The EMBO Journal Vol.17 No.24 pp.7161–7169, 1998

A viral activator of gene expression functions via the ubiquitin–proteasome pathway

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The ability of herpes simplex virus type 1 (HSV-1) to attain a latent state in sensory neurones and reactivate periodically is crucial for its biological and clinical properties. The active transcription of the entire 152 kb viral genome during lytic replication contrasts with the latent state, which is characterized by the production of a single set of nuclear-retained transcripts. Reactivation of latent genomes to re-initiate the lytic cycle therefore involves a profound change in viral transcriptional activity, but the mechanisms by which this fundamentally important process occurs are yet to be well understood. In this report we show that the stimulation of the onset of viral lytic infection mediated by the viral immediate-early (IE) protein Vmw110 is strikingly inhibited by inactivation of the ubiquitin–proteasome pathway. Similarly, the Vmw110-dependent reactivation of quiescent viral genomes in cultured cells is also dependent on proteasome activity. These results constitute the first demonstration that the transcriptional activity of a viral genome can be regulated by protein stability control pathways.

Keywords: HSV-1/latency/ubiquitin–proteasome pathway/Vmw110

Introduction

Herpes simplex virus type 1 (HSV-1) is an important human pathogen which owes its evolutionary success and clinical importance, at least in part, to its ability to attain a latent state in sensory neurones after an initial infection at the periphery (for reviews, see Fields *et al*., 1996). The latent genomes reactivate periodically, thus leading to recurrent episodes of disease. During lytic replication, the whole of the 152 kb viral genome is transcriptionally active as a consequence of the activity of viral regulatory proteins expressed during the immediate-early (IE) phase of infection. In contrast, during latency only one set of related RNAs (the latency associated transcripts) are transcribed at detectable levels (reviewed by Fraser *et al*., 1992). The bulk of the viral genome becomes repressed or quiescent during latency, a consequence which applies not only to all classes of lytic cycle viral genes, but also to many heterologous transcription units which have been introduced in order to utilize the virus as a vector. Despite the obvious significance of the regulatory mechanisms which govern the establishment of and reactivation from latency, as yet they are poorly understood.

The sequence of expression of viral genes during lytic infection is now well understood (for reviews see Fields *et al*., 1996). The virus particle includes protein VP16 which is required for the efficient activation of the IE genes. Because of this, viruses which are defective in the activation function of VP16 (such as *in*1814; Ace *et al*., 1989) have difficulty in initiating productive infection, especially at low multiplicity. The IE genes encode regulatory proteins which are required for normal activation of the early and late lytic cycle genes. The product of gene IE-3, Vmw175, is an essential transcriptional regulator. Mutations in this protein (such as the temperature-sensitive lesion *ts*K) cause a block in viral replication at the IE stage with consequent accumulation of IE gene products (Preston, 1979). The product of gene IE-1, Vmw110 or ICP0, is another important regulator of viral gene expression which forms the focus of this paper.

Several lines of evidence have indicated that Vmw110 is involved in the onset of virus infection and reactivation from latency. Vmw110 is a non-specific activator of gene expression, and viruses which express inactive forms of the protein have a defect in the onset of the lytic cycle that is dependent on the multiplicity of infection (m.o.i.) and cell type (reviewed by Everett *et al*., 1991). After low multiplicity infection of human fetal lung (HFL) fibroblast cells, Vmw110 mutant viruses preferentially attain a quiescent state of repressed genome expression from which they can be reactivated by provision of exogenous Vmw110 (Stow and Stow, 1989; Preston and Nicholl, 1997; Samaniego *et al*., 1998). This phenotype correlates with the reduced efficiency of reactivation of Vmw110 mutant viruses from latently infected mouse ganglia (Leib *et al*., 1989; Cai *et al*., 1993). Elucidation of the mechanisms by which Vmw110 stimulates lytic infection and reactivation from quiescence is therefore crucial for a proper understanding of HSV-1 biology. In addition, the use of HSV-1 as a vector for gene therapy requires a detailed understanding of how genome repression is regulated.

Vmw110 is a potent activator of expression from both viral and cellular reporter genes in transfected plasmids (reviewed by Everett *et al*., 1991). There is no evidence that it interacts specifically with either DNA or components of the RNA polymerase II machinery, and while it has been shown to increase the rate of transcription and not act at the post-transcriptional level (Jordan and Schaffer, 1997), it is not clear whether it acts directly or indirectly. On the other hand, an extensively studied and increasingly understood property of Vmw110 is its ability to localize to and then disrupt specific nuclear structures named nuclear domain 10 (ND10), promyelocytic leukemia (PML) bodies or PML oncogenic domains (PODs) (Maul

et al., 1993; Everett and Maul, 1994; Maul and Everett, 1994). The roles of ND10 are not well understood, but there is increasing evidence that PML, the prototype ND10 protein, is involved in the regulation of gene expression (Alcalay *et al*., 1998; LaMorte *et al*., 1998; Wang *et al*., 1998). ND10 are modified by stress and interferon (Maul *et al*., 1995), and become abnormal in the uncontrolled proliferating blast cells in patients with promyelocytic leukemia (Dyck *et al*., 1994; Koken *et al*., 1994; Weis *et al*., 1994). Infection with several DNA viruses, including HSV-1, human cytomegalovirus (HCMV) and adenovirus, also leads to modification of ND10 (Maul *et al*., 1993; Carvalho *et al*., 1995; Kelly *et al*., 1995; Doucas *et al*., 1996; Ahn and Hayward, 1997). Intriguingly, the parental genomes of all these viruses preferentially localize at ND10 during the earliest stages of infection, and subsequently viral transcription and replication begin at sites adjacent to ND10 (Ishov and Maul, 1996; Maul *et al*., 1996; Ishov *et al*., 1997). In the case of HSV-1, newly synthesized Vmw110 rapidly localizes to and then disrupts ND10, causing the dispersal of the constituent cellular proteins. Since the regions of the Vmw110 protein required for localization to and disruption of ND10 closely correlate with those required for its roles in the activation of gene expression and stimulation of viral replication (Everett *et al*., 1998, and references therein), it is a reasonable prediction that the mechanisms involved in ND10 disruption by Vmw110 are relevant to its role in the viral life cycle. However, hitherto there has been no direct evidence in support of this hypothesis.

Recently, we have shown that Vmw110 binds to a member of the ubiquitin-specific protease family of enzymes (Everett *et al*., 1997), and that Vmw110 induces the proteasome-dependent loss of a number high molecular weight isoforms of PML. The degradation of the PML isoforms correlates directly with the disruption of ND10 (Everett *et al*., 1998), and the endogenous PML isoforms that are affected by Vmw110 have gel mobilities which strongly resemble those of PML proteins that have been modified by covalent conjugation to the small ubiquitin-like protein known as PIC1 or SUMO-1 (Sternsdorf *et al*., 1997; Kamitani *et al*., 1998; Muller *et al*., 1998). Furthermore, Vmw110 also induces the proteasome-dependent loss of a number of uncharacterized cellular proteins that can also be modified by PIC1/SUMO-1 (Everett *et al*., 1998). These observations prompted us to examine the effect of the ubiquitin–proteasome pathway inhibitor MG132 on the ability of Vmw110 to modulate viral infection. We found that MG132 strongly inhibited the ability of Vmw110 to promote viral gene expression, replication and reactivation from quiescence. This finding is the first example of a regulator of viral gene expression functioning via the ubiquitin–proteasome pathway, and furthermore our results suggest, at least in principle, a mechanism by which reactivation of latent virus might be inhibited.

Results

The proteasome inhibitor MG132 reduces viral replication

Viruses which do not express functional Vmw110 are replication proficient if the infection is initiated at a high multiplicity of viral particles per host cell, but they are relatively uninfectious if used at low multiplicity. This multiplicity dependence varies widely with cell type. For example, the human osteosarcoma line U2OS plaques Vmw110-mutant viruses as efficiently as the wild-type virus (Yao and Schaffer, 1995), but the particle to plaque-forming unit (p.f.u.) ratio of the mutant is reduced by as much as 100-fold in baby hamster kidney (BHK) cells and by a further factor of up to 100-fold in HFL cells, although the exact extent of the defect can vary depending on the metabolic state of the cells. However, once the lytic cycle has started, Vmw110-mutant viruses express normal levels of viral proteins and produce normal yields of progeny virus particles (Stow and Stow, 1986; Sacks and Schaffer, 1987; Everett, 1989).

Table I. The affect of MG132 on virus yield in HFL and BHK cells

Cells	Virus	Input m.o.i.	Yield –MG132	Yield +MG132	Fold reduction
HFL	17+	0.5	2.3×10^8	2.6×10^5	880
HFL	17+	0.05	9.0×10^7	1.7×10^3	53 000
HFL	*dl*1403	5	4.0×10^5	1.3×10^4	31
HFL	*dl*1403	0.5	4.5×10^4	1.8×10^2	250
BHK	17+	0.05	1.7×10^8	3.7×10^7	4.6
BHK	17+	0.005	1.3×10^7	1.5×10^6	8.7
BHK	*dl*1403	0.05	1.9×10^6	9.3×10^5	2.0
BHK	*dl*1403	0.05	3.1×10^4	1.6×10^4	1.9

The multiplicity and cell-type-dependent phenotype of Vmw110-deficient viruses implies that Vmw110 affects the probability of successful onset of lytic infection, and in its absence genome shut-down preferentially occurs. Therefore, the true effect of Vmw110 is best assayed during low multiplicity infection of HFL cells, since in other situations its requirement can be bypassed. However, the cell-type and multiplicity dependence of the phenotype can be used as a tool to identify Vmw110 activity.

BHK and HFL cells were infected with wild-type virus and Vmw110-deletion mutant virus *dl*1403 (Stow and Stow, 1986) at a range of multiplicities of input virus and in the presence or absence of the proteasome inhibitor MG132. Progeny virus was harvested 20 h later (before there was any significant visible cytotoxicity of the drug on uninfected cells), then titrated on BHK cells. MG132 reduced the yield of wild-type virus from BHK cells by a factor of ~10, but in HFL cells the reduction was up to 50 000-fold (Table I). Importantly, the extent of the reduction was dependent on the multiplicity of the original infection, and MG132 inhibited the replication of *dl*1403 to a much lesser degree (Table I). These results show that inactivation of the ubiquitin–proteasome pathway inhibits viral replication in a manner that closely follows multiplicity and cell-type dependence of the requirement for Vmw110.

While it is possible that the results with the wild-type virus (but not *dl*1403) could be explained if MG132 caused greater cytotoxicity in HFL compared with BHK cells, examination of uninfected cells in the presence of the drug indicated that the two cell types exhibited signs of toxicity with approximately equal kinetics. Over the time course of these experiments, neither cell type was visibly affected by the drug (data not shown).

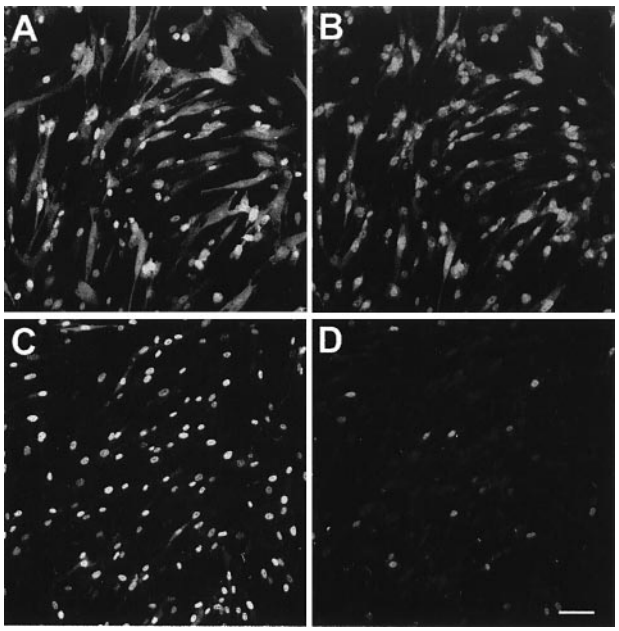

Fig. 1. Inhibition of Vmw110-stimulated viral gene expression by MG132. HFL cells were infected with wild-type HSV-1 strain 17+ at 0.5 p.f.u. per cell after no drug treatment (**A** and **B**) or in the presence of MG132 (**C** and **D**). The cells were fixed 8 h after infection and prepared for immunofluorescence. Vmw110 (left-hand panels) and UL29 (right-hand panels) were detected by simultaneous staining using Mab 11060 and serum r515 as described in Materials and methods. The bar in (D) represents 100 μm.

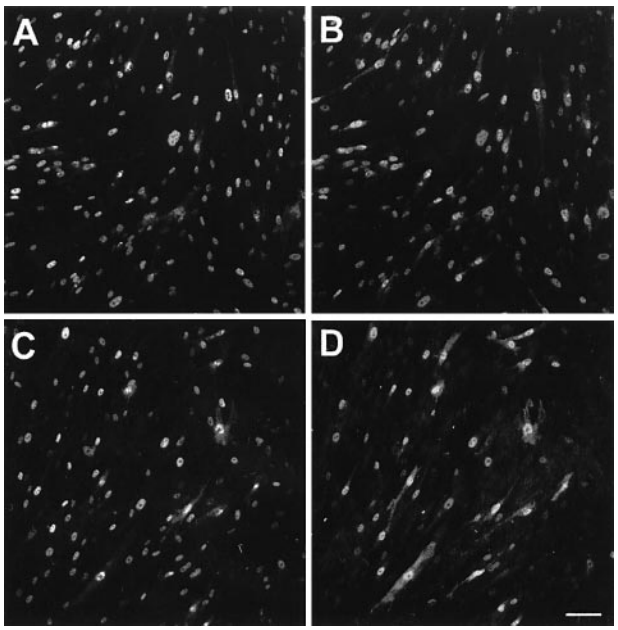

Fig. 2. MG132 has little effect on gene expression of Vmw110-null mutant *dl*1403. A similar experiment to that of Figure 1, using Vmw110 mutant virus *dl*1403 (at a nominal m.o.i. of 1 p.f.u. per cell). The cells were infected after no drug treatment (**A** and **B**) or in the presence of MG132 (**C** and **D**), then fixed 8 h after infection and prepared for immunofluorescence. Vmw175 (left-hand panels) and UL29 (right-hand panels) were detected by simultaneous staining using Mab 58S and serum r515 as described in Materials and methods. The bar in (D) represents 100 μm.

MG132 inhibits the onset of virus infection

To evaluate the role of Vmw110 more directly, we examined cells by immunofluorescence after infection at a relatively low multiplicity of 0.5 p.f.u. per cell. Eight hours after infection with wild-type virus, most HFL cells expressing Vmw110 were also expressing UL29, a DNA replication protein which is expressed during the middle and late stages of viral infection (Figure 1A and B). At this stage of infection of HFL cells, Vmw110 is present in both the nucleus and cytoplasm. In contrast, although the number of cells expressing Vmw110 was slightly reduced by MG132, the proportion of these that were expressing UL29 was drastically decreased (Figure 1C and D). Note that the MG132-induced retention of Vmw110 in punctate nuclear ND10 structures (Figure 1C) occurs as a result of inhibition of the Vmw110-induced disruption of these domains (Everett *et al*., 1998). Essentially similar results were obtained at earlier times of infection, but the proportion of cells expressing UL29 decreased with shortening periods of infection (data not shown). The fact that the drug did not cause a substantial decrease in the number of cells expressing Vmw110 implies that it does not significantly inhibit viral adsorption, penetration, uncoating or transcription of the IE-1 gene which encodes Vmw110. However, the majority of cells expressing Vmw110 did not go on to express later viral gene products, indicating that infection was becoming stalled at the IE phase. Counting positive cells showed that MG132 reduced the number of cells expressing Vmw110 and UL29 by ~1.2- and 20-fold, respectively.

After infection at even lower multiplicities, the virus spread to form plaques. However, in the presence of MG132 the number of cells expressing UL29 did not increase with time and little or no virus spreading occurred. One explanation for the failure of the virus to spread after several hours infection in the presence of drug could be that extended incubation with MG132 caused the cells to become uninfectable. However, pre-treatment with the drug for 16 h did not reduce further the proportion of cells expressing Vmw110 after virus infection (data not shown). Therefore the reduced virus yields (Table I) can be explained by a reduction in the number of cells initially engaged in productive infection, followed by subsequent reductions in the onset of secondary infections.

If MG132 was having a specific effect on Vmw110 activity, it would be expected to have lesser effects on infection of cell types which circumvent the requirement for Vmw110 and on the number of cells expressing viral proteins after infection with *dl*1403. Indeed, an analogous experiment using *dl*1403 at a multiplicity adjusted to give similar numbers of infected cells showed that MG132 had little effect on the number of cells expressing either the major IE transcriptional activator Vmw175 (the product of gene IE-3) or on the proportion of those cells expressing UL29 (Figure 2). The lack of effect of MG132 on *dl*1403 is consistent with the high particle to p.f.u. ratio of the mutant, which ensures that the infections are conducted at high multiplicity, thereby avoiding the requirement for Vmw110. Also as expected, MG132 had no significant affect on the proportion of infected U2OS cells which progress into the middle stages of infection (Figure 3). This result is consistent with the lack of requirement for Vmw110 for virus replication in this cell line (Yao and Schaffer, 1995).

While it would have been of interest to confirm that

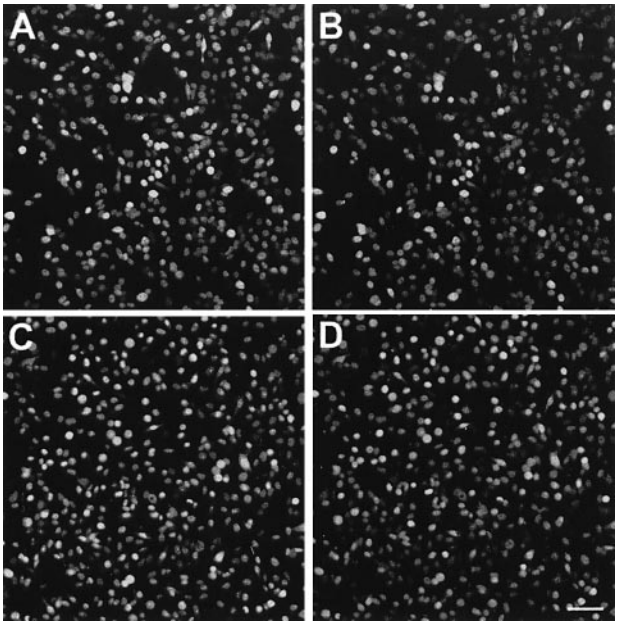

Fig. 3. MG132 has no effect on viral gene expression in a cell line that does not require Vmw110 for efficient viral infection. U2OS cells were infected by wild-type virus at a multiplicity of 0.5 p.f.u. per cell after no drug treatment (**A** and **B**) or in the presence of MG132 (**C** and **D**). The cells were fixed 8 h after infection and prepared for immunofluorescence. Vmw110 (left-hand panels) and UL29 (right-hand panels) were detected by simultaneous staining using Mab 11060 and serum r515 as described in Materials and methods. The bar in (D) represents 100 μm.

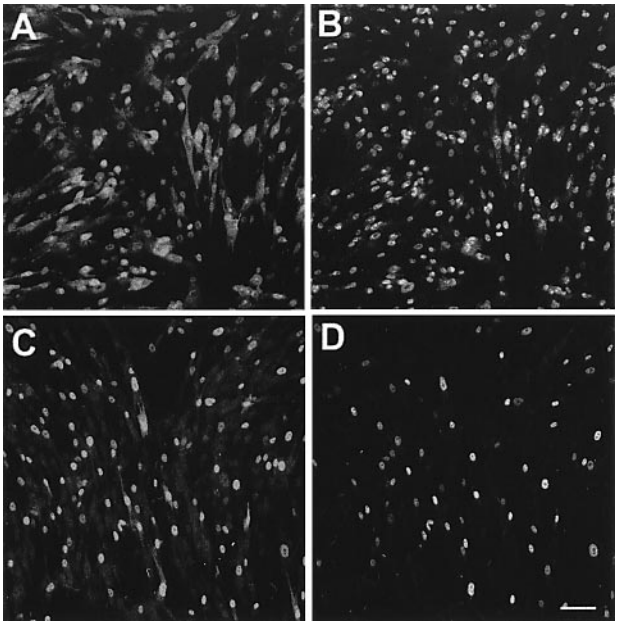

Fig. 4. MG132 decreases the proportion of Vmw110-positive infected cells also expressing Vmw175. HFL cells were infected with wild-type HSV-1 strain 17+ at 0.5 p.f.u. per cell after no drug treatment (**A** and **B**) or in the presence of MG132 (**C** and **D**). The cells were fixed 8 h after infection and prepared for immunofluorescence. Vmw110 (left-hand panels) and Vmw175 (right-hand panels) were detected by simultaneous staining using serum r190 and Mab 58S as described in Materials and methods. The bar in (D) represents 100 μm.

MG132 did not reduce progeny virus yield in U2OS cells in an experiment analogous to that shown in Table I, we found that the drug was more rapidly toxic to these cells, causing clearly detrimental effects before a round of virus replication could be completed. Therefore as a more general analysis of the effect of MG132 on virus replication, we used [^{35}S]methionine labelling and Western blotting to examine the expression levels of several viral proteins in U2OS cells infected in the presence or absence of drug. These were either unaffected or much less affected by MG132 compared with the effect in HFL cells (data not shown).

Because *dl*1403 does not express Vmw110 it was necessary to use Vmw175 as a marker for the IE phase of infection. To check whether this difference influenced the results, we repeated the experiment in Figure 1 with wild-type virus, but investigated the proportion of cells which were expressing both Vmw110 and Vmw175. Compared with the effect on Vmw110, MG132 had a significantly greater effect on the number of cells expressing Vmw175 (Figure 4). At first sight, the reduction of the number of cells expressing Vmw175 is surprising, since it would be expected to be under the same control as the IE-1 promoter. However, we note that Vmw110 can strongly stimulate the IE-3 promoter (O'Hare and Hayward, 1985), that the IE-3 promoter is less responsive to activation by the virion transactivator VP16 than the other IE promoters (Ace *et al.*, 1989), and that the phenotype of Vmw110 mutant viruses demonstrates that the probability of an infected cell expressing Vmw175 is dependent on functional Vmw110 (Stow and Stow, 1986; Everett, 1989). Therefore, the reduction in Vmw175 expression by MG132 can be explained by inhibition of the stimulation of the IE-3 promoter by Vmw110. Indeed, since Vmw175 and the other viral activators of gene expression are sufficient to induce normal levels of viral transcription in the absence of Vmw110 once infection has been properly initiated, if Vmw110 did not have a role in stimulation of IE gene expression it would be difficult to understand why *dl*1403 was defective.

To test whether MG132 had any direct effect on transcription from the IE-1 and IE-3 promoters, we utilized a pair of recombinant viruses which are multiply defective in VP16, Vmw175 and Vmw110, and carry β-galactosidase cassettes linked to the IE-1 and IE-3 promoters. As a further control, we included a similar virus with the HCMV promoter/enhancer driving the β-galactosidase cassette. Cells were infected with these viruses either alone, or in combination with UV-inactivated *ts*K virus (see below) to provide VP16. The infections were conducted in the presence or absence of MG132, then the cells were harvested for β-galactosidase assays. The results (Table II) confirmed that VP16 activates the IE-1 promoter more than the IE-3 promoter, and demonstrated that MG132 inhibited neither the basal nor the VP16-activated levels of either promoter; indeed, levels of basal gene expression from the three promoters tested were increased ~2-fold by MG132. These results are entirely consistent with the previous conclusion that the reduced expression of Vmw175 in MG132-treated infections (Figure 4) is due to inhibition of the stimulation of IE-3 expression by Vmw110, and this factor undoubtedly contributes to the reduced progression of infection into the middle phase (Figure 1).

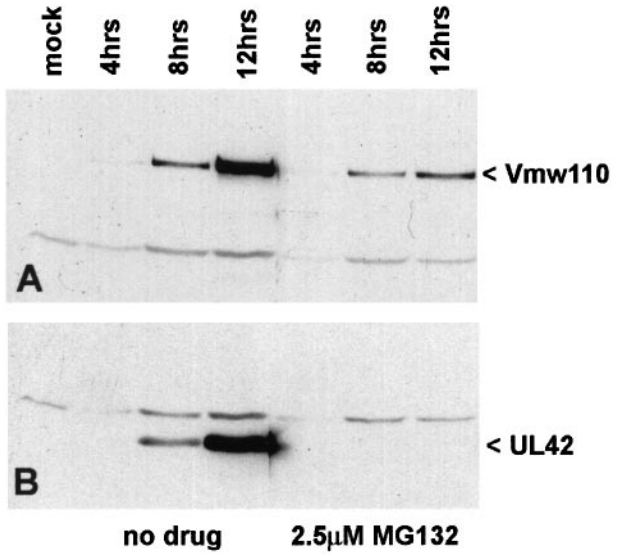

Fig. 5. MG132 inhibits the expression of UL42, a marker for the middle phase of virus infection. HFL cells were infected with HSV-1 strain 17+ at a multiplicity of 0.5 p.f.u. per cell in the absence or presence of MG132. Total cell proteins were harvested at 4, 8 and 12 h after infection, separated by electrophoresis on a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose by Western blotting. Vmw110 was detected using Mab 11060, then the blot was stripped and re-probed for UL42 using Mab Z1F11.

Table II. Effect of MG132 on IE-1, IE-3 and HCMV promoter activity

Virus	Promoter controlling lacZ	UV *ts*K	MG132	β-gal activity
*in*1354	IE-3 (−330 to +28)	−	−	84
*in*1354	IE-3 (−330 to +28)	−	+	330
*in*1354	IE-3 (−330 to +28)	+	−	1880
*in*1354	IE-3 (−330 to +28)	+	+	2590
*in*1355	IE-1 (−807 to +55)	−	−	185
*in*1355	IE-1 (−807 to +55)	−	+	337
*in*1355	IE-1 (−807 to +55)	+	−	29 500
*in*1355	IE-1 (−807 to +55)	+	+	23 600
*in*1352	HCMV (−750 to +3)	−	−	530
*in*1352	HCMV (−750 to +3)	−	+	1085
Mock	none	−	−	21

Monolayers of 1×10^6 HFL cells were infected at 38.5°C with the indicated viruses at a multiplicity corresponding to 10 infectious units per cell, in the absence or presence of UV-inactivated *ts*K at a nominal multiplicity of 1 p.f.u. per cell. 2.5 μM MG132 was present during the infections as indicated. Extracts were prepared for β-galactosidase assays after 6 h, and the means of duplicate samples are presented.

MG132 inhibits viral gene expression in a multiplicity-dependent manner

If MG132 was inhibiting the function of Vmw110, it would be predicted that the drug would have little or no effect on infections conducted at high multiplicity. To test this, we investigated total protein production by Western blotting. Following infection at the same multiplicity used for the experiment in Figure 1, we found that expression of UL42 (a viral protein which is expressed with kinetics similar to those of UL29) was not detectable in the presence of MG132, whereas the expression of Vmw110 was reduced only ~2-fold after 8 h of infection (Figure 5). The proportionately greater inhibition of Vmw110 expression seen at 12 h is due to infection spreading to neighbouring cells in the absence of drug. However, the affect

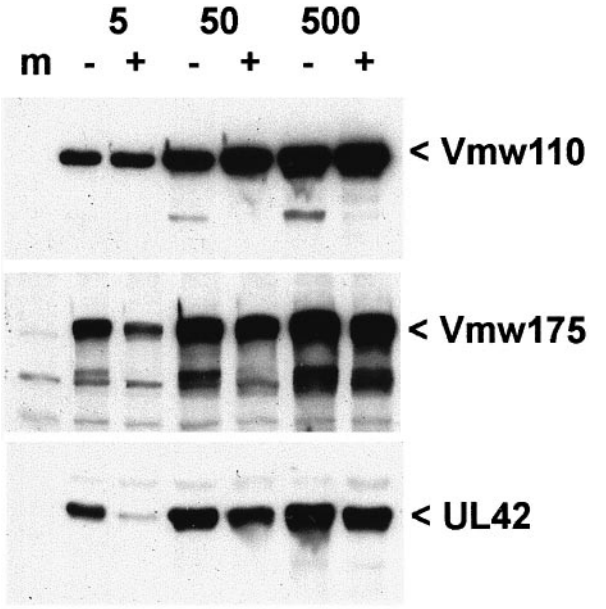

Fig. 6. Inhibition of UL42 expression by MG132 is relieved by high m.o.i. HFL cells were infected with HSV-1 strain 17+ at multiplicities of 5, 50 and 500 p.f.u. per cell in the absence or presence of MG132. Total cell proteins were harvested 4 h after infection, separated by electrophoresis on a 7.5% SDS–polyacrylamide gel and transferred to nitrocellulose by Western blotting. Vmw110 was detected using Mab 11060, then the blot was stripped and sequentially re-probed for Vmw175 and UL42 using Mabs 10176 and Z1F11 respectively.

of MG132 could be eliminated by increasing the m.o.i. (Figure 6), a result which is again entirely consistent with the multiplicity dependence of the requirement for Vmw110.

The results of this and the previous experiments show that the effect of MG132 on wild-type HSV-1 closely mirrors, in all aspects, the phenotype of Vmw110-deficient mutants. An important aspect of the interpretation of these experiments is that the inhibition of virus infection by MG132 is multiplicity-dependent in a way that reflects the requirement for Vmw110. However, it could be argued that rather than affecting Vmw110, MG132 inhibits virus infection in some other way that is bypassed at high multiplicity. To test this possibility it is necessary to use a virus which has a multiplicity-dependent defect that is independent of Vmw110. This is not a straightforward experiment. Mutations in essential IE, DNA replication or structural genes are lethal regardless of multiplicity. Viruses with lesions in the IE transactivation function of VP16 (such as *in*1814) have a defect which is overcome at high multiplicity, but their phenotype is complicated because the mutations result in reduced IE gene expression. However, if MG132 was causing a Vmw110-independent defect that can be overcome at high multiplicity, it should cause a similar result as that seen with *dl*1403 (Figure 2) and not inhibit *in*1814 gene expression. If the drug inhibits Vmw110 activity, it should inhibit *in*1814 replication in a similar way to inhibition of the wild-type virus (Figure 1). The results (not shown) indicated that MG132 resulted in similar reductions in the proportion of cells infected with *in*1814 and wild-type virus proceeding into the lytic cycle, with many *in*1814-infected cells becoming stalled at the IE stage. This result strongly suggests that MG132 is

inhibiting the activity of Vmw110, rather than some other process which can be overcome at high multiplicity.

MG132 inhibits the reactivation of quiescent viral genomes

The shut-down of viral gene expression which occurs during latent infection of animal models can in part be mimicked in cultured cells (Preston and Nicholl, 1997; Samaniego *et al*., 1998). Recombinant viruses have been constructed which carry a heterologous transgene and are defective in multiple viral IE genes (Preston *et al*., 1997, 1998). These viruses, which constitute prototype gene delivery vectors, are relatively non-cytotoxic and can be used to infect all the cells of a monolayer under conditions in which the lytic cycle fails to initiate. Such cultures maintain the viral genome for several days, but in time repression of all gene expression occurs so that even the transgene is silenced. Previous work has shown that the quiescent virus can be reactivated by provision of exogenous Vmw110 (Stow and Stow, 1989; Samaniego *et al*., 1998), so this system allows the investigation of the effect of MG132 on the ability of Vmw110 to relieve quiescent genome repression. HFL cells were infected with HSV-1 *in*1382, which is mutated in VP16, Vmw175 and Vmw110 and which expresses β-galactosidase from the HCMV promoter/enhancer. After 2 days at 38.5°C, shut-down of the viral genome had occurred and cells expressing β-galactosidase were extremely rare (~30 cells per plate; Figure 7A). To induce reactivation and test the requirement for Vmw110, parallel cultures were super-infected with HSV-1 mutants *ts*K and *in*1366 at the non-permissive temperature; *ts*K carries a temperature-sensitive lesion in Vmw175 so that it expresses only the IE gene products, including Vmw110 (Preston, 1979), while *in*1366 is a Vmw110-deficient derivative of *ts*K. Super-infection with *ts*K resulted in large numbers of cells expressing β-galactosidase (~10 000 cells per plate; Figure 7B), whereas *in*1366 had no effect (Figure 7C). MG132 strikingly reduced the reactivation induced by *ts*K, both in terms of the number of β-galactosidase-expressing cells (~200 cells per plate; Figure 7D) and β-galactosidase enzyme activity (Table III). As expected, the drug had no significant effect on the expression of Vmw110 by *ts*K (Figure 7E). In a similar manner to the lytic cycle experiments, we found that the effect of MG132 in this reactivation experiment could be overcome by using high multiplicities of the superinfecting virus, perhaps because in this situation very large amounts of Vmw110 are very rapidly synthesized.

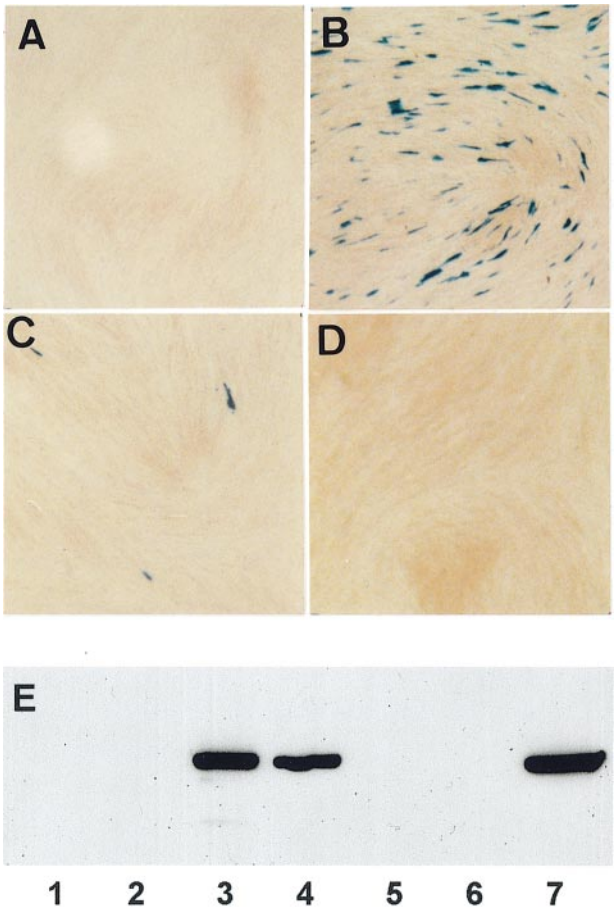

Fig. 7. MG132 inhibits the ability of Vmw110 to reactivate quiescent virus. HFL cells were infected as described in the Methods section. (A–D) Fields of cells equivalent to those used for samples 2–5, Table III. All had been initially infected with virus *in*1382, then either mock superinfected (**A**), or superinfected with *ts*K (**B**), *ts*K in the presence of MG132 (**C**) or *in*1366 (**D**). (**E**) Western blot of duplicate samples 1–7 (as detailed in Table III) probed for Vmw110 expression using Mab 11060.

Table III. Activation of β-galactosidase activity from quiescent virus in HFL cells

Sample	Virus 1	Virus 2	MG132	β-gal activity
1	mock	mock	−	1.0
2	*in*1382	mock	−	18.0
3	*in*1382	*ts*K	−	1184.0
4	*in*1382	*ts*K	+	37.0
5	*in*1382	*in*1366	−	19.0
6	*in*1382	mock	+	18.0
7	mock	*ts*K	−	2.0

Values are the mean of duplicate samples, expressed as arbitrary fluorescence units.

Discussion

These experiments show that inactivation of the ubiquitin–proteasome pathway inhibited the ability of Vmw110 to stimulate both onset of virus lytic infection and reactivation of quiescent viral genomes, and provide the first demonstration of a viral activator of gene expression functioning via the ubiquitin–proteasome pathway. The results clearly show how the activity of Vmw110 can be obstructed, and for the first time present the possibility of finding a specific inhibitor which blocks reactivation of latent HSV-1.

Previous work has shown that Vmw110 binds strongly and specifically to HAUSP, a ubiquitin-specific protease (Everett *et al*., 1997), and that it co-localizes with a number of cellular proteins in discrete nuclear structures termed ND10, PML nuclear bodies or PODs (Everett and Maul, 1994; Maul and Everett, 1994). The consequence of this co-localization is the disruption of ND10, a process which correlates with the proteasome-dependent loss of a number of modified PML isoforms (Everett *et al*., 1998). Consistent with the results presented here, Vmw110 mutants which fail to disrupt ND10 and induce the loss of the PML isoforms also exhibit reduced abilities to

stimulate the viral lytic cycle (Everett, 1989; Everett and Maul, 1994; Meredith *et al*., 1995). However, the effects of Vmw110 may not be limited to the components of ND10, since the PML isoforms which are targets for Vmw110-induced degradation are almost certainly conjugated to PIC1/SUMO-1, a protein related to ubiquitin which can be conjugated to a large number of nuclear proteins (Sternsdorf *et al*., 1997; Kamitani *et al*., 1997, 1998; Muller *et al*., 1998); many such modified proteins can be eliminated during virus infection in a proteasome- and Vmw110-dependent manner (Everett *et al*., 1998). Therefore, Vmw110 induces substantial changes in the intracellular environment which lead to increased virus infectivity and reactivation of quiescent genomes. The multiplicity of the potential targets of Vmw110 activity create problems in determining the significance of any individual examples, but it is possible to speculate on the underlying pathways.

The repression of the large segment of DNA which comprises the viral genome bears some similarity to the concept of silencing of chromosomal loci, and it is intriguing that the ubiquitin–proteasome pathway has been implicated in this process. For example, in yeast the ubiquitin-specific protease UBP3 interacts with SIR4 and regulates silencing at both telomeres and mating-type loci in yeast (Moazed and Johnson, 1996), while in *Drosophila* a ubiquitin-specific protease suppresses position-effect variegation in a dose-dependent manner (Henchoz *et al*., 1996). The simplest interpretation of the results presented here and previously is that the HSV-1 genome is being targeted for repression or silencing at the earliest stages of infection, and that Vmw110 acts to inhibit or reverse the repression. Host repression proteins may be targeting the incoming viral genome, initiating a race between shutdown and further transcriptional activation by viral *trans*-acting factors. If Vmw110 were to induce the degradation of the hypothetical repression proteins, the probability that transcriptional activation wins the race would be increased. At high multiplicities of infection, the number of viral genomes in the cell would overload the repression mechanism, thus eliminating the requirement for Vmw110. Another prediction of this model is that cell lines in which Vmw110-mutant viruses grow readily are defective in this repression mechanism; it may be significant that U2OS cells are highly transformed and very fast growing compared with HFL cells.

An extension of this model is that if the proteins required for repression are normally turned over to some extent by the proteasome pathway, treatment with MG132 may not only inhibit the activity of Vmw110, but also of itself stabilize the repression mechanism. In other words, Vmw110-deficient mutants such as *dl*1403 are also subject to repression, but this is overcome because one is forced to use a high m.o.i. However, reduction of the m.o.i. could reveal a further inhibition of *dl*1403 by MG132, a prediction supported by the results using HFL cells in Table I, and immunofluorescence experiments conducted with *dl*1403 at very low m.o.i. (data not shown).

If this model is correct, what could be the relationship between the relief from repression and the well established effect of Vmw110 on ND10? The mechanism of parental viral genome repression must be a *cis*-effect, which is occurring in their vicinity and not necessarily throughout the nucleus. Therefore it is relevant that in productive infections, parental genomes preferentially localize adjacent to ND10, the initial site of Vmw110 accumulation (Ishov and Maul, 1996; Maul *et al*., 1996). Accordingly, it is possible that silencing of the viral genomes in the absence of Vmw110 also occurs adjacent to ND10, thus providing a link between Vmw110 modification of ND10 and relief of genome repression. This hypothesis suggests that investigation of possible connections between Vmw110, ND10 and host repression or silencing proteins may be fruitful.

Materials and methods

Viruses and cells

HSV-1 strain 17+ was the wild-type virus used in these experiments, from which the Vmw110 null mutant virus *dl*1403 had been derived (Stow and Stow, 1986). Virus *ts*K contains a temperature-sensitive mutation in Vmw175, which results in failure to activate early and late gene expression and high-level production of the other IE proteins at the non-permissive temperature of 38.5°C (Preston, 1979). These viruses were propagated and titrated in BHK cells under permissive conditions. Viruses with multiple defects in IE genes and inserted transgenes were as follows:

*in*1382: Vmw175*ts*, VP16 activation negative, Vmw110 negative, HCMV lacZ.

*in*1366: Vmw175*ts*, Vmw110 negative.

*in*1354: Vmw175*ts*, VP16 activation negative, Vmw110 negative, vhs negative, IE-3 lacZ.

*in*1355: Vmw175*ts*, VP16 activation negative, Vmw110 negative, vhs negative, IE-1 lacZ.

*in*1352: Vmw175*ts*, VP16 activation negative, Vmw110 negative, vhs negative, HCMV lacZ.

The Vmw175*ts* mutation was from *ts*K. The VP16 activation-negative mutation was from *in*1814, which eliminates the IE activation function of VP16 without affecting its role in the virus particle (Ace *et al*., 1989). The Vmw110 negative mutation was a deletion inducing a frame-shift at residue 105. The vhs mutation is a deletion of HSV-1 nucleotides 91610–92545 which removes most of the coding sequences of the UL41 virion host cell shut-off factor. The lacZ cassette insertions were made in the thymidine kinase locus. The titres of these multiply defective viruses were determined by their ability to complement temperature sensitive mutant virus *ts*1201 at the non-permissive temperature. Further details of these viruses and their propagation are given in Preston *et al*. (1997, 1998) and Preston and Nicholl (1997).

All viruses were grown and titrated in BHK cells propagated in Glasgow modified Eagle's medium (GMEM) containing 100 units/ml penicillin and 100 μg/ml streptomycin, and supplemented with 10% newborn calf serum (NBCS) and 10% tryptose phosphate broth. Hep2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) and antibiotics as above. HFL cells (Imperial Laboratories) and U2OS cells (a kind gift from Tony Kouzarides) were propagated in DMEM supplemented with 5% FCS, 5% NBCS, 1% non-essential amino acids (Gibco-BRL) and antibiotics as above.

Virus replication assays

HFL and BHK cells were infected with wild-type strain 17+ and Vmw110 deletion mutant *dl*1403 viruses in the presence and absence of 2.5 μM MG132. The cells were pre-treated with drug for 1 h before infection, and where relevant continuous drug treatment was maintained. Progeny virus was harvested 20 h later by scraping the cells into the medium, the mixture was sonicated, then the titre was determined on BHK cells.

Immunofluorescence

HFL or U2OS cells (2×10^5 cells per coverslip in 24-well dishes) were infected with wild-type HSV-1 strain 17+ or Vmw110 mutant *dl*1403 as noted in the figure legends. Where relevant, 2.5 μM MG132 was present continuously, and the infections were initiated after a 30 min pre-incubation with drug. The cells were fixed with formaldehyde [5% v/v in phosphate-buffered saline (PBS) containing 2% sucrose] 8 h after infection and permeabilized with 0.5% Nonidet P-40 in PBS with

10% sucrose. The primary antibodies were diluted in PBS containing 1% NBCS. Antibodies were used at dilutions as follows: anti-Vmw110 Mab 11060: 1/1000; anti-UL29 r515 serum: 1/100; anti-Vmw175 Mab 58S supernatant: 1/100. Goat anti-mouse fluorescein isothiocyanate (FITC)-labelled (Sigma) and goat anti-rabbit Cy3-labelled secondary antibodies (Amersham) were used at 1/100 and 1/2000 respectively. After staining, the coverslips were mounted and examined in a Zeiss LSM 510 confocal microscope. Images of typical fields of cells captured using the 10× objective lens and the two channels were scanned separately to avoid channel overlap. Identical settings were used for data acquisition for pairs of FITC- or Cy3-labelled images in each figure. The images were compiled using PhotoShop.

Western blotting

SDS–polyacrylamide gels (7.5% acrylamide) were prepared and run in the Bio-Rad MiniProtean II apparatus, then proteins were electrophoretically transferred to nitrocellulose membranes according to the manufacturer's recommendations. After blocking in PBS containing 0.1% Tween-20 (PBST) and 5% dried milk overnight at 4°C, the membranes were incubated with primary antibody in PBST/5% dried milk at room temperature for 4 h, then washed in PBST at least 6 times before incubation with horseradish peroxidase-conjugated secondary antibody in PBST/2% dried milk at room temperature for 1 h. After extensive washing, the filters were soaked in Amersham ECL reagent and exposed to film. Antibodies were stripped from the membranes following the Amersham ECL protocol and the membranes were re-probed as necessary.

Antibodies

The anti-UL42 Mab Z1F11, anti-Vmw110 Mab 11060 and anti-Vmw175 Mab 10176 have been described previously (Schenk and Ludwig, 1988; Everett *et al*., 1993a,b). Rabbit polyclonal serum r515 recognizes UL29 (Marsden *et al*., 1996) and Mab 58S recognizes Vmw175 (Showalter *et al*., 1981). Anti-Vmw110 rabbit serum r190 was generated after immunization with a glutathione *S*-transferase fusion protein including residues 594–775. Horseradish peroxidase sheep anti-mouse and goat anti-rabbit antibodies were purchased from Sigma Immunochemicals.

Establishment and reactivation of quiescent viral genomes

HFL cells were infected at 38.5°C with virus *in*1382 at a multiplicity of 0.3 infectious units per cell. After incubation for 48 h at 38.5°C, the cells were superinfected with *ts*K (0.03 p.f.u. per cell) or *in*1366 (0.03 infectious units per cell) and incubation at 38.5°C was continued. Where noted, MG132 was present at 2.5 μM from the time of superinfection. After 8 h, cells were fixed and stained for β-galactosidase (Jamieson *et al*., 1995) or enzyme activities in cytoplasmic extracts measured. The values are the mean of duplicate samples, expressed as arbitrary fluorescence units.

Acknowledgements

The authors are grateful for the skilled assistance of Angela Rinaldi, the constructive criticism of Duncan McGeoch, and the production and supply of anti-Vmw110 rabbit serum r190 by Anne Cross. This work was supported by the Medical Research Council.

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Received July 2, 1998; revised September 21, 1998; accepted October 16, 1998

Note added in proof

Two recent papers provide evidence that may be relevant to the ideas put forward in the latter part of the Discussion. Lehming *et al.* (*Proc. Natl Acad. Sci. USA*, 1998, **95**, 7322–7326) and Seeler *et al.* (*Proc. Natl Acad. Sci. USA.*, 1998, **95**, 7316–7321) show that the ND10 protein sp100 interacts with heterochromatin protein (HP1), thereby suggesting for the first time a link between ND10 and chromatin dynamics.