RNase L activity does not contribute to host RNA degradation induced by herpes simplex virus infection

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In early herpes simplex virus (HSV) infection, the virion host shutoff (vhs) protein mediates the degradation of mRNA and subsequent shutoff of host protein synthesis. It is unclear whether vhs acts alone or in concert with virus-induced cellular factors for this activity. This paper examines whether RNase L, a virally induced endoribonuclease, contributes to HSV-induced mRNA decay. Results showed that RNA degradation was comparable in wild-type and RNase L−/− cells, demonstrating that HSV-mediated RNA degradation is independent of RNase L activity. Furthermore, the data show that HSV-1 does not significantly induce RNase L activity in murine embryo fibroblasts.

During herpes simplex virus (HSV) infection, the product of the UL41 gene, known as the virion host shutoff (vhs) protein, causes the rapid shutoff of macromolecular synthesis. This 58 kDa phosphoprotein, packaged within the tegument of the virus, can exert its effect immediately upon infection (Fenwick & Clark, 1982; Read et al., 1993). Viruses lacking vhs do not cause rapid RNA destabilization (Fenwick & Everett, 1990; Read & Frenkel, 1983; Read et al., 1993; Smibert et al., 1992), exhibit only modest growth reductions in growth in tissue culture (Read & Frenkel, 1983), but are profoundly attenuated in vivo (Strelow & Leib, 1995, 1996). vhs non-specifically induces the destabilization of cytoplasmic cellular and viral mRNAs leading to the preferential translation of viral messages and efficient, sequential transition between the viral gene kinetic classes (Kwong & Frenkel, 1987, 1989; Kwong et al., 1988; Oroskar & Read, 1989; Read & Frenkel, 1983). Both tRNAs and rRNAs are resistant to vhs-mediated degradation and there is a preferential degradation of mRNA at regions of translation initiation through the interaction of vhs with the cellular translation factor elf4H (Feng et al., 2001). Several non-mutually exclusive models have been proposed for the mechanism of action of vhs. First, that the vhs protein is a messenger ribonuclease (RNase) itself; second, that it is part of a ribonuclease complex requiring activation by some additional cellular targeting protein; or third that it activates a latent cellular mRNAse or modifies mRNA rendering it susceptible to cellular RNase activity (Lu et al., 2001; Zelus et al., 1996). vhs is part of an integral ribonuclease component and it functions in the absence of other HSV proteins (Doherty et al., 1996; Jones et al., 1995). Cellular factors, however, are required for vhs-mediated degradation activity in vitro and in yeast (Elgadi & Smiley, 1999; Lu et al., 2001; Zelus et al., 1996). These studies support the idea that vhs is the only HSV protein necessary to cause shutoff but that a host cell component is likely involved in this nuclease complex.

Mammalian cells contain multiple RNases with little or no apparent specificity for any type of RNA. RNase L, a ubiquitous 2′,5′-oligoadenylate (2-5A)-dependent endoribonuclease, is present at low levels in an inactive form (Silverman, 1997). When activated, it mediates both the antiviral and the anticytokine effects of interferon (IFN) through the 2-5A RNA degradation pathway by the decay of both viral and cellular ssRNA. Enhanced levels of IFN, stimulated by virus infection, increase the levels of both 2-5A synthetase and RNase L. The synthetases are activated by viral dsRNA to produce 2-5A. 2-5A then converts RNase L from its inactive to its active form. Several viruses, including HSV, have specific mechanisms to evade the antiviral effects of 2-5A and RNase L (Cayley et al., 1982, 1984; Martinand et al., 1999). Specifically, HSV activates RNase L only minimally in HSV-infected, IFN-treated human conjunctival (Chang) cells because HSV induces synthesis of 2-5A derivatives, which are only weak activators of RNase L relative to authentic 2-5A. Such derivatives would therefore be predicted to antagonize RNase L activation, although it is unknown whether other 2-5A derivatives are also synthesized in other cell types. Other studies have shown that HSV can inhibit the IFN signalling pathway, which in turn would inhibit RNase L activation (Mossman et al., 2001; Yokota et al., 2001).
Studies of HSV infection and RNase L activity are conflicting. One study of HSV infection of RNase L-deficient mice using the mouse ocular model revealed little or no alterations in growth or virulence (Leib et al., 2000). This is consistent with the ideas that either RNase L plays no role in limiting HSV replication in this infection model or more likely, as shown previously, that HSV has some mechanism to overcome the effect of RNase L (Cayley et al., 1984). Another study has shown, however, that RNase L plays an important role in controlling herpetic disease in vivo, with significantly increased mortality and stromal keratitis in RNase L knockout mice compared to controls (Zheng et al., 2001). There were two goals of this study: first, to examine the contribution of the virus-inducible RNase L to the activity of vhs; second, to examine the extent to which HSV can induce RNase L activity in primary mouse cells.

To assess the role of RNase L in mediating RNA degradation in HSV infection, total cytoplasmic RNA was prepared (Smith et al., 2000) from monolayer cultures of infected RNase L−/− murine embryo fibroblasts (MEFs) or control RNase L+/+ MEFs, as described previously (Zhou et al., 1997). Cells were mock- or virus-infected at an m.o.i. of 20 with wild-type HSV-1 (strain KOS) or UL41NHB, a vhs-null virus (Strelow & Leib, 1995), in the presence or absence of actinomycin D (10 mg ml−1, Sigma). The use of this RNA synthesis inhibitor allowed us to evaluate the degradation of finite pools of RNA induced by only preformed tegument-derived vhs. Cytoplasmic RNAs were harvested at 8 h post-infection (p.i.) and analysed for mRNA degradation by Northern blot analysis by probing for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Fort et al., 1985). GAPDH is a stable mRNA that allows the distinction between mRNA instability and vhs-induced destabilization. Other mRNAs that have been tested, however, show susceptibility to vhs (Becker et al., 1993). Filters were first probed for GAPDH, stripped and then reprobed for the 28S ribosomal subunit as a loading control. The level of GAPDH for mock-infected cells was set at 100 % and compared with the 28S-normalized GAPDH values of virus-infected cells. Vero (African green monkey kidney) cells served as a control for this assay because of the strong mRNA degradation induced by KOS at 8 h p.i. in this cell line. A representative Northern blot probed for GAPDH mRNA, stripped and then reprobed for the 28S ribosomal subunit is shown (Fig. 1A). In combined results from three independent experiments, infection with KOS led to significant and similar decreases in the amount of remaining GAPDH message irrespective of cell line (Fig. 1B). The average remaining GAPDH RNA was 57 % in Vero cells, 35 % in RNase L+/+ MEFs and 49 % in RNase L−/− MEFs, all relative to mock-treated cells. The difference between virus-induced degradation of GAPDH in RNase L+/+ and RNase L−/− MEFs was reproducible but not statistically significant (P>0.1 by Student’s t-test). As expected (Strelow & Leib, 1995), UL41NHB failed to induce degradation of GAPDH in all cell lines tested (Fig. 1B). Additional experiments in which the degradation of GAPDH was examined at 4 and 12 h p.i. in RNase L+/+ and RNase

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**Fig. 1.** RNA degradation assay by Northern blot analysis. Cytoplasmic RNA was extracted from mock-, KOS- and UL41NHB (vhs-null mutant)-infected Vero, RNase L+/+ and RNase L−/− cells at 8 h p.i. (A) Autoradiographic images show a representative Northern blot probed for GAPDH mRNA (top) and the same blot stripped and reprobed for the 28S ribosomal subunit (bottom). (B) Combined results from three independent experiments using actinomycin D (10 mg ml−1) showing the averaged per cent of GAPDH mRNA remaining relative to 28S rRNA-normalized, mock-infected cells. (C) Combined results from three independent experiments without actinomycin D showing the averaged per cent of GAPDH mRNA remaining relative to 28S rRNA-normalized, mock-infected cells. Error bars represent SD.
L−/− MEFs demonstrated no differences in the kinetics of vhs-mediated shutoff activity in the presence or absence of RNase L (data not shown). These results demonstrate that RNase L is not required for rapid shutoff mediated by the HSV-1 vhs protein. Moreover, there was no degradation of 28S rRNA observed in any of the HSV-infected cells, indicating that, as shown previously (Cayley et al., 1984; Strelow & Leib, 1995), HSV-1 is a poor inducer of RNase L in these cells in the presence or absence of actinomycin D (Fig. 1A and data not shown). Control experiments performed with transfection of poly(rI):poly(rC) resulted in almost complete degradation of 18S and 28S ribosomal subunits (data not shown), demonstrating that, as expected, RNase L activity can be robustly induced in cultured RNase L+/+ MEFs (Zhou et al., 1997).

To address the caveat that the use of actinomycin D may prevent the activation of RNase L, thereby masking its contribution to RNA degradation, the RNA degradation assay was also performed in the absence of this drug. The combined results of three independent infections performed in the absence of actinomycin D are shown (Fig. 1C). Infection with KO2G3 resulted in a significant increase in GAPDH. The average remaining levels of GADPH in KOS-infected cells were 37% in Vero cells, 21% in RNase L−/+ MEFs and 37% in RNase L−/− MEFs, all relative to mock-treated cells. As above, the difference between virus-induced degradation of GAPDH in RNase L−/+ and RNase L−/− MEFs was reproducible but not statistically significant. Again, as expected, there was little detectable degradation in UL41NB-infected cells when compared to KOS-infected cells (Fig. 1C). A comparison of the actinomyacin D-treated and untreated experiments reveals a greater level of RNA degradation in untreated cells than in treated cells infected with KO2. This is as expected because degradation in the presence of actinomycin D is mediated by tegument-derived vhs alone, whereas in the absence of actinomycin D degradation is caused by tegument and de novo-synthesized vhs protein.

The results of this study indicate that RNase L is only very modestly activated in MEFs infected with HSV-1 and that its contribution to RNA degradation mediated by vhs is minimal. There was a reproducible (although statistically non-significant, P > 0.1 by Student’s t-test) increase in RNA degradation in infected RNase L+/+ compared with infected RNase L−/− cells, and this small increase was independent of vhs. This most likely reflects RNase L activity in these cells, although this activity is low under these conditions relative to vhs in infected cells. Indeed, this activity is more noticeable in cells infected with the vhs-null virus and can only be discerned experimentally when RNA degradation is measured in RNase L+/+ in parallel with RNase L−/− cells. These are essentially consistent with previous results that showed a lack of RNase L activation in HSV-infected Chang cells due to the production of inhibitory molecules by the virus, although it was also shown that activation of RNase L prior to HSV infection can inhibit subsequent replication and disease (Cayley et al., 1984; Fujihara et al., 1989).

Relating these data to in vivo studies in RNase L−/− mice is more complex. The MEFs used in this study were derived from the same line of RNase L−/− and congenic control mice used in the previous in vivo studies. One study showed a significant increase in keratitis and mortality and decreased apoptosis in RNase L−/− mice following corneal infection with HSV-1 strain McKrae compared to congenic control mice (Zheng et al., 2001). Virus replication was not reported. In contrast, the other study using HSV-1 strain 17 showed that virus replication in corneas and trigeminal ganglia following corneal infection and lethality following intracerebral injection were comparable in RNase L−/− and congenic control mice (Leib et al., 2000). These two studies therefore measured disparate parameters using different HSV strains, perhaps explaining the apparent differences in these previous data. The present study, however, suggests that the RNA degradation activity mediated by RNase L in infected cells may not be sufficient to account for changes in pathogenesis of HSV observed in vivo (Zheng et al., 2001). The interplay between RNase L and dsRNA-dependent protein kinase, another IFN-inducible antiviral molecule, may indeed be important in this respect (Khabar et al., 2000). Further studies of mouse models using mice deficient in multiple IFN-inducible antiviral pathways may be enlightening in this respect (Zhou et al., 1999).

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