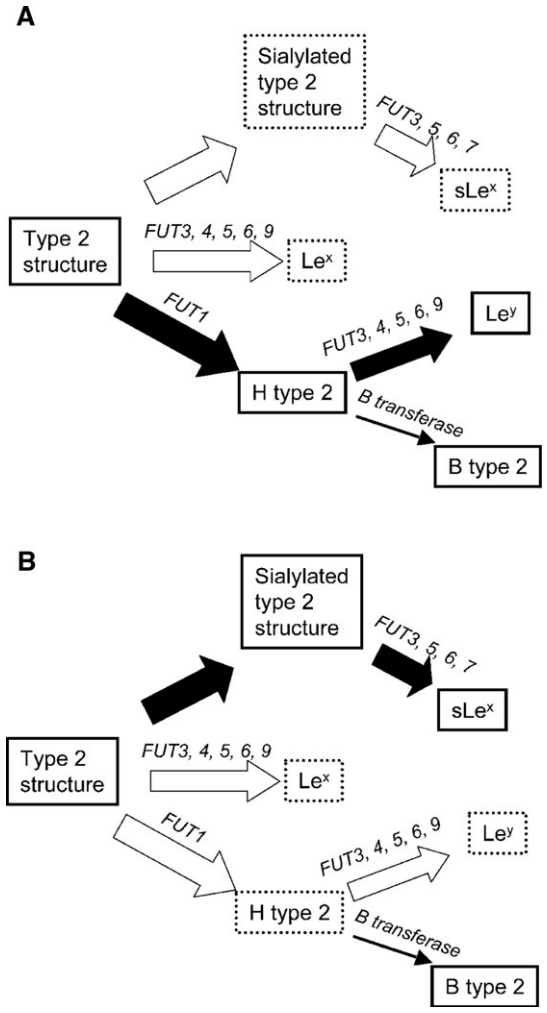

Fig. 8. Hypothetical major pathways for synthesis of fucose-containing carbohydrates in CMV-infected cells (**A**) and VZV-infected cells (**B**). For structural representations of the different glycoepitopes see Figure 1. Rectangles with solid lines indicate structures that either detected or are likely to be detectable according to the experimental data of the present study. Dotted rectangles represent antigens not detected or not likely detectable in the present study. Solid arrows represent pathways likely to take place in CMV- or VZV-infected cells. Open arrows indicate pathways not likely to be utilized in the virus-infected cells. The B transferase is indicated by a thin arrow.

involves T memory cells, infected by VZV in the tonsils for subsequent cell-bound transport to the skin [Reviewed in Ku et al. (2005)]. It is easy to imagine that the efficiency of such a viremic colonization should be further potentiated, given that the virus transiently could induce sLe^x as a tag for addressing the appropriate target skin cells. One VZV advantage in expressing sLe^x on the T memory cells used in viremia is that sLe^x does not only permit specific adhesion to the target tissue, but also offers the intricate selectin-mediated mechanism for endothelial passage and tissue extravasation. It should, however, be born in mind that the present study was performed in HEL cells and further proofs for a possible VZV use of sLe^x in viremia must await studies with VZV-infected T memory cells.

So far, very little is known about the biological functions of Le^y, in spite of the fact that it is expressed very selectively in biologically active cells. Thus, Le^y is expressed during short intervals in embryogenesis, in activated granulocytes, and in several types of neoplasia (Dettke et al. 2000; Kannagi 2002), and possible functions suggested include engagement in the apoptosis process, in activated granulocyte behavior and not least specific cellular adhesion (Hiraiwa et al. 2003; Azuma et al. 2004). Thus, polymorphonuclear leukocytes play a central role in CMV dissemination and it is therefore possible that the Le^y induction is engaged in specific adhesion of infected cells in analogy with previous observations for Le^y-expressing tumor cells (Kannagi and Hakomori 2001; Kim et al. 2005).

Interference with sLe^x and Le^y expression in tumor cells including oligosaccharide mimics and humanized anti-carbohydrate antibodies have been considered for anti-tumor therapy, and a few of these antibodies have been approved by Food and Drug Administration for use in humans [Reviewed by Saleh et al. (2000), Ramsland et al. (2004), and Brodzik et al. (2006)]. Therefore, should viral expression of sLe^x and Le^y or related epitopes prove to be of biological relevance for development of viral disease, as described for the HTLV-1 induced leukemia, it seems reasonable to assume that the therapeutical modalities, described above, could be of interest also for treatment of e.g. CMV or VZV infections in immunocompromised patients.

Materials and methods

Cells and viruses

HEL cells (Lundström et al. 1987) that were kept at a low passage level were used throughout the study. The fibroblasts were cultivated in Eagle's MEM with Penicillin, Streptomycin, and 1% L-glutamine. 10% fetal calf serum (FCS) was used during cell growth. The CMV laboratory strain Towne (ATCC-977) was used throughout the study. The virus titers for Towne were determined by plaque titration on HEL cells (Lundström et al. 1987) as previously described (Chiba et al. 1972). A patient VZV isolate C822 that was typed by PCR (Bergstrom 1996) was used for the VZV studies.

Viral infection of cells

One plaque forming unit per cell of CMV strain Towne was added to HEL cells in six-well culture plates (700 000 cells/well) and one well was used for each infectious sample or mock-infection. Cell-associated VZV (180 000 particles/cell; see *Real-time PCR*), where dimethylsulfoxide was removed prior to infection, was added to HEL cells at 500 μL/well. The viruses were allowed to attach to the cells for 3 h at 37 °C and 5% CO_2 before the inoculum was removed. The cells were washed with phosphate-buffered saline (PBS) and fresh Eagle MEM with penicillin, streptomycin, and 1% L-glutamine were added. Virus- and mock-infected cells were harvested by removing the medium from the well and washing the cells in PBS. The cells were lysed in 2 × Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA).

Immunofluorescence

Mock-, CMV-, or VZV-infected HEL cells were trypsinated and resuspended in Eagle's MEM, penicillin, streptomycin, 1% L-glutamine, and 10% FCS. Mock-infected and CMV- or VZV-infected cells (trypsinated at 48 h p.i.) were mixed and

added to teflon-coated object slides and allowed to further infect for at least 2 days to ensure infected cells at different stages. The slides were fixated in cold acetone and stored at −80 °C.

Immunofluorescence was performed on the slides by incubating the cells with human sera containing antibodies directed against CMV or VZV diluted 1:400 and 1:200, respectively. Le^x, Le^y, and sLe^x were detected using immunoglobulin (Ig)M antibodies P12, (abcam, Cambridge, UK), F3 (abcam, Cambridge, UK), and KM93 (Chemicon International, Temecula, CA), respectively. Possible cross-reactions between sLe^a and sLe^x were ruled out by the use of MCA2031 (Serotec, Oxford, UK). Le^a and Le^b were detected using IgG1 antibodies 7LE and 2-25LE (abcam, Cambridge, UK). The primary antibodies were incubated for 1 h at 37 °C. TRITC-conjugated anti-human antibody (Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated anti-mouse antibody (abcam) were used at dilutions of 1:200 and 1:50 and incubated on the cells for 45 min at 37 °C. The slides were washed thoroughly in PBS between each of the antibody incubations. Prolong Antifade Kit (Molecular Probes, Eugene, OR) was used as a mounting media on the slides. The slides were analyzed in a Zeiss LSM 510 Meta confocal microscope using a Plan-Apochromat 36x objective in oil immersion, and the possibility of cross-reactivity between TRITC and FITC was checked for in each well.

RNA and DNA extraction

The 6100 Prep Station (Applied Biosystems) was used for the isolation of RNA (Isolation of Total RNA from Cultured Cells, Applied Biosystems) and DNA (BloodPrep Chemistry, Applied Biosystems) from cells and supernatant according to the manufacturer's instructions. Briefly, 400 μL of lysed virus-infected cells or mock-infected cells were added to 96-well RNA or DNA isolations trays used as described by the manufacturer. Purified total RNA and DNA were eluded in a final volume of 100 μL. The concentration and purity of the RNA was checked using spectrophotometry, measuring the samples at 260 and 280 nm. The 260/280 nm ratio was 2.0 ± 0.2.

Real-time PCR

Real-time PCR was performed as previously described (Nystrom et al. 2004), and the primers and probes are listed in Table II. Briefly, RNA analysis was carried out using TaqMan One-Step RT-PCR Mastermix (Applied Biosystems) and 40 ng of total RNA. A volume of 2 μL of DNA extraction was added to the real-time PCR to control the infection. The reverse transcription reaction was performed at 48 °C for 30 min and the following PCR according to the manufacturer's instructions, for 40 cycles (95 °C 15 s, 60 °C 60 s) using 2 × AmpliTaq Gold DNA Polymerase mix and 40 × RT enzyme mix, forward and reverse primers (0.5 μM and probe (0.3 μM). The DNA real-time PCR was performed in the same manner, with omission of the reverse transcription step and the reverse transcription enzyme mix. All real-time PCR reactions were performed using 30 μL reaction volumes and carried out in 96-well plates, which were centrifuged for 1 min at 1000*g* in a swing-out rotor (Rotina 48R; Hettich, Tuttlingen, Germany) before the PCR. The amplification and detection was carried out with an ABI prism 7000 sequence detection system (Applied Biosystems). All RNA samples were subject to a β-actin PCR without added reverse transcriptase to check for DNA contamination. Samples where DNA was detected were DNase treated with Turbo DNAfree (Ambion, Austin, TX) according to the manufacturer's instructions.

The C_T values were normalized to 18S, which is a housekeeping gene previously shown to be constantly expressed in herpesvirus-infected cells (Nystrom et al. 2004). Linearized results were calculated using the ΔC_T method [(Livak and Schmittgen 2001); Applied Biosystem's User Bulletin #2: Relative Quantitation of Gene Expression 2001] relative 18S expression as follows. The linearized ΔC_T expression of a sample (Arbitrary unit, A) was obtained from the formula: $A = 2^{(-\Delta C_{T\text{sample}} + \Delta C_{T\text{reference}})}$, where the reference was the one sample giving the highest *FUT*ΔC_T value selected among all the *FUT* assays depicted in Figures 3, 4, and 5. This enables direct comparison of the arbitrary unit values given in these three figures. As the C_T values obtained rather than the linearized values are normally distributed, it is not possible to use the standard deviations directly in the graphs. In order to transform the standard deviation values, derived from C_T measurements, to surrogate error estimates applicable in graphs presenting the linearized results, the following formula was used for each value: $S = 2^{(-\Delta C_{T\ \text{sample}} + \Delta C_{T\ \text{reference}} + \text{std 18S} + \text{std sample}) - A}$, where S represents the length of the error bar, standard deviation values (std) obtained for 18S PCR and the sample (*FUT* PCR).

Genotyping

PCR-SSP genotyping of ABO, FUT2, FUT3, and FUT6 mutations. The PCR-SSP assay conditions and the primers for the *ABO* mutations, the *FUT2* 428G > A, 385A > T, and 571C > T, *FUT3* 59T > G, 202T > C, 314C > T, 508G > A, 1067T>A mutations have been described previously (Procter et al. 1997; Grahn et al. 2001). The *FUT6* PCR-SSP assay was optimized using DNA samples from individuals with known *FUT6* genotypes (Larson et al. 1996; Elmgren et al. 2000) and was designed to use the same PCR-program as described in *Real-time PCR*. The primers (A. Grahn, unpublished data) were selected to identify the 370C > T, 730C > G, 738C > T, 739G > A, 907C > G, 945C > A, and 977G>A mutations and to give products of 819, 462, 455, 453, 282, 245, and 212 bp not interfering in size with each other or with the *human growth factor* or *developmentally regulated RNA-binding protein* internal controls of 428 and 796 bp, respectively. All products were analyzed on UltraPURE 1% agarose gel (Gibco BRL, Paisley, Scotland) in 0.5 mg/mL ethidium bromide at 125 V and visualized under UV-light.

PCR-RFLP genotyping of FUT7 329G>A mutation. The genotyping for the *FUT7* 329G>A mutation was performed as described in Bengtson et al. (2001) using the primer pair VII-3s/VII-4as to generate a 1404 bp product further cleaved by *Not*I into 791 and 613 bp products.

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Conflict of interest statement

None declared.

Abbreviations

CMV, human cytomegalovirus; CPE, cytopathic effects; FCS, fetal calf serum; FITC, Fluorescein Iso-Thiocyanate; Fuc, fucose; *FUT*, fucosyltransferase gene; Fuc-T, fucosyltransferase enzyme; HEL, human embryonic diploid fibroblats; HTLV, human T-cell leukemia virus type 1; Le^x, Lewis X; Le^y, Lewis Y; p.i., postinfection; PBS, phosphate-buffered saline; PCR-RFLP, PCR-restriction fragment length polymorphism; PCR-SSP, PCR-sequence specific primers; PFU, plaque forming unit; ST3Gal III, α(2, 3)-sialyltransferase III; ST3Gal IV, α(2, 3)-sialyltransferase IV; TRITC, Tetramethyl Rhodamine Iso-Thiocyanate; VZV, varicella-zoster virus.

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IV

Herpes simplex virus type 1 ICP0-activated transcription of host fucosyltransferase genes resulting in neo-expression of sialyl-Le^x in virus-infected cells

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Abstract

We previously showed that herpesviruses varicella-zoster virus (VZV) and cytomegalovirus (CMV) infection of diploid human fibroblasts (HEL) resulted in neo-expression of Lewis antigens sialyl Lewis x (sLe^x) and Lewis y (Le^y), respectively, by transcriptional activation of different combinations of normal dormant human fucosyltransferase genes (FUT1, FUT3, FUT5, and FUT6), whose gene products are responsible for synthesis of Le antigens. Here, we showed that herpes simplex virus type 1 (HSV-1) induced a similar sLe^x expression as VZV caused by a similar induction pattern of FUT3, FUT5 and FUT6 transcription in HSV-1 infected cells. HSV-1 induction of FUT5 was used as a model system for analyzing viral activation of dormant fucosyltransferase genes. This was an extremely rapid process, giving rise to elevated FUT5 RNA levels at one hour post infection. Using a set of conditional and non-conditional HSV-1 mutants, defective in expression of selected viral factors we demonstrated that expression of ICP0, an immediate early viral factor, was responsible for the HSV-1 effects on FUT5 transcription. In addition, our results suggested that ICP0 RNA rather than the translated gene product was directly responsible for FUT5 activation, although the known ubiquitinylating activity of the ICP0 protein also seemed to effect transcription of FUT5, after the direct activation event of this gene.

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Introduction

Several viruses including human immunodeficiency virus type 1 (HIV-1), human T cell lymphotropic virus type 1 (HTLV-1), human cytomegalovirus (CMV) and varicella-zoster virus (VZV) induce formation of carbohydrate neoantigens, that are exposed at the infected cell surface and sometimes also at the viral envelope (1, 4, 15, 16, 25, 26, Hansen, 1990 #37). Most of these virus-induced carbohydrate antigens belong to the Lewis families of fucose-containing histo-blood group antigens. Thus, HIV-1 infection may result in neo-expression of

Le^y (for structural representations, see Fig 1) (1), CMV induces expression of sialyl-Le^x (sLe^x) and Le^y; (4, 26), and VZV induces expression of sLe^x (26). The HTLV-1 induced sLe^x participates in lymphoma pathophysiology by catalyzing homing of virus-transformed cells to their skin localization (17). Besides this, little is known so far of the specific functions of sLe^x and Le^y during the viral colonization of its host, though homing of virus-infected cells to the skin has been shown to play a central role in VZV pathogenesis (18).

The important factor behind the CMV- and VZV-induced increase in sLe^x appears to be a prominent induction of transcription of at least three fucosyltransferase genes that are normally restrictedly expressed in many cell types, i.e. FUT3, FUT5 and FUT6 (26), where FUT3 and FUT5 encodes α1,3/4-fucosyltransferases and FUT6 encodes an α1,3-fucosyltransferase, all of which are involved in the last step in synthesis of sLe^x (Characterized in (9)). Le^y is expressed in CMV-infected cells because this virus in addition to the VZV-induced FUT genes mentioned above also induces transcription of FUT1 (26), encoding an α1,2-fucosyltransferase whose catalytic activity is necessary for formation of Le^y, but is incompatible with formation of sLe^x (7). Hence, sLe^x is only expressed in a sub-fraction of CMV-infected cells, where the Fuc-TI levels are too low to permit a complete switch to Le^y synthesis, resulting in a larger proportion of the infected cells expressing Le^y and a smaller fraction expressing sLe^x.

In the present paper we have focused on the consequences of HSV-1 induced changes in the transcriptional rates of fucosyltransferase genes mainly for three reasons: (i) Expression of fucosylated carbohydrates such as Le^x are necessary for the functional status of CNS regions (24), regions of significance for which are targets in HSV-1 pathogenesis (28)**.** (ii) Owing to its short replication cycle compared with CMV and VZV, HSV-1 provides a more straight-forward and well characterized model for exploring the molecular mechanism behind the viral induction of transcription of selected human fucosyltransferase genes. The most important findings here were that (i) HSV-1 induced a similar transcription pattern of fucosyltransferase genes as did another α-herpesvirus, VZV, resulting in sLe^x expression, distinct from that of the β-herpesvirus CMV, and (ii) ICP0 RNA rather than the translated viral gene product was responsible for transcriptional induction of at least the host FUT5 gene, thereby providing an extremely rapid viral mechanism for activation of dormant host genes in the newly infected cell.

Materials and methods

Viruses and cells. The wild type HSV-1 virus Syn17+ and mutant HSV-1 viruses *dl*1403, tsS and tsK have been previously described (3, 6, 21, 31, Davison, 1984 #12, Blumel, 1995 #33). The virus titers were determined by plaque titration on GMK (Green monkey kidney) cells (13) at 37°C for Syn17+ and *dl*1403 whereas temperature sensitive mutants were plaque titrated at 32°C. Human diploid embryonic lung fibroblasts (HEL cells) (20) were used throughout the study. The fibroblasts were cultivated in Eagle's MEM with 1% penicillin-streptomycin and 1% L-glutamine. 2% fetal calf serum was used during cell growth.

Immunofluorescence. HEL cells were trypsinated and resuspended in Eagle's MEM, penicillin, streptomycin, 1% L-glutamine and 10% FCS. The cells were allowed to adhere to teflon-coated object slides for 24h. The cells were infected with HSV-1 for 4, 6, 9 or 24 hours and 200µg/ml Cycloheximide (CHX, Sigma Aldrich, St. Louis, MO) or 50µM MG-132 (Merck Bioscience Ltd., Nottingham, UK) (5) was added to certain wells. Subsequently, the slides were fixated in cold acetone and stored at -80°C. Immunofluorescence was performed on the slides by incubating with human sera containing antibodies directed against HSV-1 diluted 1:600, and/or IgM antibodies against Le^x, Le^y and sLe^x (P12, (abcam, Cambridge, UK), F3 (abcam, Cambridge, UK), and KM93 (Chemicon International, Temecula, CA) respectively) diluted 1:40. The primary antibodies were incubated for overnight at 4ºC. TRITC conjugated anti human antibody (Jackson ImmunoResearch, West Grove, PA) and FITC-conjugated anti mouse antibody (Dako, Glostrup, Denmark) were used at dilutions of 1:200 and 1:100 and incubated 1h at 37°C. All dilutions of antibodies were performed in 3% BSA in Phosphate Buffer Solution (PBS). Prolong Antifade Kit (Molecular Probes, Eugene, OR) was used as a mounting media on the slides. The slides were analyzed in a Zeiss LSM 510 Meta confocal microscope using a Plan-Apochromat 63x objective in oil immersion.

Cell culture experiments. HSV at a multiplicity of infection of 8-10 PFU of virus/cell were added to HEL cells in 6-well culture plates (700,000 cells/well). Virus was allowed to attach to the cells for one hour at 37°C and 5% CO_2 before the inoculum was removed. After different intervals of infection each well was harvested and extracted for RNA or DNA separately. The cells were then washed in PBS and new medium was added. CHX (Sigma Aldrich, St. Louis, MO) was added at 200µg/ml and 50µM MG-132 (5) to certain wells. In experiments involving tsS and tsK mutants, cells were incubated at 32°C, permissive, and 39°C, non-permissive temperatures, respectively. In the experiments with synchronized infections, virus was allowed to attach to the cells at 4°C and the infection process was initiated by transferring the cells to 37°C. UV inactivated virus for certain experiments was created by UV radiation of 2250, 4500 or 9000 J, (λ_{max}=252nm), respectively. Cells were harvested by washing with PBS and lysed with 300 µl 2x Nucleic Acid Purification Lysis Solution (Applied Biosystems, Foster City, CA) and PBS, respectively. An experiment to control the rate of uptake of *dl*1403 into the infected cell was performed as previously described (32). Briefly, cells were infected with *dl*1403 and HSV-1 and allowed to attach to cells at 37ºC. The cells were subsequently washed 5 times with PBS and medium was added and the cells were incubated for 1hour and 5 hours. The cells were put on ice and washed with PBS prior to 1mg/ml proteinase K treatment in the presence of fluoride and 30mg BSA. The cells were harvested and processed as described above.

RNA and DNA extraction. The 6100 Nucleic Acid Prepstation, (Applied Biosystems, Foster City, CA) was used for purification of total RNA and DNA according to manufacturer's instructions including DNase treatment for total RNA extraction.

Real time PCR. Real time PCR was performed as previously described (26) according to manufacturer's instructions regarding Taqman chemistry. Primers and probes for FUT1-7 and 9 were previously described (26) and primers for HSV-1 genes are as follows: ICP0 (located in the nondeleted section of the ICP0 gene of deletion mutant *dl*1403) forward primer TCCGGGTTATGCTAATTGCTTT, ICP0 reverse primer TTCCCGAAGAAACTCA-TTACCATA, ICP0 probe FAMCCCG-CGGGTCGCTCAATGAATAMRA, ICP4 forward primer TGTGGATGAGGAACA-GGAGTTG, ICP4 reverse primer GCTGCCACAGGTGAAACCA, ICP4 probe FAM-TGAGTTGTGTTGTTGGGC-AGGTGTGGT-TAMRA, gB forward primer TTTGTGTACATGTCCCCGTTT-TAC, gB reverse primer AGAAGCCGT-CGACCTGCTT, gB probe FAM-ACACC-GAACACACCAGCTACGCCG-TAMRA (Thermo Electron, Ulm Germany) and were designed according to the guidelines of Applied Biosystems, using Primer Express 2.0 (Applied Biosystems, Foster City, CA). The C_T values were normalized to 18S as described previously (26).

Results

Neo-expression of sLe^x on HSV-1 infected human fibroblasts

We previously showed that HSV-1 infection of HEL cells resulted in a dramatic increase in transcription of the α-1,3/4 fucosyltransferase gene designated FUT5 (25), whose gene product, Fuc-TV, is of relevance for synthesis of sLe^x as well as Le^y (7). First, we investigated whether this change was associated with neoexpression of any or both of these neoantigens. HSV-1 infected and mock-infected HEL cells were examined in immunofluorescence using antibodies to viral antigens and/or specific monoclonal antibodies to sLe^x, Le^x, and Le^y (Fig 1). HSV-1 positive cells demonstrated a predominantly Golgi-like granular staining with the sLe^x antibody, but no signal was detected in uninfected cells demonstrating that HSV-1 infection of HEL cells indeed resulted in neoexpression of a carbohydrate epitope, sLe^x. We were only able to detect little if any expression of Le^x or Le^y in the HEL cells independent of whether they were HSV-1 positive or not. In conclusion, the expressional pattern of Lewis antigens in HSV-1-infected HEL cells therefore resembled that of VZV-infected cells, i.e. neoexpression of sLe^x exclusively, which is in contrast to CMV-infected HEL cells with a predominant Le^y expression (26).

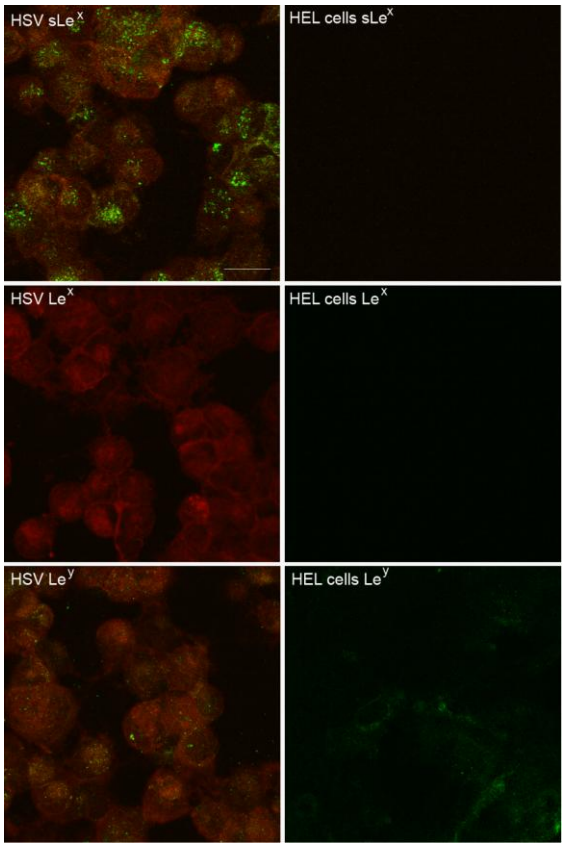
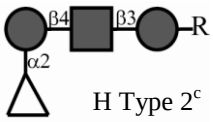
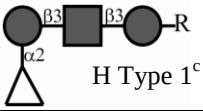
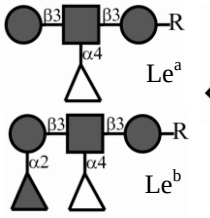
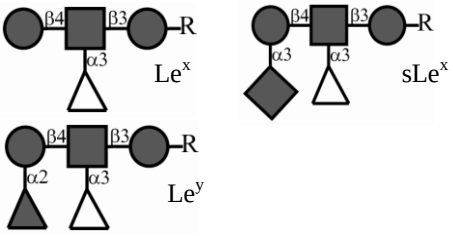
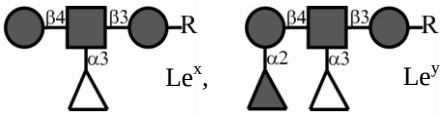
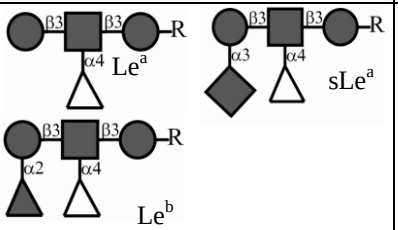
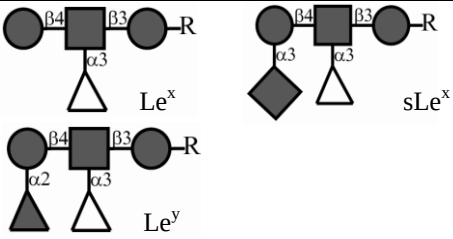
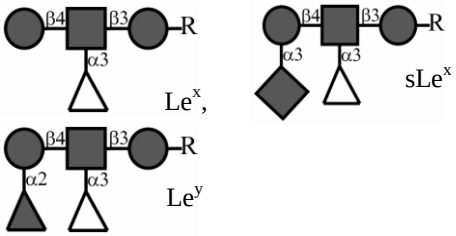
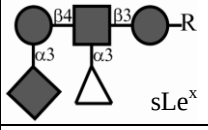
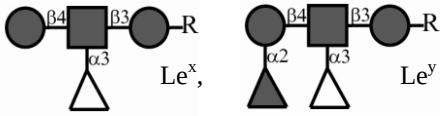


Figure 1. Immunofluorescence images from HSV-1 infected cells. A human IgM-positive serum to HSV-1 and monoclonal mouse IgM antibodies to Le^y, Le^x and sLe^x were allowed to bind to the cells. TRITC-conjugated (red) secondary antibodies were used for detection of HSV-1 positive cells and FITC-conjugated (green) secondary antibodies were used for detection of the glycoepitopes. The white bar represents 20µ.

Influence of HSV-1 on α-3/4 fucosyltransferase genes

Next, we explored possible effects of HSV-1 infection on the transcription of seven other fucosyltransferase genes than the previously investigated FUT5 (25), encoding enzymes known to be involved in the synthesis either sLe^x or related fucosylated carbohydrate antigens. A brief characterization of these enzymes with respect to specificity and tissue expression patterns is given in Table 1. HSV-1 infected and mock-infected cells were harvested at different time intervals post infection. Total RNA was isolated and real time RT-PCR was performed according to previously published methods (25). Relative concentrations of transcripts from different fucosyltransferase genes were determined using the ΔC_T method, as optimized for herpes virus-infected cells (26). The transcription rates of four FUT genes, i.e. FUT3, FUT5, FUT6, and FUT9, were enhanced by HSV-1 infection by at least one order of magnitude, (Fig 2). Except for FUT9, whose transcription was detectable only at 12 h p.i., the maximal transcription rates of these genes were reached already at 7h p.i., with a decrease in the RNA concentrations later in infection (Fig 2). The expression of FUT1, FUT2, FUT4 and FUT7 was only marginally changed after HSV-1 infection compared with expression of FUT3, FUT5, FUT6, and FUT9 (data not shown). These observations were in accordance with the previously published FUT expression pattern induced by VZV, but differing from that of CMV regarding the ability of the latter to induce transcription of FUT1, compatible with Le^y expression (26).

Table 1. Properties of the human fucosyltransferases

Gene (GenBank)	Enzyme abbreviation /Linkage[a]	Synthesized Type 1 Structures[a]	Synthesized Type 2 Structures[a]
FUT1 (H) (M35531)	Fuc-TI Fuc α(1,2)		 H Type 2[c]
FUT2 (Se) (U17894)	Fuc-TII Fuc α(1,2)	 H Type 1[c]	
FUT3 (Le) (X53578)	Fuc-TIII Fuc α(1,3/4)	 Le[a] sLe[a] Le[b]	 Le[x] sLe[x] Le[y]
FUT4 (Le[x]) (M58596)	Fuc-TIV Fuc α(1,3)		 Le[x], Le[y]
FUT5 (M81485)	Fuc-TV Fuc α(1,3/4)	 Le[a] sLe[a] Le[b]	 Le[x] sLe[x] Le[y]
FUT6 (M98825)	Fuc-TVI Fuc α(1,3)		 Le[x], sLe[x] Le[y]
FUT7 (X78031)	Fuc-TVII Fuc α(1,3)		 sLe[x]
FUT9 (AJ238701)	Fuc-TIX Fuc α(1,3)		 Le[x], Le[y]

[a] Based on the reference: (7)

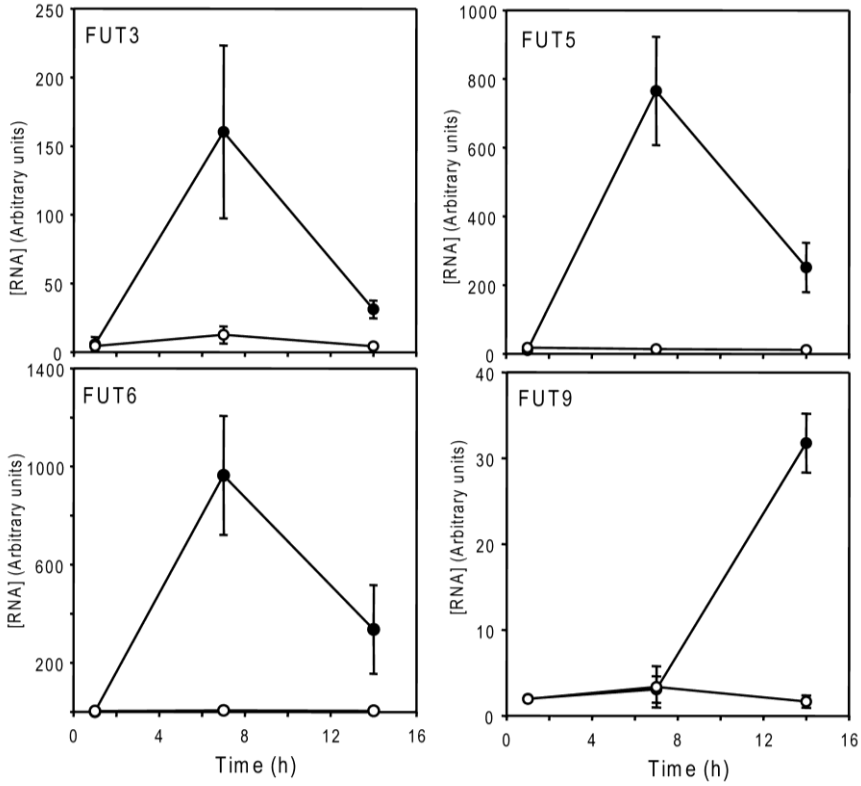


Figure 2. RNA expression of *FUT* genes induced by HSV-1 (●) compared to mock-infected HEL cells (○). The arbitrary unit for RNA concentration is identical for all four panels.

Viral gene transcription essential for FUT5 induction

The next series of experiments were designed to identify the viral factor(s) responsible for virus induced induction of FUT genes. We chose FUT5 as a model gene for these studies because the transcription kinetics of this gene in HSV-1 infected cells was characterized in detail previously (25). As transcriptional induction of FUT5 obviously was an early event, we first determined whether the mere binding of virions to the cells, and/or newly delivered virion proteins (e.g. tegument protein VP16, VHS) alone were sufficient for this process, owing to their significance for early transcriptional activation of viral and cellular genes (reviewed in (33)). Thus, HEL cells were incubated with HSV-1 particles, whose DNA was inactivated with different doses of UV radiation (2) (Fig 3). UV radiation of HSV-1 efficiently destroyed the viral ability to induce FUT5 transcription in the infected cells as recorded at 5 and 8 hours post infection, respectively. The finding that borderline doses of UV radiation (2250 J) suppressed early induction of FUT5 transcription to baseline levels emphasized the necessity of expression of intact HSV-1 genes for FUT5 activation. The slight, but reproducible increase in the late FUT5 transcription at 24h p.i. (second run of viral multiplication) confirmed that the virus suspension indeed was treated with a borderline UV dose, not sufficient for inactivation of all viral genomes in the virus suspension. Altogether these results indicated that the mere presence of virion proteins in the infected cell, including

VP16 or other structural components known to influence the transcriptional activity, was not sufficient to enhance transcription of FUT5 in the absence of a functional genome.

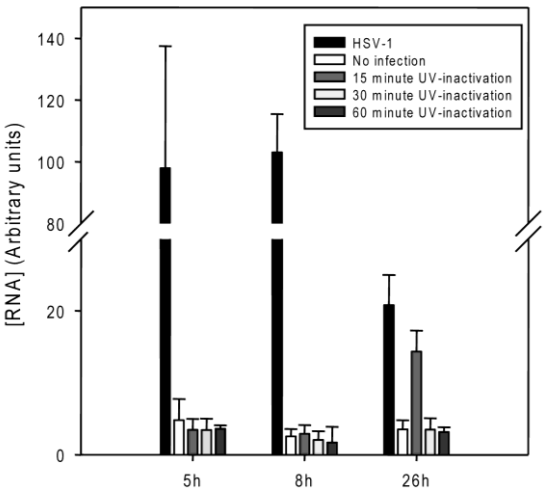


Figure 3. Effect of UV-radiation of HSV-1 on expression of FUT5 in virus-infected cells harvested at the times indicated p.i. Virus was exposed to UV-radiation for the time periods indicated. Total RNA was isolated and FUT5 RNA levels were detected by Real-time PCR.

Immediate early gene ICP0 essential for FUT5 induction

Next, we determined whether HSV-1 induction of FUT5 transcription was controlled by immediate early protein ICP0. This protein is the first to be transcribed during lytic HSV-1 infection and plays a key role in the interactions with the host cell (10, 14). The studies were carried out with a deletion mutant HSV-1 mutant virus *dl*1403 containing deleted segments in both copies of ICP0 (31). HEL cells were infected with 10 PFU/cell of wild type virus and a corresponding amount of virus particles of the ICP0 deletion mutant, respectively. Total RNA was isolated from lysed cells at different time intervals, and the material was assayed using real time RT-PCR for FUT5, the transcribed part of the ICP0 gene and as a further control also the ICP4 gene (Fig 4), the latter encoding another immediate early (IE) protein. No early onset of FUT5 transcription was detected in *dl*1403-infected cells, whereas the wild type virus infection resulted in significantly increased levels of FUT5 RNA as early as 5h p.i. As expected, no detectable amounts of ICP4 or ICP0 RNA were found in the *dl*1403-infected cells. We controlled that these differences between mutant *dl*1403 and wild type virus did not reflect an unspecific defectiveness of *dl*1403 virus particles to perform early virus-cell interactions including attachment and entry, using proteinase K sensitivity of attached extracellular virus (32) (Fig 5). Thus, equal amounts of physical virus particles of *dl*1403 and wild type virus, respectively, were added to HEL cell and similar amounts of proteinase K-resistant intracellular viral DNA were detected at 1h p.i. for both *dl*1403 and wild type virus. The late increase of intracellular wild type but not *dl*1403 DNA at 5h p.i. was expected, owing to inefficient early HSV-1 DNA replication in the absence of functioning ICP0 (10, 29). Altogether these results indicated that induction of FUT5 in HSV-infected cells is dependent on a functional ICP0 gene.

Immediate early gene ICP4 not essential for FUT5 induction

The possible significance of ICP4 in the induction of FUT5 was investigated, seeing as this protein together with ICP0 plays a central role for regulation of early HSV-1 transcription (10, 29). We used two temperature sensitive mutant for these studies: tsK, conditionally defective in ICP4 (6), and tsS, defective in the origin-binding protein, the latter to control for

non-permissive conditions (3) (Fig 6). This control was possible because HSV-1 induced FUT5 transcription is super-induced by inhibition of viral DNA synthesis (25), and a conditional mutant in the *ori*-binding protein should therefore be expected to constitute a super-inductor of FUT5 at non-permissive temperature. In accordance, tsS infected cells expressed five times higher levels of FUT5 RNA than did wild type virus-infected cells at 39°C, confirming non-permissive conditions. In contrast, both tsK and wild type virus infected cells were equally efficient producers of FUT5 RNA at non-permissive temperature, approximately 100 times than did the uninfected cells. At permissive temperature (32°C), no differences in FUT5 RNA levels could be detected between HSV-1, tsS and tsK-infected cells. The somewhat high level of FUT5 RNA in uninfected cells most likely reflected sub-thermal stress. Altogether, no role of ICP4 could be identified in HSV-1 induction of FUT5 transcription.

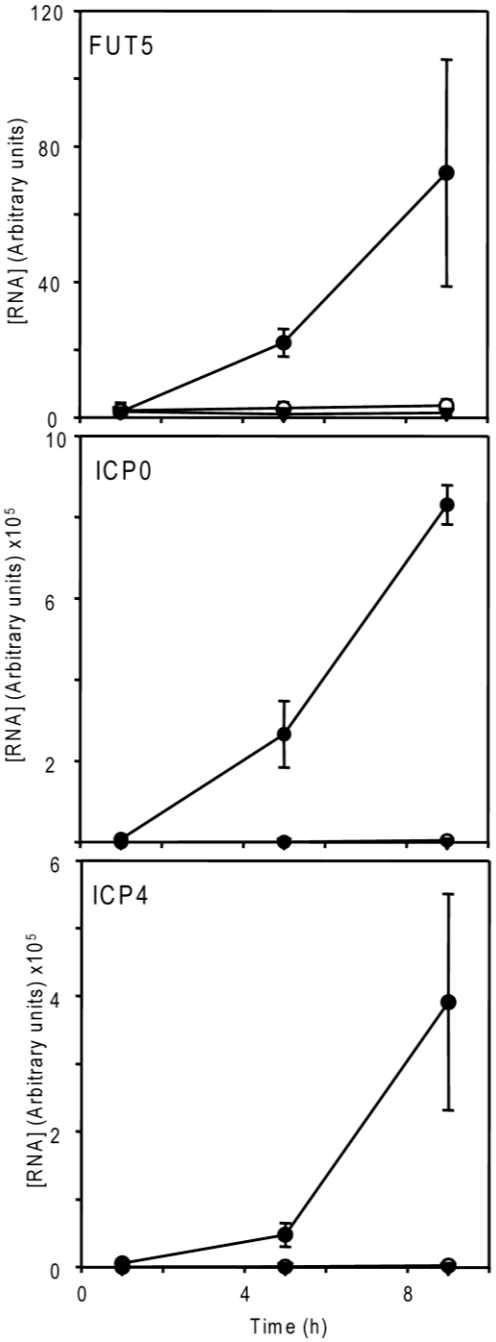


Figure 4. Expression of ICP4, ICP0, and FUT5 in mock-infected cells (▼) and cells infected with *dl*1403 (○) or wild type virus (●). The ICP0 PCR system was designed to detect the non-deleted portion of ICP0 in the *dl*1403 genome.

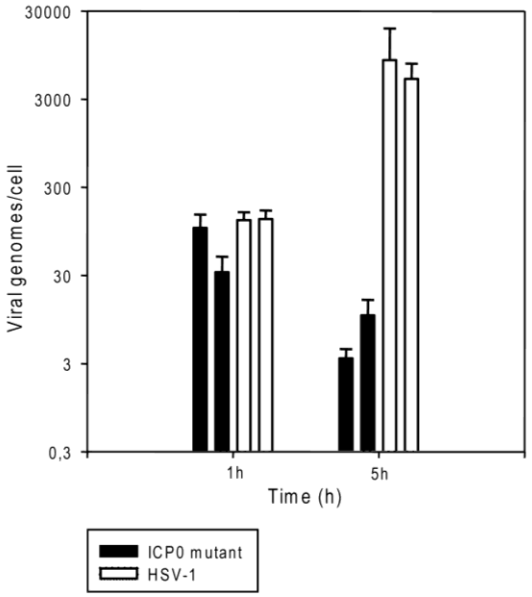


Figure 5. Accumulation of Proteinas K-resistant *dl*1403 and wild type virus in infected HEL cells. Cells were treated with Porteinase K at 1h and 5h p.i., respectively to remove attached but not engulfed virus. Viral DNA copies were assayed using Real-time PCR.

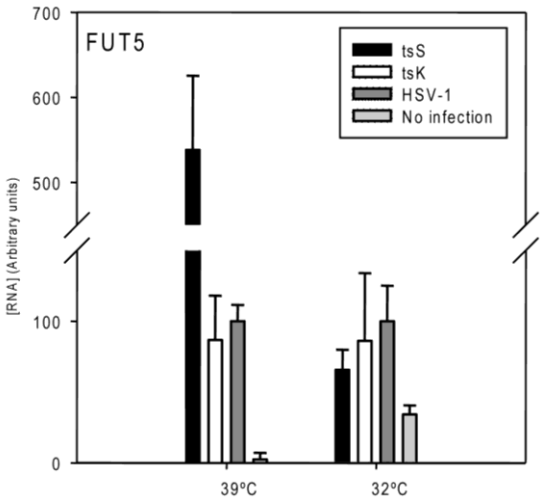


Figure 6. FUT5 RNA expression in HEL cells infected with HSV-1, ori binding protein mutant tsS and ICP4 temperature sensitive mutant tsK. FUT5 RNA was assayed at permissive (32ºC) and non-permissive (39ºC) temperature.

ICP0 gene products and FUT5 induction

Besides regulation of viral gene transcription, the ICP0 gene is involved in modulation of host cell activities by at least two mechanisms: (i) the ICP0 RNA in absence of the translated gene product is the ICP0 protein exerts a ubiquitinylating activity that interferes with the function of ND10 (PML, POD), a nuclear storage compartment for cellular transcription factors (8). The next series of experiments was to determine if any or both of these mechanisms were involved in induction of FUT5 transcription. First we investigated the early transcription kinetics of ICP0 and FUT5 was studied in synchronously infected cells at a high multiplicity to determine whether appearance of FUT5 and ICP0 RNA coincided. Under these conditions no ICP0 RNA was detected and only a background level of FUT5 RNA was detected at 30 minutes p.i. (Fig 7). At 1h substantial amounts of ICP0 RNA was detected accompanied by an about fivefold increase in the amount of FUT5 RNA, indicating a similar early transcription kinetics of both genes. However, newly translated ICP0 protein is detectable only at 2 h at the earliest, even in cells infected at a high multiplicity of infection of HSV-1 (Reviewed in (28)), favoring the notion that ICP0 RNA rather than the translated gene product is responsible for HSV-1 induced transcriptional activation of FUT5

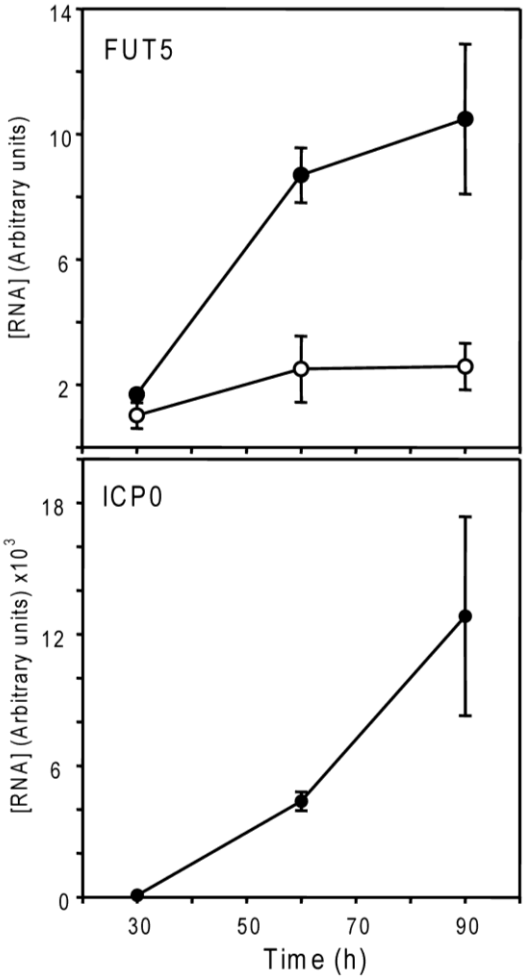


Figure 7. FUT5 RNA expression in synchronized HSV-1 infected (●) and uninfected (○) HEL cells 30, 60 and 90 minutes, respectively, post viral binding.

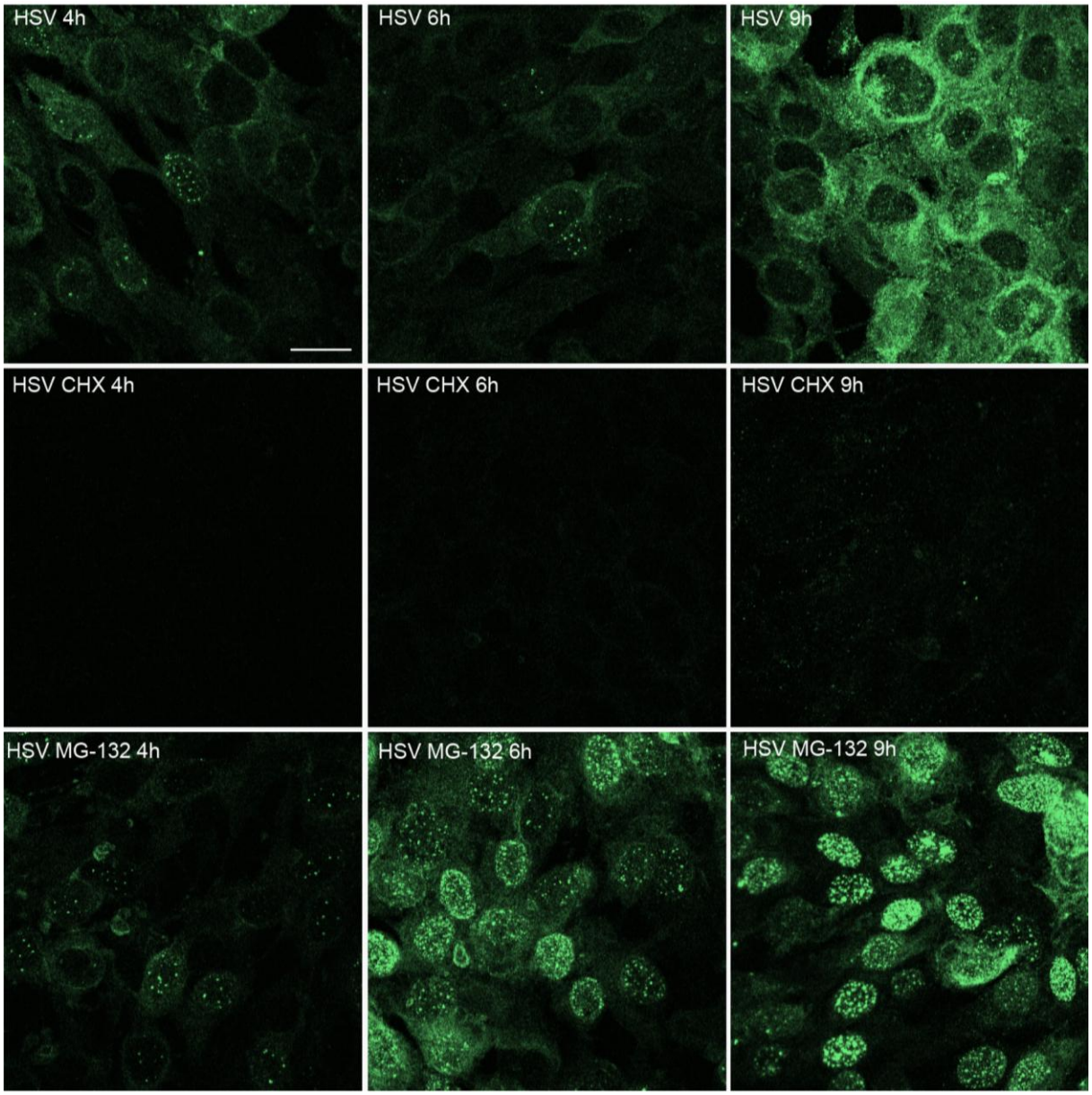


Figure 8. Distribution of ICP0 in HSV-infected and mock-infected HEL cells or infected and uninfected cells treated with CHX and/or MG-132. Cells on Teflon-coated cultures were fixed and processed for immunofluorescence at 4h, 6h, and 9h p.i., respectively. ICP0 was detected by indirect immunofluorescence using a mouse monoclonal antibody to ICP0 and a FITC-conjugated second antibody. The white bar represents 20μm.

Next we intended to confirm the role of ICP0 RNA using CHX for inhibition of viral gene translation. In addition MG-132 was used to inhibit the possible role of ND10 involvement in FUT5 induction. The effect on ICP0 appearance of these two inhibitors was assayed at different time points, using indirect immune-fluorescence with ICP0-specific monoclonal antibodies (Fig 8). In untreated, HSV-1 infected cells the ICP0 staining was associated with nuclear granulae resembling ND-10, known to be involved in early transcription of DNA viruses (22). These structures were observed at 4h and 6h p.i., though at 9h p.i., the ICP0 stain was localized in the cytoplasm, which is in accordance with previous reports (14, 19). In contrast, no

ICP0 staining was found in the HSV-1 infected cells treated with CHX, confirming that translation of the ICP0 RNA indeed was inhibited. We found that MG-132 treatment had dramatic effects on the distribution of ICP0 in HSV-1-infected cells, especially from 6h p.i. and onwards. Thus at 4 h p.i. ICP0 in MG-132 treated cells appeared as nuclear granulae with the same morphology and the same intensity as described for the untreated HSV-1-infected cells. However, whereas the number of fluorescent granulae tended to decrease in the untreated cells we observed a prominent increase of such intranuclear structures in the presence of MG-132 at 6h p.i. This difference was even more obvious at 9 h p.i. where the ICP0 stain still was associated with nuclear granulae in the MG-132 treated cells whereas the ICP0 stain at 9 h p.i. was totally cytoplasmatic. This difference in ICP0 behavior between MG-132 treated and untreated HSV-1-infected cells at 6 and 9 h p.i., respectively, is in line with results reviewed by (14),(19) indicating that MG-132 induced inhibition of the ubiquitinylating activity of ICP0 results in ICP0 accumulation in ND10 like structures and subsequent impairment of ND10 function.

The effect of CHX and MG-132 on FUT5 induction was explored in RNA from HSV-infected cells harvested at 2.5 and 9h p.i., respectively (Fig 9). The main result from the 9h p.i. experiments was that HSV-1 induced a prominent transcription of FUT5 also in the presence of CHX (Fig 9, upper panel). The higher transcription rate of FUT5 in CHX treated, HSV-1-infected cells compared with untreated, HSV-1-infected cells reflected a general higher FUT5 transcription background in the presence of CHX. This is underscored by a corresponding difference in FUT5 transcription between CHX treated and untreated uninfected cells. In contrast, MG-132 abolished the ICP0 induced FUT5 induction at 9h p.i. (Fig 9, upper panel). This was not expected from the results above that ICP0 in the absence of ICP0 protein is responsible for FUT5 induction. However, the results that MG-132 treatment resulted in an abnormal, prolonged accumulation of ICP0 protein in ND10-like structures could reflect malfunctioning ND10 like structures incompatible with normally functioning host cell transcription later than 6h p.i. (See Fig 8). To explore this possibility, we investigated the effect of MG-132 and CHX at 2.5h p.i., i.e. before abnormal accumulation of ICP0 protein in the ND10 structures (Fig 9, lower panel). As expected, we found a prominent induction of FUT5 in the presence of CHX and a modest HSV-1 induction of FUT5 in the absence of MG-132 and CHX. However, MG-132 as well as a combination of CHX and MG-132 resulted in an inhibition of FUT5 transcription. These results demonstrated that presence of ICP0 RNA as well as proteasome function is necessary for early induction of FUT5 transcription in HSV-infected cells.

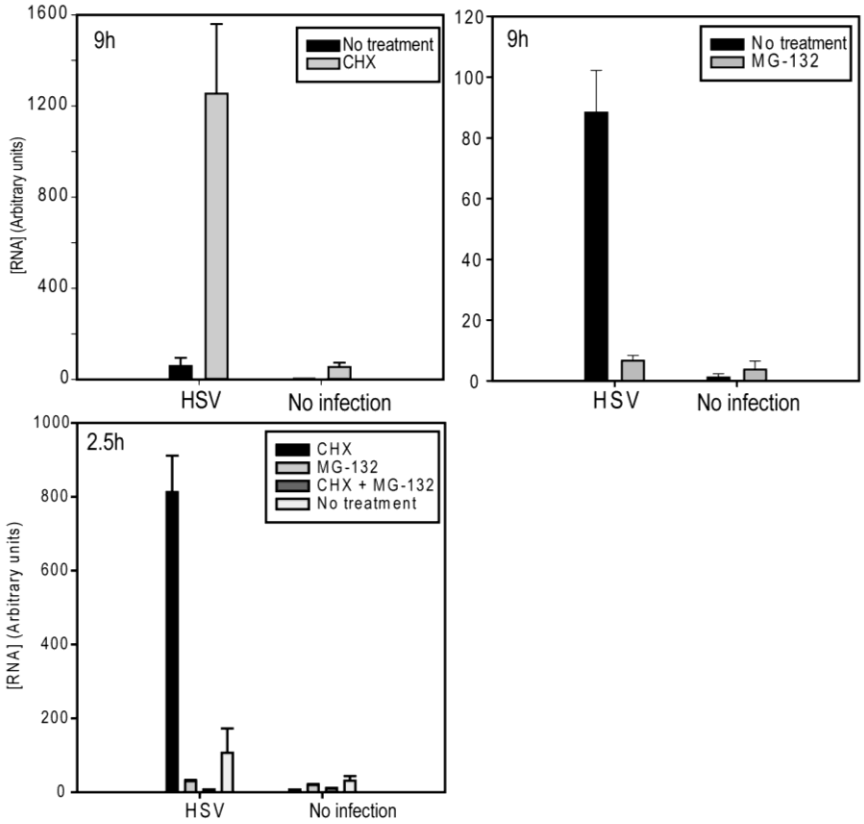


Figure 9. FUT5 RNA expression in HSV-1 infected and mock-infected HEL cells treated with cycloheximide and/or MG-132.

Discussion

The selectin ligand s-Le^x is induced by a few representatives of herpesvirideae and retroviruses including VZV, HSV-1, HTLV-1, and during certain circumstances also CMV. In line with the natural function of sLe^x as a selectin ligand that participates in e.g. leukocyte homing there is some evidence that sLe^x also participates in viral host colonisation at least with regards to HTLV-1 and CMV (4, 17). The mechanism by which HTLV-1 induces sLe^x is known in detail: The viral gene product Tax activates a dormant host gene, FUT7, which encodes a fucosyltransferase of relevance for the critical steps in sLe^x synthesis. We explored the process by which a representative for herpesviruses, HSV-1, induced neoexpression of sLe^x, and here, we show that this represent a viral strategy for controlling host gene expression both with respect to the cellular genes involved and the viral mechanism for activation. Thus, herpesviruses including CMV, VZV (26) and HSV-1 (present data) induce expression of sLe^x by one or more of the fucosyltransferases (Fuc-TIII, Fuc-TV, and Fuc-TVI) encoded by FUT3, FUT5, or FUT6, respectively, whereas HTLV-1 activates solely FUT7, whose gene product Fuc-TVII in fact is an activation marker of several types T cells, being the target for HTLV-1.

Fuc-TVII differs from the fucosyl-transferases encoded by FUT3, FUT5, and FUT6 in the sense that the Fuc-TVII only is able to fucosylate the sialylated

precursor (See Table 1) whereas the other fucosyltransferases mentioned have the capacity to fucosylate a wider variety of precursors. In other words, Fuc-TVII is tailor-made to form sLe^x even if the cell should contain immediate precursors to e.g. Le^x or Le^y. This means that the FUT1 dependent expression of Le^y in CMV-infected cells should not have been possible, should herpesviruses induce a Fuc-TVII in place of Fuc-TIII, Fuc-TV, and Fuc-TVI.

The exploration of the nature viral factors responsible for HSV-1 induced transcription of host fucosyltransferase genes was carried out using FUT5 and a model gene. From these studies it was clear that the most important difference as to viral factors engaged in induction of sLe^x between HTLV-1 and HSV-1 is that the former utilises a classical retroviral transactivator protein, Tax, which induces transcription via a CRE sequence in the human FUT7 promoter. This is in contrast to somewhat surprising mechanism used by HSV-1 for activation of FUT5 demonstrated here, requiring specific viral RNA and thus active also in the absence of viral protein synthesis. The inhibitory effect of proteasome inhibition of FUT5 transcription is probably not an effect of ICP0 function, because it takes place also in the presence of CHX at both time points studied. Thus, the most probable explanation for the inhibition is an MG-132 effect on RNA polymerase II transcription (11, 27). The conclusion that ICP0 RNA is a key actor was based on the finding that FUT5 transcription was induced during cycloheximide treatment of infected cells and by the fact that FUT5 transcription was an immediate early process with a kinetics congruent with ICP0 transcription. It could be argued that ICP0 is an inducer of other early viral factors and that our finding only reflected an indirect role of ICP0 paving the way for synthesis of other viral factors being the real inducers of FUT5. However, the cycloheximide resistance of FUT5 induction indicated that this is not a likely scenario as it implies that ICP0 RNA is able to induce transcription of other viral genes, whose RNA should be able to FUT5 induction in the absence of protein synthesis. The notion that ICP0 RNA rather than the translated gene product is responsible for inducing cellular activities is not new. Thus, ICP0 RNA alone may induce apoptosis in HSV-1-infected cells (30), a process later reversed via expression of several different HSV-1 proteins (Reviewed by (12, 23)). The present study is, however, the first demonstration of a viral RNA, acting as a specific inducer of a dormant host glycosyltransferase gene.

The HSV-1 strategy to use a viral RNA, and in particular an RNA encoded by an IE gene, for the initial step of FUT5 induction offers advantages for the virus compared with the Tax-dependent HTLV-1 induced activation of FUT7. Firstly, this is a prerequisite for a very rapid activation of the host FUT5 gene as the concept of an RNA inducer eliminates delays caused by RNA transport to the cytoplasm, translation, and transport of the transported protein to the nucleus, inevitable and time-consuming processes associated with immediate early viral proteins as inducers of intranuclear activities. Moreover, the RNA concept also permits that at least the first steps of the virus-induced activation of FUT5 takes place without any presentation of viral B and T cell epitopes

in the newly infected cells. This latter aspect may be of relevance for the latent or persistent state of HSV-1 in the infected neuron, where it at least theoretically should be possible for the viral genome to influence the cell surface glycotopes of the latently infected neuron by a short pulse of ICP0 transcription in the absence of any expression of viral proteins. This possibility is intriguing in view of the multitude of surface glycotopes of relevance for the functional status of neural cells (34).

It is important to emphasize that the ICP0-dependent activation of host fucosyltransferase genes, here exemplified by FUT5, represents only one of several steps in the virus-induced regulation of sLe^x expression over time. Thus, inhibition of viral DNA synthesis is associated with hyperexpression of FUT5 RNA, compatible with the notion that viral late proteins also are engaged in regulation of FUT5 transcription (25). It is therefore reasonable assume that virus-induced regulation of sLe^x expression over time involving several points for viral control of which the ICP0 RNA-dependent FUT5 activation represents the first one. At present no specific role for the HSV-1 induced sLe^x can be assigned in analogy with the defined role for skin colonisation of HTLV-1 leukemia cells (17) or for endothelial adhesion of infected cells as suggested for CMV (4). Although its biological relevance still remains enigmatic a possible function of HSV-1 induced expression of sLe^x or similar glycotopes may be found in interactions between HSV-1 and its many target cells, including epithelial cells, leukocytes, and neuronal tissue.

Acknowledgements

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Abbreviations

CHX – cycloheximide
CMV - cytomegalovirus
*dl*1403 – HSV-1 ICP0 deletion mutant
FITC – Fluorescein Iso-Thiocyanate
FUT – fucosyltransferase gene
HEL – human embryonic lung fibroblast
HSV-1 – herpes simplex virus type 1
HTLV-1 - human T cell lymphotropic virus type 1
ICP0 – infected cell protein 0
ICP4 – infected cell protein 4
IE – immediate early
Le^a – Lewis a
Le^b – Lewis b
Le^x – Lewis x
Le^y – Lewis y
p. i. – post infection
sLe^a – sialyl Lewis a
sLe^x – sialyl Lewis x
PBS – Phosphate Buffer Solution
tsK – temperature sensitive mutant in ICP4
tsS – temperature sensitive mutant in ori binding protein
TRITC – Tetramethyl Rhodamine Iso-Thiocyanate
VZV – varicella-zoster virus

References

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