Activation of host antiviral RNA-sensing factors necessary for herpes simplex virus type 1-activated transcription of host cell fucosyltransferase genes FUT3, FUT5, and FUT6 and subsequent expression of sLeα in virus-infected cells

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Herpes simplex virus type 1 (HSV-1) induces expression of a selectin receptor, the carbohydrate epitope sialyl Lewis X (sLeα), at the surface of infected cells. The molecular background to this phenomenon is that a viral immediate early RNA interacts with as yet unidentified host factors, eventually resulting in transcription of three dormant host fucosyltransferase genes (FUT3, FUT5, and FUT6), whose gene products are rate-limiting for synthesis of sLeα. The aim of the present study was to define the immediate targets for the viral RNA in this process. We found that the Protein Kinase R (PKR) inhibitors 2-aminopurine (2-AP) and C16 inhibited FUT3, FUT5, and FUT6 expression as well as HSV-1-induced expression of sLeα, indicating a primary role of PKR as a viral RNA target. The PKR-dependent activation of the FUT genes seemed neither to involve PKR effects on translation nor to involve NF-κB- or JNK-dependent activation. IMD-0354, known as an inhibitor of the NF-κB-activating factor IKK-2, induced FUT transcription via a novel IKK-2-independent mechanism, irrespective of whether the cells were virus-infected or not. Altogether, the results suggested that PKR is the primary target for HSV-1 early RNA during induction of FUT3, FUT5, and FUT6, and that the subsequent steps in the transcriptional activation of these host genes involve a hitherto unknown IMD-0354, yet IKK-2-independent, pathway.

Keywords: dsRNA/ICP0/IMD-0354/PKR/Selectin

Introduction

Retroviruses, including human immunodeficiency virus type 1 (HIV-1) and human T-cell leukaemia virus type 1 (HTLV-1), and human herpesviruses, including herpes simplex virus type 1 (HSV-1), varicella-zoster virus (VZV), and cytomegalovirus (CMV), induce expression of cell surface carbohydrate epitopes that are only restrictedly expressed in most types of uninfected human cells. These structures include selectin receptors such as sialyl Lewis X (sLeα) and related structures exemplified by Lewis Y (Leα) (Adachi et al. 1988; Cebulla et al. 2000; Hiraïwa et al. 2003; Nyström et al. 2007). Many of the different human glycosyltransferases needed for sLeα and Leα synthesis are expressed in most types of cells, with exception for the gene products of a few critical, often quiescent, fucosyltransferase genes, whose activation is a rate-limiting step for sLeα formation (de Vries et al. 2001; Lofling and Holgersson 2009). Fucosyltransferases relevant for sLeα are encoded by the human genes FUT3, FUT5, FUT6, and FUT7, and each of them may add a fucose residue in an α1,3-linkage, whereas synthesis of Leα requires previous addition of an α1,2-linked fucose via the fucosyltransferase encoded by FUT1 (Figure 1, Panel A). All these viruses induce sLeα by activating transcription of one or more of the Lewis antigen-associated FUT genes mentioned above (Hiraïwa et al. 2003; Nyström et al. 2004, 2007, 2009), but in contrast to the other viruses, CMV has an additional capacity to also activate transcription of FUT1 resulting in a unique ability to induce also Leα in the infected cells (Nyström et al. 2007) (Figure 1, Panel A).

As a selectin ligand, sLeα resides at the surface of activated neutrophils from where it may initialize extravasation of the neutrophil to the site of inflammation as a consequence of sLeα binding to P selectin at the endothelial wall in the vicinity of inflammatory foci (Muramatsu 2000; Kannagi 2002; Hiraïwa et al. 2003; Uchimura et al. 2005). The selectin-dependent mechanism is hijacked during metastatic spread of several tumors, often depending on appearance of sLeα at the tumor cell surface and subsequent selectin-dependent extravasation to surrounding tissue (reviewed in Magnani (2004)). The same strategy has been confirmed for HTLV-1, a virus with capacity to activate the cellular sLeα-forming machinery, and the surface appearance of sLeα in virus-transformed lymphoma is clinically correlated to the severity of skin manifestations (Kannagi 2001; Hiraïwa et al. 2003). So far our knowledge of the function of herpesvirus-induced expression of sLeα at the surface of infected cells is scarce, although circumstantial data suggest a role in CMV-induced endothelial injury (Cebulla et al. 2000). CMV- and VZV-infected white blood cells are important for viremic spread of these viruses (Ku et al. 2005; Kullberg-Lindh et al. 2008), and the possibility of facilitated tissue colonization via selectin-dependent extravasation should not be overlooked.

Herpesviruses and HTLV-1 have evolved totally different mechanisms for induction of surface sLeα, not only with respect to the identity of FUT genes activated but also to the nature of the viral factors involved. Thus, HTLV-1 induces sLeα via the virus-encoded transactivating protein, Tax, that activates transcription of a dormant FUT7 gene. In contrast, herpesviruses induce sLeα by activating three clustered host genes, FUT3,
inhibitor (Hu and Conway 1993; Thomis and Samuel 1993; Jammi et al. 2003; Gray et al. 2008). We found that 10 mM 2-AP reduced the level of FUT3, FUT5, and FUT6 transcription by more than 90% (Figure 2, left panels), suggesting a role for PKR in HSV-1 induction of FUT genes. Under certain circumstances, 2-AP may also inhibit other cellular kinases, and to estimate the possible influence of such side effects, we analyzed the effects of a newly developed highly specific PKR inhibitor, C16 (Jammi et al. 2003; Gray et al. 2008) (Figure 2, right panels). Partial inhibition on FUT3, FUT5, and FUT6 transcription was found at submicromolar concentrations of C16, and complete inhibition of FUT5 transcription was achieved at 1 μM. No cell toxicity was observed at these concentrations and the C16 inert analog had no detectable effect on FUT5 transcription. A function control of 2-AP and C16, based on the fact that activation of PKR contributes to increased IL-6 production (Carpentier et al. 2007), was positive (Figure 3, upper panel), demonstrating an impaired IL-6 production by more than 60% by 2-AP and almost 50% by C16 in HSV-1-infected cells. Altogether, these results indicated involvement of PKR in HSV-1-induced FUT3, FUT5, and FUT6 in HSV-1-infected cells.

We also investigated whether 2-AP or C16 affected expression of α(2,3)-sialyltransferase III (ST3GalIII), a host gene that is transcribed at high rates constitutively in uninfected as well as herpesvirus-infected HEL cells (Nyström et al. 2007). The level of RNA from the host cell ST3GalIII gene was equally high in mock-treated as in 2-AP-treated or C16-treated HSV-1-infected cells (Figure 3, lower panels). These results demonstrated that the PKR involvement in HSV-1 sLeα induction is selective, not including all genes encoding glycosyltransferases engaged in sLeα formation. We also found that blocking of HSV-1-induced FUT3, FUT5, and FUT6 transcription by C16 resulted in the expected inhibition of sLeα expression as detected by immunofluorescence, whereas the sLeα intensity in HSV-1-infected cells treated with the C16 inert analog was similar to that in mock-treated, infected cells (Figure 4). The C16 treatment had no effect on the intensity or distribution of gC-1-associated fluorescence.

Next, we determined whether other dsRNA-sensing proteins were involved in induction of host FUT genes, using poly(I:C) to stimulate HEL cells. The rationale for this approach was that poly(I:C) activates not only TLR3-dependent responses (reviewed by Vercammen et al. (2008)), but often also responses mediated by other cytoplasmic dsRNA-sensing proteins although less frequently PKR (Paludan 2001; Dong et al. 2008). Thus, we incubated HEL cells with poly(I:C), and IL-6 release was used as a marker for activation of the poly(I:C)-treated cells. The addition of poly(I:C) induced a 20-fold increase in the concentration of IL-6 already at 5 h after addition (Figure 5, upper panel), confirming that the HEL cells indeed was activated via TLR3 or cytoplasmic dsRNA-sensing proteins. However, no transcriptional induction of FUT3 or FUT5 above the baseline level of untreated cells was observed even at the highest concentrations of poly(I:C) (Figure 5, middle panels), indicating that activation via poly(I:C)-susceptible factors was not sufficient to induce transcription of FUT3 and FUT5. Moreover, antibodies to TLR3 were not able to inhibit HSV-1-induced transcription of FUT3 and FUT5 (Figure 5, lower panels), and negligible TLR3 was detected by immunofluorescence (data not shown). Altogether these results support a conclusion that PKR but not poly(I:C)-susceptible dsRNA-sensing proteins

Results

PKR function essential for FUT3, FUT5, and FUT6 induction and sLeα expression in HSV-1-infected cells

The previous finding that an HSV-1 IE RNA is a key factor for induction of FUT5 transcription in virus-infected cells (Nyström et al. 2009) prompted us to investigate whether antiviral cellular dsRNA-sensing factors were involved in this process. Possible involvement of the cytosolic dsRNA sensor PKR was assayed by treating HSV-1-infected cells with 2-AP, a widely used PKR

Fig. 1. Involvement of different FUT gene products in synthesis of Leα, Leβ, and sLeα. Panel A: FUT gene product capable of creating the Fuc(α1,3)GlcNAc linkage, and their involvement in Leα, Leβ, and sLeα synthesis are depicted. The activity of two sialyltransferases (ST3Gal III and ST3Gal IV), necessary for creating the direct precursor to sLeα, “Sialylated Type 2 precursor”, is indicated. Virus abbreviations in open boxes denote pathways activated by different Herpesviruses. For a more detailed account of catalytic properties and expression patterns of the FUT gene products, see Nyström et al. (2007). Panel B: Tandem organization of FUT3, FUT5, and FUT6 on the human chromosome 19p13.3. The arrows denote the position of the start codon of each gene and the direction of transcription. Data were taken from McCurley et al. (1995) and Reguigne-Arnould et al. (1995).
including TLR3 were responsible for induction of FUT3 and FUT5 transcription.

**Effect of IKKβ-inhibitors on FUT5 transcription in uninfected and HSV-infected HEL cells**

NF-κB is an inducible transcription factor crucial for regulation of divergent genes involved in a multitude of immune and non-immune responses (Perkins 2007). Moreover, dsRNA-activated PKR may be engaged in activation of NF-κB both via IKK activation and via interactions with members of the TRAF family (Garcia et al. 2007). To explore the possible involvement of NF-κB in the HSV-1-induced FUT transcription, we analyzed the effects of IMD-0354, a selective inhibitor of IKK-2 that is necessary for induction of NF-κB p65 nuclear translocation (Kamon et al. 2004). These experiments resulted in two unexpected findings. First, we found that rather than acting as an inhibitor, IMD-0354 caused a 3-fold augmentation of HSV-1 induction of FUT3 and FUT5 transcription (Figure 6, upper panels). Second, IMD-0354 was able to induce FUT5

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**Fig. 2.** Effects of 2-AP (left panels) and C16 (right panels) on FUT3, FUT5, and FUT6 transcription in HSV-1 (10 PFU/cell) and mock-infected cells. Cells were treated with 2-AP, C16, or the negative control analog of C16 at the concentrations as indicated, starting 1 h preinfection, and the cells were harvested at 5 h postinfection.
transcription at the same magnitude in mock-infected cells as in HSV-1-infected cells (Figure 6, upper panels). The positive effects on FUT3 and FUT5 transcription in uninfected cells were dose dependent (Figure 6, lower panels) and detectable as early as 3 h after initiation of IMD-0354 treatment (Figure 7, upper panels). To further explore whether these IMD-0354 effects indeed involved inhibition of IKK-2, we also investigated the effects of BAY11-7085, an independent IKK-2 inhibitor (Pierce et al. 1997; Mabuchi et al. 2004; Ding et al. 2007) on FUT transcription (Figure 6, lower panels). In contrast to the findings for IMD-0354, no significant increase in FUT3 or FUT5 transcription above the baseline was observed during BAY11-7085 treatment.

We also assayed the effects of TNF-α, a frequently used experimental NF-κB activator (Karin and Ben-Neriah 2000), in conjunction with IMD-0354 or BAY11-7085 on the transcriptional activation of FUT5 and FUT3. Treatment of uninfected HEL cells with 1 ng/mL of TNF-α indeed resulted in a 10-fold increase in IL-6 production, detectable after 5 h, an effect that was inhibited by 50% and 30%, respectively, by the addition of IMD-0354 and BAY11-7085 (Figure 7, middle panels). However, in spite of its NF-κB-dependent effects on IL-6 production, TNF-α treatment was not able to induce transcription of FUT3 or FUT5 (Figure 7, upper panels), suggesting that the control of FUT3 and FUT5 transcription did not involve the normal function of IKK-2 in NF-κB activation, i.e., via inducing degradation of IκBα. Accordingly, the addition of TNF-α had only minor effects on the prominent IMD-0354-induced transcription of FUT5 (Figure 7, upper panels). Altogether the results suggested that IMD-0354 acted as a bona fide IKK-2 inhibitor in its relationship with TNF-α-induced activation of NF-κB, but that the IMD-0354-induced augmentation of FUT3 and FUT5 transcription represented a novel and separate activity, not involving IKK-2.

In contrast to VZV and HSV-1, CMV has an additional capability to induce transcription of FUT1, encoding an enzyme adding α1,2-linked fucose, necessary for Le^y synthesis (Nyström et al. 2007, 2009). We found that IMD-0354 activates FUT6 in addition to FUT3 and FUT5, but not FUT1 (Figure 7, lower panel). Thus, the IMD-induced FUT expression pattern in uninfected HEL cells resembled that of HSV-1 and VZV rather than that of CMV.

**IMD-0354 as a noncanonical inducer of FUT3 and FUT5 transcription**

HSV-1 induction of FUT3 and FUT5 transcription is repressed by the proteasome inhibitor MG-132 but augmented by
inhibitors of protein synthesis such as cycloheximide (CHX) (Nyström et al. 2009). We found that MG-132 inhibited the IMD-0354-dependent transcription of FUT5 in a dose-dependent manner (Figure 8, Panel A), but the IMD-0354-induced FUT5 transcription was not further augmented even by high concentrations of CHX (Figure 8, panel B). Thus, the sensitivity to MG-132 and resistance to CHX are in accordance with previous observations for HSV-1-induced transcription of FUT3 and FUT5 (Nyström et al. 2009). From the above results it is unlikely that the IMD-0354-induction of FUT3, FUT5, and FUT6 transcription was caused via effects on NF-κB by IKK-2 inhibition. Owing to its significance for HSV-1 activation of FUT transcription, one intriguing question is whether PKR is a hitherto unidentified target for IMD-0354. To evaluate this possibility, we analyzed the effects of 2-AP on IMD-0354-induced transcription of FUT5, indicating that functional PKR was not necessary for IMD-0354-dependent FUT5 transcription. These results indicated that the IMD-0354 effects on FUT5 transcription was due to an event downstream PKR activation or that IMD-0354 activation of FUT5 transcription represented a totally different activation pathway for regulation of FUT5 expression.

It was also investigated whether IMD-0354 induction of FUT transcription was associated with sLe\(^\alpha\) formation in HEL cells (Figure 9). No sLe\(^\alpha\) was detected by immunofluorescence in cells treated with IMD-0354, whereas the positive control, HSV-1-infected cells were distinctly stained by the sLe\(^\alpha\) antibody. This is in line with previous results that all sLe\(^\alpha\) detectable by confocal microscopy are associated with a mucin stretch of the HSV-1-specific glycoprotein gC-1. Thus, studies with glycoprotein-deficient HSV-1 mutants demonstrated that in spite of unimpaired FUT expression, neither other HSV-1 glycoproteins than gC-1 nor HEL cell glycoconjugates could support sLe\(^\alpha\) expression in HSV-1-infected cells, where expression of gC-1 or its mucin-like region was prevented (Nordén et al. submitted).

### JNK is not involved in HSV-1 or IMD-0354-induced transcription of FUT5 in HEL cells

PKR-dependent responses may result in activation of the JNK pathway, which could be involved in transcription enhancement of selected human genes (Chakrabarti et al. 2008). To explore the possible significance of this phenomenon, IMD-0354-treated cells were treated with SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase of the JNK pathway (Bennett et al. 2001) (Figure 10, upper panel). First, we found that the TNF-α-induced production of IL-6 was impaired by SP600125 treatment. Thus, the JNK pathway was activated, and the SP600125 concentration applied was sufficient to interfere with Jun N-terminal kinase of HEL cells. However, SP600125 had only minor effects on the IMD-0354-induced FUT3 or FUT5 transcription (Figure 10, lower panels), indicating a marginal role of the JNK pathway in IMD-0354-induced FUT5 transcription.

### Discussion

A viral IE RNA, independent of newly synthesized viral proteins, plays a key role as initiator of HSV-1-induced transcription of the quiescent host fucosyltransferase genes necessary for expression of sLe\(^\alpha\) in virus-infected cells (Nyström et al. 2009). The results here strongly suggested that the primary cellular target for this viral IE RNA is PKR, a cytosolic antiviral dsRNA protein (reviewed in Garcia et al. (2006)). This conclusion is based on results with the classical PKR inhibitor 2-AP (Tiwari et al. 1988; Hu and Conway 1993; Thomis and Samuel
HSV-1-activated transcription of host fucosyltransferases

Fig. 5. Poly(I:C) induced effects on IL-6 and FUT gene expression. HEL cells were incubated with poly(I:C) at a final concentration of 10 μg/mL for 5 h. The growth medium was assayed for IL-6 content; maximum concentration is denoted as 100%. (Top panel) HEL cells were treated with 10 μg/mL poly(I:C) and/or infected with HSV-1 at 10 PFU/cell. The total RNA was extracted after 5 h, and FUT3 and FUT5 expression levels were determined (middle panels). The bottom panel depicts effects of anti-TLR3 antibodies on HSV-1-induced transcription. HEL cells were incubated with 10 μg/mL TLR3.7 Mab for 1 h before infection with 10 PFU/cell HSV-1. The cells were harvested 5 h postinfection, and FUT3 and FUT5 expression was assayed by real-time PCR.

1993) and a newly developed highly specific PKR inhibitor, C16, lacking inhibitory effects on other protein kinases with similar or overlapping signaling patterns as PKR (Jammi et al. 2003; Ingrand et al. 2007; Gray et al. 2008), ruling out that the 2-AP results reflected such side effects. Moreover, the inert C16 analog, used as a control, did not inhibit transcription of the FUT genes. No evidence was found that TLR3 or other poly(I:C)-inducible antiviral dsRNA sensing proteins (Paludan 2001; Dong et al. 2008) contributed to the FUT3 and FUT5 induction.

Although as yet not exactly mapped, the HSV-1 dsRNA responsible for FUT3 and FUT5 induction is a HSV-1 IE RNA,
Fig. 6. Effects of IMD-0354 and BAY 11-7085 on FUT3 and FUT5 transcription in mock or HSV-1-infected cells. HEL cells incubated with IMD-0354 for 1 h before mock or HSV-1 (10 PFU/cell) infected. The cells were harvested 5 h postinfection and respective FUT gene expression was determined (upper panels). Lower panels depict a dose-response analysis of IMD-0354 and BAY 11-7085, where the inhibitors at the concentrations indicated were administered to uninfected HEL cells for 5 h.

although not from the larger part of the IE gene α0 (Nyström et al. 2009), and it might be that any viral IE dsRNA fulfilling certain basic requirements for PKR-binding will function. PKR-binding dsRNAs defined for large DNA viruses may originate from transcripts of viral ORFs that run in the opposite direction (Melchjorsen et al. 2002; Garcia et al. 2006). In general, the optimal dsRNA size for PKR activation is 80 bp, and smaller dsRNA molecules than 30 bp are not active, excluding short interfering RNAs (Manche et al. 1992; Garcia et al. 2006). HSV-1 envelope glycoprotein–receptor interactions activate different cellular signal pathways, and viral tegument proteins, released after HSV-1 entry, affect the transcription status of the infected cell (LaBoissiere and O’Hare 2000; Santoro et al. 2003; Herrera and Triezenberg 2004; Taylor et al. 2007; Satoh et al. 2008; Sciortino, Medici, Marino-Merlo, Zaccaria, Giuffre-Cuculletto, Venuti, Grelli, Bramanti, et al. 2008; Sciortino, Medici, Marino-Merlo, Zaccaria, Giuffre-Cuculletto, Venuti, Grelli, Mastino 2008; Sedy et al. 2008), but these events alone without HSV-1 gene expression are not sufficient to induce transcription of the FUT genes (Nyström et al. 2009).

PKR is a multifunctional protein, affecting translation as well as transcription of human genes. The present data suggest that HSV-1-induced transcription of FUT3 and FUT5 involves an unusual PKR-dependent pathway in several aspects. Firstly, the CHX-resistance of the FUT induction process excluded PKR-dependent effects on translation, which is one major PKR target via eIF-2α (Garcia et al. 2006, 2007). Secondly, other PKR effects resulting in transcriptional activation of cellular genes often involve the JNK (Chakrabarti et al. 2008) or NF-κB, primarily via the IKK complex (Garcia et al. 2007; Karehed
HSV-1-activated transcription of host fucosyltransferases

Fig. 7. Effects of TNF-α and IMD-0354 on FUT transcription and IL-6 production. HEL cells were incubated with TNF-α and/or IMD-0354 at concentrations of 1 ng/mL or 10 μM, respectively, for indicated time intervals before they were harvested and transcription measured (top panels). IL-6 release into the growth media was determined after incubation of HEL cells with TNF-α (1 ng/mL), BAY 11-7085 (10 μM), and IMD-0354 (10 μM) as indicated, for 5 h. Hundred percent corresponds to the highest IL-6 concentration determined in each assay (middle panels). Uninfected HEL cells were treated with IMD-0354 (10 μM) for time intervals indicated prior to analysis of FUT1 and FUT6 RNA levels by real-time PCR (bottom panel).

et al. 2007), but no evidence was obtained that these pathways were active in HSV-1-induced FUT transcription. Thus, neither the JNK inhibitor SP600125 nor the IKK-2 inhibitor BAY11-7085 interfered with FUT3 and FUT5 expression. Moreover, the NF-κB activator TNF-α (Karin and Ben-Neriah 2000) induced the expected IKK-dependent production of IL-6 in HEL cells without activating transcription of FUT3 and FUT5 (Figure 7). Therefore, activation of FUT3 and FUT5 may involve other
signaling intermediates including other known PKR-activated transcription factors, e.g. IRF-1, STAT, or p53 (Garcia et al. 2006, 2007), but as yet unidentified pathways must also be considered.

One observation supporting the notion that activation of FUT3, FUT5, and FUT6 may reflect a previously unknown PKR-dependent pathway was that the IKK-2 inhibitor IMD-0354 had a dual role in HSV-1-infected cells. The expected inhibitory effect of both IMD-0354 and BAY11-7085 on TNF-α-induced IL-6 production indicated that IMD-0354 functioned as an IKK-2 inhibitor in this context. But the high levels of FUT3 and FUT5 RNA induced by IMD-0354 in HEL cells, whether HSV-1 infected or not, are difficult to reconcile with inhibition of IKK-2, primarily because this effect was observed neither for BAY11-7085 (cf. Figure 6) nor for SN50, a cell-penetrating peptide inhibitor of IKK-2 (D’Acquisto and Ianaro 2006) (data not shown). Hence, IMD-0354 but none of the other IKK-2 inhibitors must have an alternative, as yet unknown, target other than IKK-2 in HEL cells, with a key function for FUT induction.

The relationship between HSV-1 induction and the IMD-0354 induction of FUT3, FUT5, and FUT6 is intriguing from another perspective. Thus, the 2-AP insensitivity indicated that PKR is not involved in IMD-0354 induction, and this opens two possibilities: HSV-1 infection and IMD-0354 may activate two totally separate ways for inducing transcription of the cluster of fucosyltransferase genes, or they activate one and the same pathway for activation of the clustered FUT genes. If this latter scenario is true, IMD-0354 must exert its effect at a step downstream the HSV-1 RNA activation of PKR. Further studies are needed to clarify the novel function of IMD-0354 in this context.

FUT5, FUT3, and FUT6 are arranged in tandem on the human chromosome 19p13.3 (Figure 1, Panel B), but their transcription seems to be regulated independently, and each and every one of them is expressed in a tissue-dependent manner (Oulmouden et al. 1997; Escrevente et al. 2006). But HSV-1 infection (Nyström et al. 2007) as well as IMD-0354 treatment (present data) of HEL cells reveals that all three genes can be activated simultaneously, an option probably facilitated by their clustered tandem arrangement that is a result of gene duplication events (Dupuy et al. 2002). The concept of PKR as a primary cellular target for early viral RNA-dependent transcriptional activation of human genes is attractive because PKR is constitutively present in the cytoplasm of low-active cells such as density-inhibited HEL cells used in the present study (Garcia et al. 2006, 2007; Vercammen et al. 2008). Hence, PKR should be accessible for HSV-1 IE transcripts sufficiently early to permit the rapid induction of FUT3 and FUT5 transcription detectable at 90 min postinfection (Nyström et al. 2009). The constitutively transcribed ST3GalIII (Nyström et al. 2007), encoding an α(2,3)-sialyltransferase relevant for sLe^a^, was resistant to PKR inhibitors, indicating that transcription of FUT3, FUT5, and FUT6 on one hand and that of ST3GalIII on the other are regulated by separate mechanisms. Moreover, IMD-0354 did not induce transcription of the Le^a^-essential gene FUT1, selectively induced by CMV but not by VZV and HSV-1 (Nyström et al. 2007), possibly suggesting a third regulatory mechanism.

PKR mediates an antiviral response against both DNA and RNA viruses (Garcia et al. 2006, 2007; Vercammen et al. 2008). Herpesviruses have evolved strategies aimed at turning such antiviral cellular responses against the host itself. For example, the early phases of HSV-1-induced apoptosis of infected cells...
promote in fact viral replication, but apoptosis is later reversed by viral late proteins to prevent the detrimental outcome of cell destruction before the production of viral progeny (Sanfilippo and Blaho 2006; Nguyen and Blaho 2007). The HSV-1-mediated induction of sLe\(^{\alpha}\) via FUT gene activation may therefore not necessarily participate in any functional antiviral host mechanism. The PKR-dependent antiviral programme is efficiently interrupted later during infection by at least two HSV-1 gene products (Roizman 1999; Mohr 2004), explaining why sLe\(^{\alpha}\) synthesis is possible without disturbing the HSV-1 replication cycle (Nyström et al. 2009). The appearance of sLe\(^{\alpha}\) in HSV-1-infected cells may thus result from a viral strategy where early and late viral gene products cooperate in inducing the relevant host genes by initiating and interrupting a PKR-dependent antiviral response.

**Material and methods**

**Viruses and cells**
The wild-type HSV-1 virus Syn17+ was used throughout the study and the virus titers were determined by plaque titration on GMK (Green monkey kidney) cells (Günalp 1965). Human diploid embryonic lung fibroblasts (HEL cells) (Lundström et al. 1987) were used throughout the study. The fibroblasts were cultivated in Eagle’s MEM with 1% penicillin–streptomycin and 1% L-glutamine. Ten percent of fetal calf serum (FCS) was used during cell growth.

**Inhibitors and agonists**
2-Aminopurine (2-AP), used for inhibition of PKR (Hu and Conway 1993; Thomis and Samuel 1993), polyriboinosinic polyribocytidylic acid (poly(I:C)); the IKK-2 inhibitor IMD-0354 (N-(3,5-bis-trifluoromethylphenyl)-5-chloro-2-hydroxybenzamide); CHX; and SP600125 (1,9-pyrazoloanthrone), an inhibitor of Jun N-terminal kinase (Bennett et al. 2001), were purchased from Sigma Aldrich, St Louis, MO. The PKR inhibitor C16 (8-(1H-imidazol-4-ylmethylene)-6,8-dihydro-thiazolo[5,4-E]indol-7-one) (Jammi et al. 2003), its negative control (5-chloro-3-(3,5-dichloro-4-hydroxybenzylidene)-1,3-dihydro-indol-2-one), TNF-α, and MG-132 were obtained from Calbiochem-Merck Bioscience Ltd, Nottingham, UK.

**Cell toxicity test**
For determination of cell viability after drug treatment, a MTS ((4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium salt) reduction assay (Promega, Madison, WI), based on a standard method (Bakand et al. 2007), was performed according to manufacturer’s instructions. Briefly, cells in a 96-well plate were incubated with the compounds mentioned above for 5–14 h. Twenty microliters of CellTiter 96® aqueous one solution reagent was added to each well, and the plate was incubated at 37°C and 5% CO\(_2\) in a humid chamber for 2 h. The absorbance at 490 nm was determined using a 96-well plate reader.

**Immunofluorescence**
The procedures for immunofluorescence with slight modifications have been published previously (Nyström et al. 2007). Briefly, HEL cells were trypsinized and resuspended in Eagle’s MEM, supplemented with penicillin, streptomycin, 1% L-glutamine, and 10% FCS. The cells were allowed to adhere to teflon-coated object slides for 24 h. The cells were infected with HSV-1 at different time intervals and treated with various inhibitors (see Inhibitors and agonists). Subsequently, the slides were fixed in 4% paraformaldehyde for 10 min, washed in PBS (phosphate-buffered saline), dried, and stored at −80°C. Immunofluorescence was performed on the slides with human sera containing antibodies directed against HSV-1 diluted 1:600, human antibodies to the HSV-1 glycoprotein gC-1 obtained by an immunosorbent, containing purified gC-1 (Lundström et al. 1987), and IgG antibodies to TLR3 diluted 1:50, and/or IgM antibodies against sLe\(^{\alpha}\) (KM93; Chemicon International, Temecula, CA) respectively diluted 1:40. The primary antibodies were incubated overnight at 4°C or 37°C for 1 h (TLR3 antibody). A TRITC-conjugated antihuman antibody (Jackson ImmunoResearch, West Grove, PA) and a FITC-conjugated antimouse antibody (Dako, Glostrup, Denmark) were used at dilutions of 1:400 and 1:100, respectively, and incubated for 1 h at 37°C. All dilutions of antibodies were performed in 3% BSA in PBS. A Prolong Gold Antifade Kit with DAPI (4′,6-diamidino-2-phenylindole; Invitrogen Molecular Probes, Eugene, OR) was used as mounting fluid on the slides. The slides were analyzed under a Zeiss LSM 510 Meta confocal microscope using a Plan-Apochromat 63× objective in oil immersion.
Cell culture experiments

HSV-1 at a multiplicity of infection of 10 PFU (plaque forming unit) of virus/cell was added to HEL cells in 6-well culture plates (700,000 cells/well). Virus was allowed to attach to the cells for 1 h at 37°C and 5% CO2 before the inoculum was removed. The cells were then washed in PBS and a new medium was added. Different inhibitors (see Inhibitors and agonists) were added as indicated in the figure legends. After different intervals of infection, each well was harvested and extracted for RNA.

RNA extraction

Prior to RNA extraction, the cells were washed with PBS and lysed with a 300 μL 2× nucleic acid purification lysis solution (Applied Biosystems, Foster City, CA) mixed with 300 μL PBS. The 6100 Nucleic Acid PrepStation (Applied Biosystems) was used for purification of total RNA according to manufacturer’s instructions including DNase treatment for total RNA extraction as previously described (Nyström et al. 2009).

Real-time PCR

Relative concentrations of transcripts from different fucosyltransferase genes were determined using the ΔCT method, as optimized for herpesvirus-infected cells (Nyström et al. 2004, 2007), according to manufacturer’s instructions regarding Taqman chemistry. Primers and probes for ST3Gal III, FUT1, and FUT6 were previously described (Nyström et al. 2007, 2009). Primers and probes for FUT3 and FUT5 were revised in order to enhance the assay performance. The new sequences were: FUT3 forward (5′-GCT CAG AGT TCA GAC AGG TCC AA-3′), reverse (5′-CCA GAA GTC CTC ATT TAC AGT CGA T-3′), probe (FAM 5′-TCA AGC CCA GGA CCA CCA CTT ATA GGG T-3′ TAMRA); FUT5 forward (5′-GGT GTC ACA AAT GCA TCA CTA TGG-3′), reverse 5′-ACA TCC ACA GGC ACT ATC AGA GT-3′), probe (CAC GCC GAG GTC ACA C (MGB)). The error bars in the figures represent the standard deviation.

Measurements of IL-6 production

IL-6 was assayed essentially as described by Mogensen et al. (2004). Briefly, HSV-1-infected or uninfected HEL cells were stimulated with the substances as indicated in the text. The supernatant was harvested and cell debris was removed by centrifugation. The samples were stored at −20°C until ELISA was performed. The IL-6 concentration in the growth medium was determined by ELISA according to manufacturer’s instructions (BD Bioscience, San Jose, CA). The highest IL-6 concentration determined for each individual experiment served as a reference for calculation of the percentage and was subsequently denoted 100% in the graphs.

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Conflict of interest statement
None declared.

Abbreviations
CHX, cycloheximide; CMV, cytomegalovirus; Fuc, fucose; Fuc-T, fucosyltransferase, Fuc-TI, etc.; FUT, fucosyltransferase gene, FUT1, etc.; HEL, human embryonic diploid fibroblasts; HTLV, human T-cell leukemia virus type 1; ICP, infected cell protein, ICPO, etc.; IE, immediate early, belonging to transcriptional class α; Leα, Lewis X; Leβ, Lewis Y; PBS, phosphate-buffered saline; FFU, plaque forming unit; sLeα, sialyl Lewis X; ST3Gal III, α(2,3)-sialyltransferase III; ST3Gal IV, α(2,3)-sialyltransferase IV; VZV, varicella-zoster virus; α0, α4, genes encoding ICPO and ICPO, respectively.

References


