Simian TRIM5α proteins reduce replication of herpes simplex virus

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Old World monkey TRIM5α proteins are known to block the replication of human immunodeficiency virus and other retroviruses in a species-specific fashion. In this report, we show that specific forms of simian TRIM5α proteins can restrict herpes simplex virus (HSV) infection. To define the effect of TRIM5α on HSV replication, we examined HSV infection in HeLa cell lines that stably express simian and human orthologs of TRIM5α proteins. We demonstrated that several simian TRIM5α proteins can restrict HSV replication, with the TRIM5α protein of rhesus macaques showing the strongest inhibition of HSV infection. We also found that the level of the inhibition of virus replication was viral strain-specific. TRIM5α is likely to inhibit HSV at the early stage of infection; however, at later times of infection, the levels of TRIM5α are significantly decreased. Thus, some TRIM5α proteins exhibit antiviral effects that extend beyond retroviral infections, but HSV may be able to reduce this restriction by reducing TRIM5α levels during the later phases of virus replication. Our results also argue that TRIM5α is only part of the reduced level of HSV replication in rhesus macaques, which are known to be less susceptible to HSV infection than other primates.

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Introduction

Herpes simplex virus (HSV), a human nuclear DNA virus, is capable of replicating in a variety of cell types of many species (Roizman et al., 2007). This broad host range has several possible explanations. First, the HSV virion has approximately 12 envelope surface glycoproteins that can facilitate entry into a range of cell types (Roizman et al., 2007). Second, HSV uses the broadly distributed heparan moiety of cell surface glycosaminoglycans for attachment to cells and at least three different receptors for entry (Spear, 2004). Third, HSV has multiple mechanisms for overcoming intracellular blocks to replication. These include (1) degradation of the TRIM19/PML protein (Chelbi-Alix and de The, 1999) and disruption of nuclear domain 10 (ND-10) nuclear bodies (Maul et al., 1993) to prevent their restriction of HSV infection (Everett et al., 2006), (2) blocking host cell chromatin-silencing mechanisms to allow active lytic gene transcription (Cliffe and Knipe, 2008; Gu and Roizman, 2007; Knipe and Cliffe, 2006), and (3) blocking interferon induction (Lin et al., 2004; Melroe et al., 2004). These and likely other mechanisms allow a broad host range for HSV replication.

Nevertheless, HSV infection of certain animal species such as rhesus macaques does not lead to disease (Juan-Sallés et al., 1997; London et al., 1974), but the reasons for the avirulence of HSV in rhesus macaques are not known. Rhesus macaques are used as a host for simian immunodeficiency virus (Desrosiers and Ringler, 1989), and this is considered to be an excellent nonhuman primate model for acquired immunodeficiency syndrome (AIDS). HSV-based vaccine vectors expressing SIV proteins have been tested in rhesus macaques (Kaur et al., 2007; Murphy et al., 2000). Because the rhesus-SIV model often serves as a measure of AIDS vaccine vector efficacy (Barouch, 2008), it is important to understand the interactions of the viral vectors with rhesus cells.

Several host factors have been shown to regulate retroviral infection (Wolf and Goff, 2008). The tripartite motif 5α (TRIM5α) protein was identified as a major factor involved in restricting human immunodeficiency virus type 1 (HIV-1) infection in Old World monkey cells (Stremlau et al., 2004; Zheng et al., 2005). TRIM5α is believed to restrict infection at an early, postentry stage, likely by specifically recognizing the capsid and promoting its premature disassembly (Owens et al., 2003, 2004; Stremlau et al., 2006). Studies with TRIM5α orthologs cloned from different primates, including Old and New World monkeys and hominoids, showed that TRIM5α inhibits retroviral infection in a species-specific fashion (Song et al., 2005b; Yap et al., 2004).

TRIM5α is a member of the tripartite motif (TRIM) family of proteins which contain a RING domain, one or two B-box domains, and coiled-coil (CC) domains (Reymond et al., 2001). Some TRIM proteins including TRIM5α contain a C-terminal B30.2 or SPRY domain. The TRIM5α primary transcript can be spliced into several isoforms but only the largest product of splicing, TRIM5α, which contains the B.30.2 domain (493 amino acids in humans), has the...
ability to mediate retroviral restriction (Stremlau et al., 2004). To date, about 69 members of the TRIM family have been identified in the human genome and some of their homologs have been found in primates and other species.

Members of the TRIM protein family are involved in various cellular processes including cell proliferation, differentiation, development, oncogenesis and apoptosis (Nisole et al., 2005; Reymond et al., 2001). Depending on their function, localization and cellular partner, TRIM proteins can act in the nucleus or in the cytoplasm and affect different stages of viral life cycles. TRIM19/PML, a major component of nuclear domain 10 (ND10) bodies (Everett and Maul, 1994), interferes with replication of several DNA and RNA viruses including HSV-1 (Everett and Maul, 1994), Ebola (Björndal et al., 2003), SIV (Bonilla et al., 2002; Djavani et al., 2001), and arenaviruses (Asper et al., 2004) among others (Blondel et al., 2002; Borden et al., 1998), interferes with replication of several DNA and RNA viruses depending on their function, localization and cellular processes including cell proliferation, differentiation, development, oncogenesis and apoptosis (Nisole et al., 2005; Reymond et al., 2001). Depending on their function, localization and cellular processes including cell proliferation, differentiation, development, oncogenesis and apoptosis (Nisole et al., 2005; Reymond et al., 2001).

TRIM19/PML is an antiviral molecule involved in innate resistance to viral infection (Regad and Chelbi-Alix, 2001). The definition of the precise role of TRIM19/PML is complicated because of the number of proteins that interact with this protein and the fact that IFN I up-regulates other proteins (for example, PKR, 2′,5′OAS and Mx protein) directly involved in inhibition of virus replication (Nisole et al., 2005).

Based on the structural and functional similarities between TRIM19/PML and other TRIM proteins, we asked if TRIM5α could influence HSV replication. Our results show that TRIM5 proteins from Old World monkeys (OWM) can restrict HSV infection, and this inhibition is viral strain-specific. We therefore hypothesize that TRIM5 protein may exhibit antiviral effects that inhibit viruses outside of the retrovirus family. These antiviral effects may be counted in some cases, such as late times in cells infected with HSV, by downregulation of TRIM5α levels during the late phase of infection.

**Results**

**HSV-1 and HSV-2 replication is reduced in rhesus macaque fibroblasts**

Several reports have indicated that rhesus macaques are less susceptible to HSV infection than other primates and are poor non-human primate models of HSV-1 and 2 infection (Juan-Sallés et al., 1995; Levine et al., 1980; London et al., 1974). However, little is known about the basis for this low susceptibility. To test directly the ability of HSV to grow in rhesus cells, we investigated the replication of HSV-1 and 2 in rhesus monkey fibroblasts and compared this with the replication of those viruses in permissive HeLa cells. HeLa cells and rhesus macaque fibroblast cell lines were infected with HSV-1 and HSV-2 at an MOI of 3 PFU/cell. Infected cells were harvested at various times post-infection, and the yields of progeny viruses were determined on Vero cells. We found that the HSV-1 and HSV-2 yields obtained from rhesus monkey fibroblasts were significantly reduced when compared to yields from HeLa cells (p < 0.05) (Fig. 1). Both reached maximal values by 40 hpi, and yields in rhesus monkey fibroblasts were about 100-fold lower than the yields from HeLa cells (Fig. 1). The reduced replication of HSV in rhesus cells led us to investigate whether the rhesus TRIM5α protein contributed to the reduced permissiveness.

**Rhesus TRIM5α protein restricts HSV infection**

To determine if rhesus TRIM5α, a factor known to restrict HIV infection, contributed to the restriction of HSV-1 and 2 in rhesus cells, we examined HSV infection of HeLa cells that constitutively express the rhesus monkey TRIM5α protein (H-R cells). H-R cells have been used to investigate the restricting activity of TRIM5α on different retroviruses, including HIV-1, SIVmac and SIVagm, and have demonstrated virus-specific inhibitory activities (Song et al., 2005b). We infected H-R cells and the control H-L cells (that contain the empty pLPCX vector) with HSV-1 or HSV-2 at MOIs ranging from 1–30 PFU/cell. At 24 hpi the cells were harvested, and yields of HSV were determined by titration on Vero cells. In H-R cells infected at low MOIs (1 and 3), we observed significant reductions in viral yield for both HSV-1 and HSV-2 (HSV-1 KOS MOI = 1: p = 0.04; HSV-1 KOS MOI = 3: p = 0.003; HSV-2 186 MOI = 1: p = 0.014) compared with the yields obtained from the control H-L line (Fig. 2). HSV-1 KOS virus yields following infection of H-R cells were reduced by as much as 2-fold (Fig. 2A), and HSV-2 186 replication was reduced by as much as 5-fold compared to infection of H-L cells (Fig. 2B). At MOI = 30, the viral yields from the H-R cells were similar to the H-L cells. Therefore, rhesus monkey TRIM5α can reduce HSV replication at low MOIs, but the effect of rhesus TRIM5α protein was overcome at higher MOIs.

**Effect of other primate TRIM5α molecules on HSV infection**

Because the effects of TRIM5α protein on retrovirus infection depend on the species origin of the TRIM5α protein (Song et al., 2005b), we asked if the source of TRIM5α influenced the effects on HSV infection. We therefore compared the effects of TRIM5α molecules from African green monkey, squirrel monkey, rhesus monkey and human. HSV-2 replication in H-H cells expressing human TRIM5α was similar to that in H-L cells containing the empty expression vector (Fig. 3). Viral replication in H-AGM cells expressing African green monkey (AGM) TRIM5α was reduced by 3.6-fold relative to the control H-L cells (Fig. 3). Similarly, viral replication in H-R cells expressing rhesus TRIM5α was reduced by 4.5-fold (Fig. 3), as observed above. In contrast, viral replication in H-Sq cells expressing squirrel monkey TRIM5α was reduced by only 35%. HSV-2 infection of...
HeLa cells expressing TRIM5α from both OWM tested (rhesus and AGM) resulted in significantly lower virus yields (H-R: \( p = 0.04 \); H-AGM: \( p = 0.04 \)) of all the tested cell lines. Expression of squirrel monkey (NWM) TRIM5α modestly inhibited HSV-2 replication, and human TRIM5α only slightly suppressed HSV-2 replication; however, the reductions in virus yields in these cells were not statistically significant (\( P < 0.05 \)). Therefore, of the TRIM5α molecules that we examined, OWM TRIM5α molecules demonstrated the strongest inhibitory activity on HSV replication.

**Effects of rhesus TRIM5α protein on HSV-1 and HSV-2 protein synthesis**

Because the restrictive activity of TRIM5α on retroviruses is thought to occur at an early stage of infection (Stremlau et al., 2006; Wu et al., 2006), we hypothesized that rhesus monkey TRIM5α might also exert its restrictive activity on HSV infection at an early stage. If this were the case, HSV IE viral protein synthesis would be decreased in rhesus monkey TRIM5α-expressing cell lines. To test this hypothesis, we infected H-L control cells and H-R cells expressing rhesus monkey TRIM5α with HSV-1 and HSV-2 and assayed viral protein synthesis by Western blot analysis. The HSV-1 immediate-early ICP4 protein was detectable at 4 hpi in both cell lines and peaked at 6 hpi, at which time approximately 2-fold lower levels of ICP4 were observed in the H-R cells (Fig. 4A). Expression of the IE ICP27 protein was reduced by 3-fold in H-R cells compared to H-L cells as early as at 4 hpi. Expression of the early ICP8 protein in the H-R cell line was reduced by 4-fold in H-R cells compared to H-L cells at 4 hpi. Therefore, the reduction in HSV-1 replication in H-R cells was apparent at the level of IE gene expression.

In HSV-2 infected cells, the reduction of viral protein synthesis in H-R cells was greater compared to the reduction in HSV-1 gene expression. ICP8 and ICP27 were reduced by 5–7-fold at 4–10 hpi in H-R cells compared to H-L cells (Fig. 4B). Therefore, the reduction in HSV-2 replication in H-R cells could be explained by a similar reduction in IE and E gene expression.

**Effects of rhesus TRIM5α on different HSV strains**

Because replication of HSV-1 strain KOS and HSV-2 strain 186 syn+ was reduced by rhesus TRIM5α, we investigated whether the restriction extended to other HSV strains, including clinical isolates. First, we infected H-R cells and the control H-L cells with the low passage HSV-2 clinical isolate SD90-3P or the low passage laboratory strain HSV-2 G. H-R cells showed reduced yields of HSV-2 laboratory
strains (186 syn+ and G) as well as the HSV-2 clinical isolate (Fig. 5A). HSV-2 strain 186 syn+ was reduced by 12-fold, HSV-2 strain G was reduced by 4-fold, and the SD90-3P clinical isolate was reduced by 2.5-fold in rhesus TRIM5α cells. In the second set of experiments we infected H-R cells and the control H-L cells with the HSV-1 low passage laboratory strain F or the laboratory strain 17 syn+. In H-R cells we observed a 3.6-fold reduction of HSV-1 strain F in H-R cells compared to H-L control cells (Fig. 5B). In contrast, replication of HSV-1 strain 17syn+ was not significantly inhibited (p>0.05) in rhesus-TRIM5α cells. Therefore, both HSV-1 and HSV-2 strains were inhibited by TRIM5α, and the inhibition of HSV replication in TRIM5 expressing cells appeared to be more viral strain-specific than species-specific.

Effect of TRIM5α on HSV ICP0 distribution

It was reported recently that a cytoplasmic PML retained the HSV infected cell protein 0 (ICP0) in the cytoplasm and reduced HSV-1 replication (McNally et al., 2008). Therefore, it was conceivable that TRIM5α acted in a similar fashion. To test this hypothesis, we examined the distribution of ICP0 in HSV-1 KOS virus-infected H-L, H-R, and H-H cells. In H-L and H-H cells, we detected nuclear ICP0 by 1 hpi (results not shown). By 4 hpi, nuclear ICP0 had accumulated in H-L and H-H cells, and small numbers of cytoplasmic punctate structures containing ICP0 were observed (Fig. 6). By 8 hpi, more punctate ICP0 was observed in infected H-L and H-H cells (Fig. 6, panels G and I). In infected H-R cells, we observed increased numbers of cells with cytoplasmic ICP0 by 4 hpi (Fig. 6, panel E), and by 8 hpi, there was decreased nuclear ICP0 and more cytoplasmic ICP0 (Fig. 6, panel H). Therefore, rhesus TRIM5α appeared to increase the amounts...
of ICP0 in the cytoplasm. Furthermore, when we dual stained the cells to detect ICP0 and TRIM5α, we observed that, in HSV-1-infected H-R cells, ICP0 localized to punctate structures near punctate TRIM5α-containing structures (Fig. 7, panels A and C). In contrast, in HSV-infected H-H cells, there were few ICP0-containing structures localized near the TRIM5α cytoplasmic punctate structures (Fig. 7, panels B and D). Similar changes in ICP0 distribution were observed in H-R cells infected with another HSV-1 strain, HSV-1 strain 17. In total, these results suggested that TRIM5α might sequester ICP0 in the cytoplasm, preventing its nuclear functions, and thereby inhibiting HSV replication.

Effect of TRIM5α on HSV-1 ICP0-mutant replication

The increased cytoplasmic ICP0 in H-R cells suggested that the inhibition might be due to cytoplasmic retention of ICP0 at early times of infection. If this were the case, the reduction would be dependent on ICP0, as observed previously for cytoplasmic PML (McNally et al., 2008). We therefore determined if an ICP0 mutant virus showed decreased replication in H-R cells relative to H-L cells. To this end, we infected H-L and H-R cells with the ICP0 null and rescued viruses and measured the viral yields at 24 hpi. The ICP0 null mutant virus yields were 1.9-fold lower in H-R cells as compared with H-L cells while the rescued virus (Fig. 8). In HSV-2-infected cells, TRIM5α decreased by 4 hpi and was undetectable by 24 hpi. In HSV-1-infected cells, TRIM5α decreased more slowly but was also undetectable by 24 hpi. The 80 kDa band was likely a cellular protein that cross reacts with anti-HA tag antibodies but provides a loading control on the blot.

To determine if the loss of TRIM5α was related to the restriction of HSV replication, we infected or mock-infected cells that express different TRIM5α molecules (H-L, H-R, H-Sq, H-H, H-AGM) with HSV-2 virus and harvested the infected cultures at 4, 6 or 16 hpi. In all cell lines tested, loss of TRIM5α started at 6 hpi, which coincided with ICP8 expression (Fig. 9) and the protein was undetectable by 16 hpi (data not shown). Therefore, for all TRIM5α variants tested, we observed approximately the same kinetics of TRIM5α loss. However, viral gene expression, as observed by ICP8 expression, obtained from the infected cultures (Fig. 3) was different for these cell lines. These results suggested that the level of TRIM5α-mediated restriction of HSV replication by different TRIM5α proteins was not determined primarily by TRIM5α protein levels in the infected cells. The ability of HSV to promote loss of TRIM5α in infected cells may, however, reduce the overall effect of all of the TRIM5α molecules.

Discussion

HSV has long been known to be unable to cause serious disease in rhesus macaques (London et al., 1974), but little is known about the mechanism(s) of this avirulence. In this study we observed that at least certain HSV-1 and HSV-2 strains grow relatively poorly in immortalized rhesus monkey fibroblast cells, providing a potential explanation for the lack of disease in HSV-infected macaques. We therefore explored the effect of TRIM5α proteins on HSV replication following the demonstration that rhesus monkey TRIM5α can inhibit HIV-1 replication. In our study we tested different TRIM5α orthologs from Old World and New World monkeys and from humans for their restriction of HSV replication in HeLa cells. Among the TRIM5α proteins that were tested, TRIM5α from rhesus macaques (OWM) exhibited the strongest inhibitory effect on herpes virus infection, and TRIM5α of AGM also shows a significant inhibitory effect on HSV growth and protein expression. In contrast, the squirrel monkey TRIM5α exhibited only a modest effect on HSV replication, and human TRIM5α had little to no effect. These results indicated that TRIM5α from certain monkey species can significantly diminish replication of HSV. This is only a part of the mechanism of restriction for HSV in rhesus monkey cells because the reduced viral replication in rhesus monkey cells relative to that in human cells is much greater than the degree of HSV inhibition observed for rhesus monkey TRIM5α expressed in human cells.

Mechanism of inhibition

We observed that viral IE gene expression is reduced in cells expressing rhesus monkey TRIM5α. Therefore, TRIM5α acts at a very early stage in HSV infection. Because TRIM5α is located in the cytoplasm, it could be affecting viral entry, nuclear targeting of the nucleocapsid, uncoating at the nuclear pores, or translation of IE gene mRNA in the cytoplasm. TRIM5α could also be affecting IE and later gene transcription, but this would have to be indirect because we found no evidence that TRIM5α is re-directed into the nucleus in HSV-

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**Table 1**

<table>
<thead>
<tr>
<th>Virus</th>
<th>Yield on H-L and H-R cell lines*</th>
<th>Relative yields on H-L cells versus H-R cells</th>
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</thead>
<tbody>
<tr>
<td>H-R</td>
<td>H-L</td>
<td></td>
</tr>
<tr>
<td>HSV-1 KOS 7134 (ICP0-)</td>
<td>5.8 × 10^4</td>
<td>1.9</td>
</tr>
<tr>
<td>HSV-1 KOS 7134 (ICP0+)</td>
<td>6.8 × 10^4</td>
<td>2.4</td>
</tr>
</tbody>
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* The H-R and H-L cell lines were infected with HSV-1 KOS ICP0 null mutant or the 7134R rescued virus at an MOI of 1 PFU per cell and harvested after 24 h. Viral yields were measured on Vero cells.
infected cells. Rhesus monkey TRIM5α may alternatively prevent the VP16 virion transactivator from reaching the nucleus or prevent HSV ICP0 from entering the nucleus, as recently postulated for a cytoplasmic PML isoform (McNally et al., 2008). McNally et al. (2008) reported that cytoplasmic PML exerts its antiviral activity by decreasing viral gene expression via the cytoplasmic sequestration of ICP0. ICP0 is a multifunctional viral protein involved in several processes including the disruption of PML bodies (Everett et al., 2006) and modification of viral chromatin (Cliffe and Knipe, 2008; Gu and Roizman, 2007). ICP0 can shuttle between the cytoplasm and nucleus at later times postinfection (Kalamvoki et al., 2008; Lopez et al., 2001). Our immunofluorescence studies showed that cytoplasmic ICP0 co-localizes with rhesus-monkey TRIM5α suggesting that ICP0 may interact with the TRIM5α molecule. Thus it was conceivable that similar to cytoplasmic PML, TRIM5α may sequester ICP0 in the cytoplasm, preventing it from reaching the nucleus. However, replication of an ICP0-null mutant virus was reduced by TRIM5α to nearly the same extent as an ICP0+ virus; therefore, the mechanism of TRIM5α inhibition of HSV replication does not appear to require ICP0.

HIV-1 is inhibited by Old World monkey TRIM5α molecules (Song et al., 2005b; Yap et al., 2004), and this effect is believed to be due to altered uncoating of the HIV genome, by either accelerating uncoating (Strumlauf et al., 2006) or causing degradation of the capsid (Wu et al., 2006). If HSV uncoating is altered, it will be interesting to determine if the HSV major capsid protein has flexible loops similar to the HIV capsid cyclophilin-binding loop that can bind TRIM5α. This would be a candidate site for interaction between TRIM5α and the HSV infection process.

We observed that TRIM5α protein is lost during HSV infection, and HSV may have evolved to do this to limit the restrictive ability of TRIM5α. Because all forms of TRIM5α were reduced in HSV-infected cells regardless of their level of restrictive activity, the level of reduction seems to not be the primary determinant of activity. In addition, ICP0 was not required for the loss of TRIM5α from infected cells (not shown); thus, the reduction in levels of TRIM5α appears to occur by a different mechanism from the mechanism of PML degradation promoted by ICP0.

TRIM5α and PML

The PML protein may contribute to an antiviral mechanism that restricts HSV replication because depletion of PML increases viral gene expression (Everett et al., 2006), although overexpression of PML does not affect HSV replication (Lopez et al., 2002). However, the mechanism of this restriction due to PML has not been elucidated. PML is a component of nuclear domain 10 structures, which have been postulated to affect chromatin structure (Ishov et al., 1999). Based on the current evidence that it is a cytoplasmic protein, TRIM5α seems unlikely to affect nuclear processes directly, except that it may retain nuclear molecules like ICP0 or VP16 in the cytoplasm, as discussed above. Thus, the mechanisms of restriction of HSV by PML and TRIM5α may be very different.

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**Fig. 8.** Levels of TRIM5α in cells infected with HSV-1 or HSV-2. The H-R or H-L cells were infected with HSV-1 KOS strain (panel A) or HSV-2 186 syn+ strain (panel B) at an MOI of 3 PFU/cell or mock-infected. The infected cells were harvested at the times indicated, and Western blots were performed to detect TRIM5α.

**Fig. 9.** Levels of different TRIM5α proteins in cells infected with HSV-2 186 syn+ strain. The H-R, H-H, H-AGM, H-S and H-L cell lines were infected with HSV-2 186 syn+ strain at an MOI of 3 PFU/cell or mock-infected. The infected cells were harvested at 4 or 6 hpi. The resulting lysates were resolved by SDS-PAGE and a Western blot was performed with anti-ICP8 and HA antibodies. β-actin served as the loading control.
Host species specificity of the restriction

Based on our results, rhesus monkey and other Old World monkey TRIM5α proteins comprise a part of the explanation for the limited replication of HIV-1 observed in these cells. Replication of HIV-1 is also restricted by Old World monkey TRIM5α proteins, and the major changes in the B30.2 domain of Old World monkey TRIM5α proteins occurred millions of years before humans evolved (Song et al., 2005a). This report documents that TRIM5α affects DNA virus replication in addition to the previously well-documented role in affecting retrovirus replication. Therefore, TRIM5α may be part of a broader antiviral resistance mechanism that evolved very early to prevent viruses from crossing from one host species to another.

Implications for HSV studies in rhesus macaques

These results also have implications for the use of HSV vaccines and vaccine vectors in rhesus macaques. Infection of rhesus macaques with simian immunodeficiency virus (SIV) is one of the best nonhuman primate models for AIDS (Desrosiers and Ringer, 1989), and AIDS vaccines are being tested experimentally in rhesus macaques (Desrosiers, 2004; Feinberg and Moore, 2002; Morgan et al., 2008). We have tested HSV vectors expressing SIV proteins as AIDS vaccines in rhesus macaques and have found that they induce cellular and humoral responses that reduce SIV challenge viral loads and disease (Kaur et al., 2007; Murphy et al., 2000; Kaur et al., in preparation). The results from this study show that HSV infects rhesus monkey cells poorly and that part of the restriction in rhesus monkey cells is due to the TRIM5α protein. Other studies in progress have shown that HSV vectors show reduced protein expression in rhesus monkey cells (J. Sen and D.M. Knipe, manuscript in preparation). These results in total suggest that tests of HSV vectors in rhesus macaques may underestimate their immunogenicity in humans.

Materials and methods

Cells and viruses

Vero (ATCC) and U2OS (ATCC) cells were grown as described (Taylor et al., 2003). HeLa-derived cell lines that stably express TRIM5α variants (Song et al., 2005b; Stremlau et al., 2004) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco-BRL) with 5% heat-inactivated fetal calf serum (FCS), 5% heat-inactivated bovine calf serum (BCS), streptomycin (100 μg/ml) and penicillin (100 μg/ml) and supplemented with 1 μg/ml puromycin (Sigma). The HeLa-LPCX (H-L) cells contain the empty pLPCX vector; HeLa-R (H-R) cells express rhesus monkey TRIM5α; HeLa-H (H-H) cells express human TRIM5α; HeLa-Sq (H-Sq) cells express squirrel monkey TRIM5α; and HeLa-AGM (H-AGM) cells express African green monkey (Cercopithecus aethiops tantalus) TRIM5α. The TRIM5α orthologs are fused to epitope tags derived from influenza virus hemaglutinin (HA) for detection. With the exception of human TRIM5α, all TRIM5α proteins possess the HA tag at the N-terminus. In the human TRIM5α construct the HA tag is at the C-terminus.

The wild-type HSV-1 KOS strain and HSV-2 186 syn- were propagated on Vero cells. The HSV-1 7134 (ICP0-) and 7134 R (rescued) viruses (Cai et al., 1993) were propagated and titrated on U2OS cells. Vero cells were also used for plaque assays to measure virus yields obtained from HeLa cells that stably express TRIM5α variants.

Antibodies

Rabbit polyclonal anti-HA tag antibody (ab13834), mouse monoclonal: anti-ICP4 HSV-1 (ab6514), anti-ICP27 HSV-1 + HSV-2 (ab31631), and anti-β-actin (ab 8226) antibodies were purchased from Abcam, Inc. Mouse monoclonal anti-ICP27 antibody was purchased from Virxsys Corporation. Mouse monoclonal anti-ICP5 and mouse monoclonal anti-ICP0 HSV-1 antibodies were purchased from East Coast Bio, Inc. The 3-83 rabbit anti-ICP8 serum has been described (Knipe et al., 1987). Secondary antibodies conjugated to horseradish peroxidase were obtained from Santa Cruz Biotechnology Inc. Secondary antibodies conjugated to Alexa 594 and Alexa 488 dyes were obtained from Molecular Probes Inc.

Virus infection and viral yield assay

Cells were plated into culture dishes 24 h prior to virus infection to obtain 100% confluence at the time of infection. Cells were infected with the viruses in cold phosphate-buffered sodium (PBS) containing 0.1% glucose and 1% heat-inactivated bovine calf serum (FCS). After 1 h adsorption at 37 °C, the cells were washed as follows: once with PBS, once with low pH solution (40 mM citric acid, 10 mM KCl, 135 mM NaCl pH 3.0) to inactivate extracellular virus, twelve with PBS, once with DMEM containing 1% heat-inactivated fetal calf serum (FCS) and then the cells were maintained in the same medium for 24 h. At the time of harvesting, an equal volume of sterile milk was added to the infections. Cells were lysed by freeze–thawing and sonication (30 s), and the lysate was clarified by centrifugation (Taylor and Knipe, 2003). The cell lysate was stored at −80 °C until titration by plaque assay on Vero or U2OS cells.

Analysis of protein expression by western blotting

 Cultures were infected at a multiplicity of infection (MOI) of 3 plaque forming units (PFU)/ml. Cells infected with HSV-1 KOS were harvested at 4, 6 or 8 hpi. Cells infected with HSV-2 186 syn- were harvested at 4, 6 or 10 hpi. Cell lysates were prepared by lysis in SDS-PAGE sample buffer with Complete™ protease inhibitors (Roche). Equal amounts of cell lysate from each infection were analyzed by SDS-PAGE, and subsequent Western blot was performed as described previously (Gao and Knipe, 1989; Simpson-Holley et al., 2004).

Statistical analysis

Statistical analysis was performed using two-sided Student’s t-tests.

Immunofluorescence

H-L, H-R and H-H cells were seeded for immunofluorescence at 1.9 × 10^5 cells per well on glass coverslips in 24-well plates. Twenty-four hours later, cells were infected with HSV-1 KOS strain at an MOI of 3. At 4 and 8 hpi the cells were fixed and stained with antibodies specific for the HA tag or ICP0 as described previously (Simpson-Holley et al., 2004).

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