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The ubiquitin–proteasome machinery is essential for nuclear translocation of incoming minute virus of mice

Carlos Ros* and Christoph Kempf

Department of Chemistry and Biochemistry, University of Bern, CH-3012, Bern, Switzerland
ZLB Behring, Wankdorfstrasse 10, 3000, Bern, 22, Switzerland

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Abstract

Minute virus of mice (MVM) infection is disrupted by proteasome inhibitors. Here, we show that inhibition of the ubiquitin–proteasome pathway did not affect viral entry and had influence neither on the natural proteolytic cleavage of VP2 to VP3 nor on the externalization of the N terminal of VP1. In both MG132-treated and untreated cells, MVM particles accumulated progressively in the perinuclear region. However, in MG132-treated cells, MVM was not able to penetrate into the nuclei, remaining blocked in the perinuclear region without capsid disassembly. MVM was similarly sensitive to MG132 in the two cell lines tested, A9 and NB324K. After releasing from the reversible MG132 block, MVM recovered the ability to translocate to the nuclei and replicate. Analysis of viral capsid proteins during internalization showed no evidence of capsid ubiquitination or degradation. We examined the effect of MG132 on two other parvoviruses, canine (CPV) and bovine parvovirus (BPV). Similarly to MVM, CPV infection was sensitive to MG132; however, BPV infection, as previously shown for adeno-associated viruses (AAVs), was not disturbed. These findings suggest that parvoviruses follow divergent strategies for nuclear transport, some of them requiring active proteasomes.

Keywords: Parvovirus; MVM; Proteasomes; Ubiquitin; Cytoplasmic trafficking; Proteasome inhibitors; Nuclear transport; Viral protein (VP)

Introduction

Minute virus of mice (MVM) is a small, nonenveloped, and icosahedral parvovirus that replicates in the nucleus of actively dividing cells (Cotmore and Tattersall, 1987). The genome consists of a linear, single-stranded DNA of approximately 5 kb organized into two overlapping transcription units. The left-hand unit, driven by the P4 promoter, encodes the nonstructural proteins NS1 and NS2, and the right-hand unit, driven by the P38 promoter, encodes the structural proteins VP1 and VP2 (Clemens and Pintel, 1988).

The cytoplasmic trafficking of MVM involves the endosomal network. We have previously shown that MVM entry involves a low pH-dependent entry pathway and that escape from the endosomal network is slow (Ros et al., 2002). Phospholipase A2 motifs have been found in the VP1 protein, which might be responsible for parvovirus entry (Dorsch et al., 2002; Li et al., 2001; Zadori et al., 2001), although it has been recently shown that there should be other factors involved (Suikkanen et al., 2003). The process by which the viral particles, once released into the cytoplasm, translocate to the nucleus remains unknown. There are nuclear localization signals present in VP1 and VP2 sequences (Lombardo et al., 2000; Tullis et al., 1993), which are thought to be essential for the transport of the intact or partially uncoated capsids to the nucleus for viral replication (Vihinen-Ranta et al., 1997, 2000, 2002). It is also known that components of the cytoskeleton are required (Ros et al., 2002; Vihinen-Ranta et al., 1998). During the cytosolic trafficking, adeno-associated viruses (AAVs) are degraded by the cellular proteasome machinery (Douar et al., 2001; Duan et al., 2000; Yan et al., 2002). Consequently, under proteasome blocking conditions, a significant enhancement of recombinant AAV transduction was observed. In addition, it was found that AAV-2 and -5 capsids are ubiquitinated during the infection (Yan et al., 2002). These observations gave rise to the hypothesis that

* Corresponding author. Department of Chemistry and Biochemistry, University of Bern, Freiestrasse 3, CH-3012, Bern, Switzerland. Fax: +41-31-6314887.
E-mail address: carlos.ros@ibc.unibe.ch (C. Ros).

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the cellular proteasomes decrease the efficiency of AAV transduction as they degrade a large proportion of AAV incoming particles. We have previously shown that differently to AAVs, proteasome inhibitors abolished MVM infection (Ros et al., 2002).

The interrelationships between proteasomes and viruses are very complex. Proteasomes do not always represent a degradative factor for viruses. There are various examples of unrelated viruses in which the proteasomes are required during the replication cycle. Some retroviruses, such as human immunodeficiency virus, require the proteasome for budding from cells. In contrast, the release of other retroviruses like equine infectious anemia virus or mouse mammary tumor virus does not require the proteasomes (Ott et al., 2002, 2003; Patnaik et al., 2000, 2002; Schubert et al., 2000; Strack et al., 2000). For HIV-1 and -2, the processing of the Gag polyprotein into its mature proteins by the viral protease is also reduced when it is produced from cells with inactivated proteasomes (Schubert et al., 2000). There are various examples of viruses that modulate proteasome activity for their own benefits. Those modulations are carried out by viral gene products. The human cytomegalovirus pp71 protein stimulates quiescent cells to enter the cell cycle by targeting the hypophosphorylated forms of the Rb family for proteasome-dependent degradation inducing cell cycle progression (Kalejta and Shenk, 2003; Kalejta et al., 2003). The V protein of simian virus 5 and human parainfluenza virus type 2 blocks IFN signaling by targeting STAT1 or STAT2 (host cell transcription factors essential for both IFN-α/β and IFN-γ signaling) for proteasome-mediated degradation (Andrejeva et al., 2002; Didcock et al., 1999). Three viral proteins of Epstein–Barr virus, the nuclear antigen 1 and the latent membrane proteins-1 and -2A, regulate viral latency by manipulating ubiquitin-dependent proteolysis (Dantuma and Masucci, 2003). The proteasome activity is important for the establishment of latency of herpes simplex virus 1 in neuronal cells (Eom and Lehman, 2003). Adenovirus E4-34 kDa requires active proteasomes to promote late gene expression (Corbin-Lickfett and Bridge, 2003). Proteasome inhibition reduces coxsackievirus replication through inhibition of viral RNA transcription and protein synthesis (Luo et al., 2003).

Using Western blotting, real-time PCR, and immunofluorescence microscopy, we have studied the role of the proteasomes in MVM infection. In MG132-treated cells, MVM virions move through the cytoplasm and gather as intact particles in the perinuclear region in a way similar to that of untreated cells, including the cleavage of VP2 to VP3 and the externalization of the N-terminal of VP1. However, in cells with inactive proteasomes, the perinuclear intact particles form aggregates without sings of capsid ubiquitination or degradation. From this point, the virions do not progress and the infection is blocked. Similarly to MVM, CPV but not BPV is sensitive to MG132, suggesting that CPV and MVM have a similar nuclear translocation strategy involving the ubiquitin–proteasome machinery, which clearly differs from that of AAV and BPV.

Results

MVM requires the activity of the proteasomes early after internalization

To clarify the role of the proteasomes in MVM infection, it was important to elucidate whether they are required early or late after viral internalization. We have previously shown in asynchronized A9 cells that MVM-DNA replication starts only 8–10 h after internalization, and that MVM endosomal trafficking is slow and can take up to 6–8 h to be complete for the bulk of viral particles (Ros et al., 2002). Therefore, if the proteasomes were required during the first hours of internalization, it would point out to a role during the cytoplasmic trafficking and before nuclear translocation and most probably involving the cytoplasmic proteasomes. A late requirement would point out to a role in MVM nuclear activity, like final steps of the uncoating process, replication, and transcription. To address this question, A9 cells were infected with MVM and MG132 (25 μM) was added and kept in the media for three different intervals, as shown in Fig. 1. The cells were incubated until 18 h post-infection, collected, and their total DNA extracted as specified in Materials and methods. Real-time PCR was used to quantify the amount of viral DNA. The results showed that MG132 was more efficient in blocking the infection when present during the first interval after internalization ($t = 0–6$ h). In contrast, a very limited effect was observed when

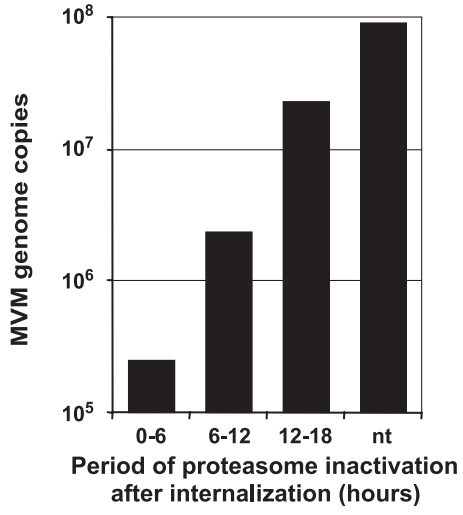

Fig. 1. Effect of proteasome inactivation at increasing periods of time after internalization. A9 cells (10^5) were infected with MVM at an MOI of 1000 DNA-containing particles/cell. Proteasomes were blocked at the indicated times by the reversible MG132 (25 μM). The cells were washed to remove the drug and further incubated until 18 h. Total DNA was extracted and MVM genome copies were detected and quantified by real-time PCR. Values represent the mean of three independent experiments. nt, not treated.

applied in the last interval (t = 12–18 h) (Fig. 1). Considering this result, it can be assumed that the requirement of the proteasomes is linked to the cytoplasmic trafficking of the virus and involving the cytosolic proteasomes.

Proteasomes are involved neither in VP2 proteolytic processing nor in the externalization of the N-terminal of VP1

During the cytoplasmic trafficking of MVM, two poorly characterized viral protein modifications occur, namely the cleavage of the N-terminal of VP2 and the externalization of the N-terminal of VP1. Because the 26S proteasome is involved not only in protein degradation but also in the proteolytic processing of proteins (Rape and Jentsch, 2002), it was therefore reasonable to investigate the potential role of the proteasomes in these two events. A9 cells were infected with MVM without detectable levels of VP3 (Fig. 2A) with and without MG132 (25 μM) as indicated above. After 5 h, cells were lysed in protein loading buffer and the proteins were resolved by SDS-PAGE. The results showed that the proteasome inhibitor MG132 did not prevent the cleavage of VP2 to VP3 (Fig. 2A). Because the virus used to infect the cells contained both full and empty capsids, a proportion of VP2 was not processed. To confirm this result, the VP2 cleavage was mimicked in vitro by digesting MVM-purified full capsids, without detectable levels of VP3, with α-chymotrypsin. The resulting viral capsids had only VP1 and VP3 proteins (Fig. 2B). The sensitivity of precleaved (VP1/VP3) and uncleaved (VP1/VP2) viral particles to MG132 was analyzed and compared. As it is shown in Fig. 2B, both viruses were similarly sensitive to MG132 and similarly infectious in untreated cells. These results indicated that the cellular proteasomes play no role in the natural cleavage of VP2 to VP3.

In addition, the possible role of proteasomes in the externalization of the N-terminal of VP1 was investigated. A double immunostaining experiment with an antibody against intact capsids (MAb B7) and an antibody against the N-terminal of VP1 was performed on MG132-treated cells. As it is shown in Fig. 2C, the N-terminal of VP1, which was not exposed 10 min after the infection, became exposed and accessible for the antibody at 8 h post-infection.

Proteasome inactivation blocks the nuclear penetration of MVM particles

To clarify the reasons for the blocking effect of MG132 in MVM infection, the intracellular distribution of MVM particles in A9 cells treated with MG132 was analyzed by

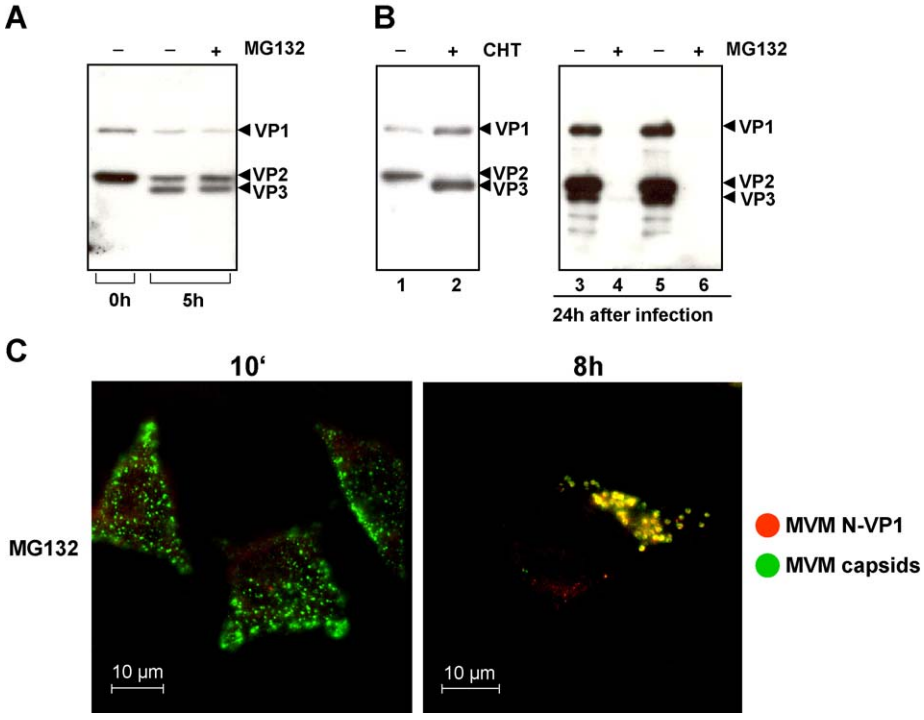

Fig. 2. Effect of proteasome inactivation on VP2 cleavage and N-VP1 externalization. (A) A9 cells (10^5) were infected with MVM at an MOI of 1000 DNA-containing particles/cell in the presence or absence of 25 μM MG132. At 0 and 5 h after infection, cells were lysed and viral proteins were resolved by 10% SDS-PAGE and detected with a rabbit anti-VPs polyclonal antibody (1:2000 dilution). (B) MVM capsids without detectable levels of VP3 (lane 1); MVM full capsids without detectable levels of VP2 generated by in vitro cleavage with chymotrypsin (lane 2); MVM from lane 1 (VP3 negative) after 24 h infection in A9 cells with and without MG132 (lanes 3 and 4); MVM from lane 2 (VP2 negative) after 24 h infection in A9 cells with and without MG132 (lanes 5 and 6). CHT; chymotrypsin. (C) A9 cells (10^5) were infected with MVM at an MOI of 5000 DNA-containing particles/cell in the presence of 25 μM MG132. Cells were fixed 10 min and 8 h after infection in a methanol–acetone solution (1:1) and stained with a mouse anti-capsid (detecting intact capsids) antibody (MAb B7; 1:200 dilution, in green) and a rabbit antibody against the N-terminal of VP1 (1:100 dilution, in red).

immunostaining and compared to untreated cells. In treated cells, MVM was able to internalize and to reach a perinuclear region with no difference to untreated cells, from the scattered distribution at 30 min post-infection to a polar perinuclear accumulation 4 h after infection. However, in MG132-treated cells, MVM was unable to penetrate into the nuclei and remained blocked in the perinuclear region (Fig. 3A). The strong signal observed at 24 h post-infection in untreated cells reflects the expected emergence of progeny virions. To exclude that the observed blocking effect of MG132 on MVM infection is due to a direct effect on the capsids by MG132 and not to the inactivation of the proteasomes, two other proteasome inhibitors, epoxomicin (15 μM) and aclarubicin (0.5 μM), were used. The results showed that both proteasome inhibitors were similarly able to block the nuclear penetration of MVM (data not shown). Because it was observed that in many cells, MVM appeared in a perinuclear polar region of the cytoplasm, resembling that of the Golgi apparatus, the possible colocalization of MVM and Golgi was investigated in A9 cells. Double immunostaining with an antibody against MVM VPs and an antibody against giantin (marker for the Golgi apparatus) did not show any area of colocalization after 3 and 8 h of internalization neither in cells treated with MG132 nor in untreated cells (data not shown).

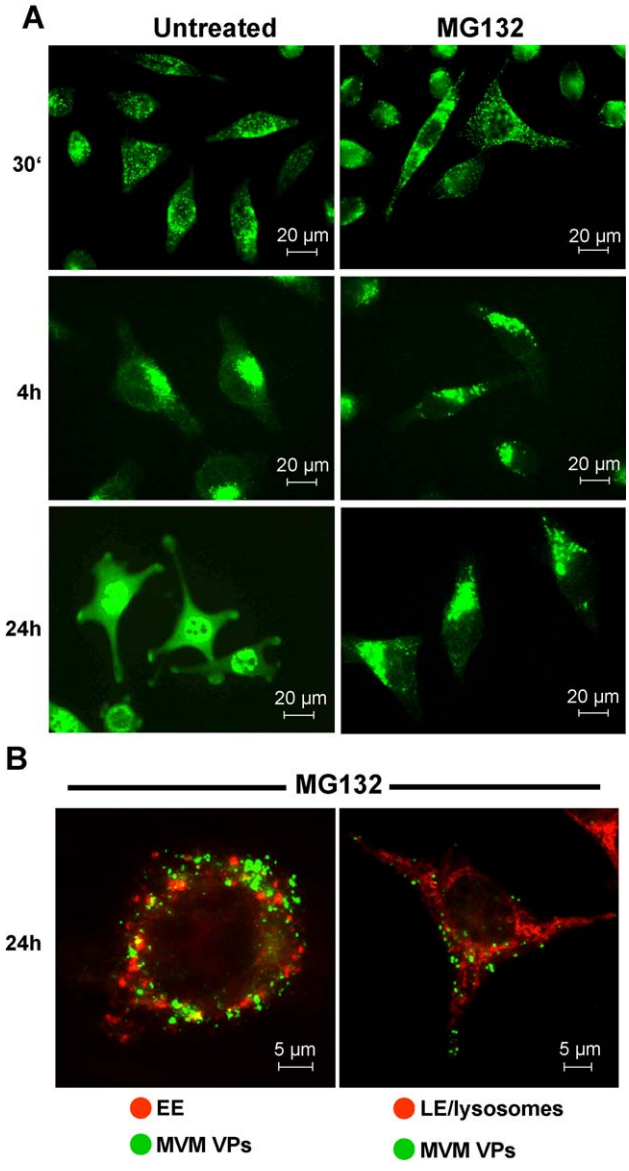

Fig. 3. Subcellular distribution of MVM under natural and proteasome blocking conditions. (A) Cells were infected with MVM in the presence or absence of 25 μM MG132. At different times, cells were washed, fixed, and stained with an antibody against MVM structural proteins (anti-VPs; 1:200 dilution, in green). (B) A9 cells were infected with MVM, treated with MG132, and fixed as indicated above. A double immunostaining was performed with a rabbit anti-VPs (1:200 dilution, in green) and a mouse antibody against early endosomes (EEA1; 1:100 dilution, in red) or a rat antibody against late endosomes or lysosomes (Lamp2; 1:200 dilution, in red).

The localization of MVM concerning endosomal vesicles in MG132-treated cells was examined. A double-labeling immunofluorescence analysis with an antibody against MVM and an antibody against EEA1, used as marker for early endosomes, and Lamp2, used as marker for late endosomes and lysosomes, was performed. The results showed that 24 h p.i. MVM did not colocalize with any of the two endosomal or lysosomal markers (Fig. 3B).

MVM incoming particles remain intact after 8 h post-internalization in both untreated and MG132-treated cells

With the aim of elucidating if the accumulated perinuclear particles are still intact or otherwise partially or totally uncoated, a double immunostaining experiment with a polyclonal antibody against MVM capsid proteins (VPs) and a monoclonal antibody against intact capsids (MAb B7) was performed. MAb B7 specifically recognizes MVM intact capsids as previously demonstrated by different immunological tests (Lombardo et al., 2000; Lopez-Bueno et al., 2003). We also tested the differential reactivity of these two antibodies in our studies. As it is shown in Fig. 4A, at 24 h post-infection in untreated cells, antibody B7 only recognizes the newly assembled particles inside the nuclei, although antibody against MVM VPs recognizes the assembled nuclear particles as well as the newly synthesized viral capsid proteins in the cytosol. Double immunostaining with these two antibodies at 8 h of internalization showed that MVM particles accumulated as intact particles in a perinuclear region in both untreated and MG132-treated cells (Fig. 4B). Interestingly, in MG132-treated cells, MVM intact particles were grouped around the nuclei as ring-like aggregates (Fig. 4B). Similar aggregates were also present in untreated cells but to a much lesser extent. In all cases, the rings were not present at the beginning of the infection, but became increasingly evident with the progression of the internalization (data not shown). Fig. 4C shows that the

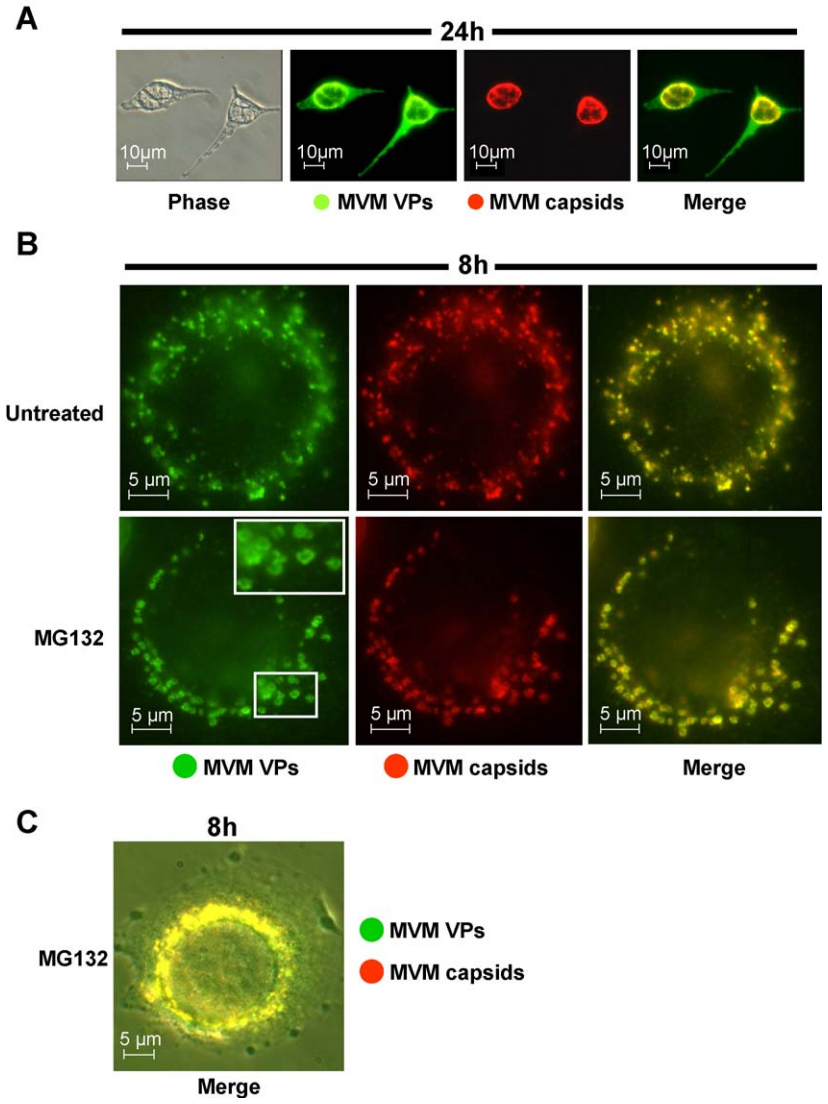

Fig. 4. Intact MVM particles accumulate around the nuclei at 8 h post-infection and aggregate in ring-like structures in MG132-treated cells. A9 cells were infected with MVM, fixed, and stained with a rabbit antibody against MVM structural proteins (anti-VPs) or a mouse antibody against intact capsids (anti-capsids). (A) Double immunostaining with anti-VPs (detecting structural proteins) and anti-capsid (detecting intact capsids) antibodies showing their differential reactivity, 24 h after infection in nontreated cells. (B) Nontreated and MG132-treated cells, 8 h after infection showing the perinuclear accumulation and the ring-like aggregates (C) Merge picture of anti-VPs, in green, anti-intact capsids, in red, and phase-contrast of an MG132-treated cell 8 h after infection, revealing the perinuclear accumulation of MVM.

accumulated perinuclear intact capsids are at the cytosolic side of the nuclear membrane.

The effect of MG132 on nuclear translocation of MVM is reversible

The action of MG132 on the proteasome can be fully reversed within 1 h after the withdrawal of the compound (Coux et al., 1996; Palombella et al., 1994). As to ascertain whether the effect of MG132 on MVM nuclear translocation can also be reversible, the drug was removed 8 h after MVM internalization and the incubation continued in MG132-free media until 24 h. Western blot analysis of MVM structural proteins after MG132 release showed the progressive recovery of MVM infection from 18 to 30 h post-infection (Fig. 5A). Double immunostaining with anti-VP and anti-capsid antibodies showed that in cells where MG132 was removed, MVM gained the ability to translocate to the nuclei and replicate. However, when MG132 was kept in the media, MVM did not penetrate into the nuclei remaining as intact particles in the perinuclear region (Fig. 5B). The recovery of the proteasome activity after withdrawal of MG132 was verified by analyzing the amount of the accumulated polyubiquitinated cellular proteins. Interestingly, polyubiquitinated proteins increased progressively up to 4 h after MG132 withdrawal, and subsequently decreased slowly reaching control levels by 24 h post-release (Fig. 5C).

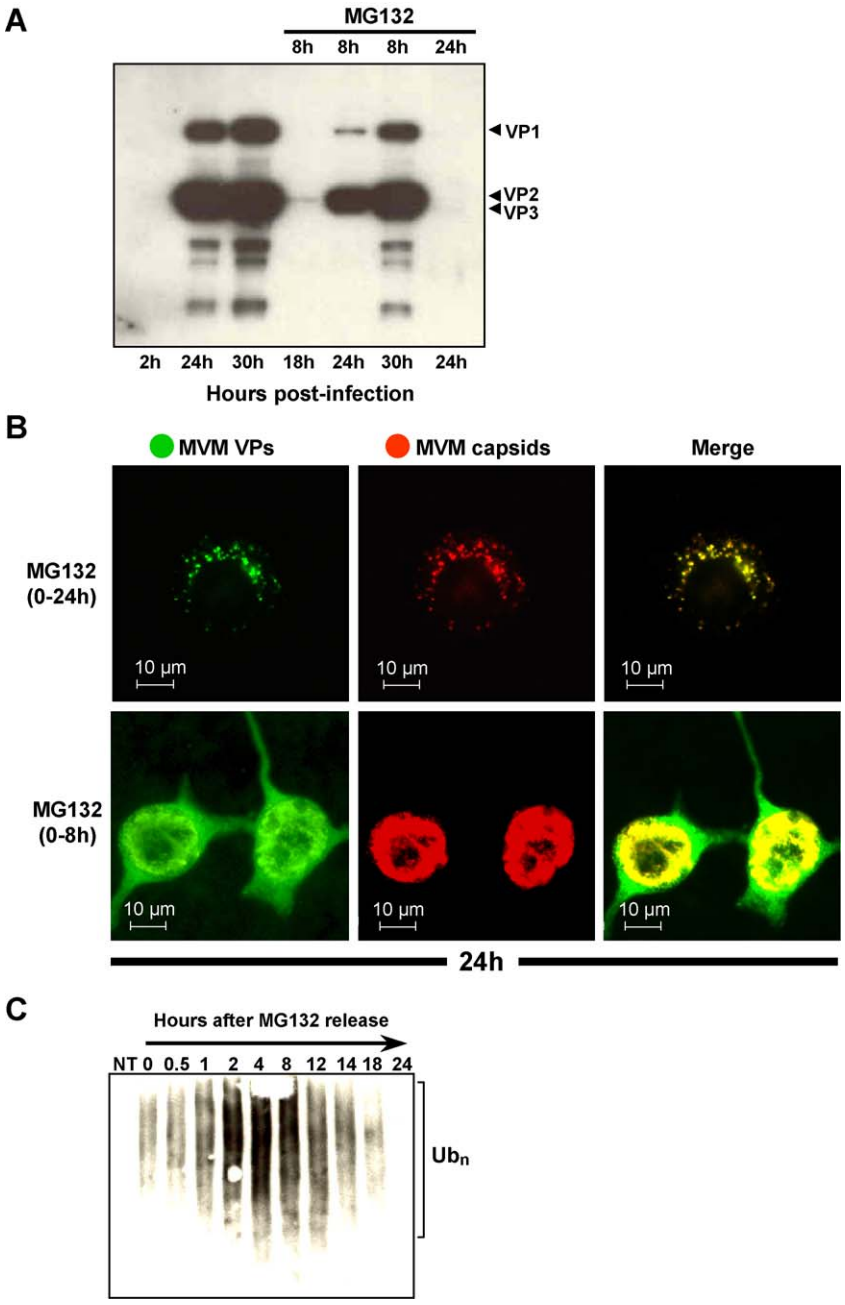

Fig. 5. The effect of MG132 on nuclear translocation of MVM is reversible. MG132 (25 µm) was removed 8 h post-infection and the incubation continued in MG132-free media. At various times after MG132-release, viral proteins were examined by Western blot (A) and immunofluorescence microscopy (B). (C) The recovery of proteasome activity after MG132 withdrawal was verified by detecting the amount of accumulated polyubiquitinated proteins at progressive times. NT; nontreated.

MVM capsids are neither ubiquitinated nor degraded during the intracellular trafficking

Proteins to be targeted to the proteasome are polyubiquitinated by the covalent attachment of ubiquitin. Monoubiquitination regulates internalization of receptors and endosomal sorting. However, there are few examples of proteins that target the proteasome by an ubiquitin-independent pathway (Asher et al., 2002; Kalejta and Shenk, 2003; Krappmann et al., 1996; Michalek et al., 1996; Miller and Pintel, 2001; Sheaff et al., 2000). We examined the possibility that MVM capsid proteins are ubiquitinated during the cytoplasmic trafficking of the virus. Immunoprecipitation experiments with an antibody against MVM VPs and Western blot with an antibody against ubiquitin (P4D1) did not demonstrate that capsid proteins from MVM were ubiquitinated in A9 cells treated with MG132 (Fig. 6A). The massive accumulation of polyubiquitinated protein conjugates observed in Fig. 6A demonstrates that the MG132-treatment effectively inactivated the proteasome function. In

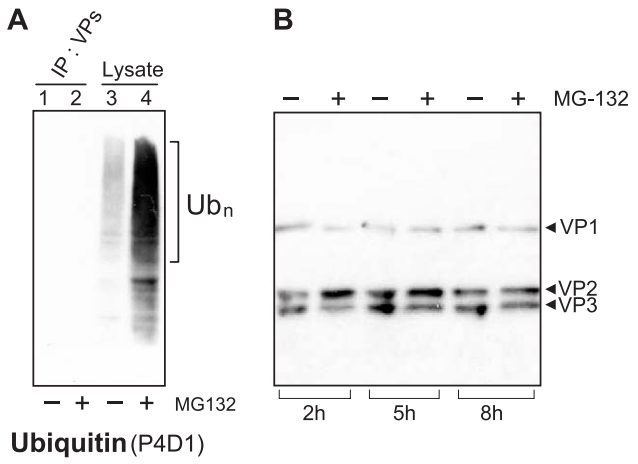

Fig. 6. MVM incoming capsids are neither ubiquitinated nor degraded during the cytoplasmic trafficking. (A) Detection of ubiquitin in samples immunoprecipitated with anti-VPs (lanes 1 and 2) and in total cell lysates (lanes 3 and 4). (B) Analysis of viral structural proteins in the presence and absence of MG132 at increasing periods of time after internalization.

addition, if virions would be ubiquitinated, then one would expect that treatment with proteasome inhibitors would cause an accumulation of ubiquitinated viral proteins, which would be readily identified by the increased molecular mass, as it has been shown for AAVs (Yan et al., 2002). However, change in the molecular mass of the MVM capsid proteins was not detectable in MG132-treated cells at any of the examined time points after internalization (Fig. 6B).

It has been previously shown that proteasomes disturb the infection of AAV-2 and -5 and thus they are considered as degradative factors for these viruses (Douar et al., 2001; Duan et al., 2000; Yan et al., 2002). It was therefore interesting to examine whether the proteasomes, although required for MVM infection, could still be able to degrade or proteolytically process a proportion of MVM incoming capsids. To address this question, the stability of the MVM structural proteins was examined at various periods of time during the intracellular trafficking of the virus, in the presence or absence of MG132. However, as it is shown in Fig. 6B, no evidence of protein loss or modification was observed, apart from the natural VP2 cleavage. This result emphasizes that the proteasome does not represent a degradative factor or a barrier but an essential part of the infectious pathway of MVM.

MVM is sensitive to MG132 in different cell lines

The effect of MG132 on MVM infection was examined in parallel in two different cells lines, the murine A9 and the human transformed NB324K cells, both permissive for MVM. The results showed that similarly to A9 cells, MVM infection was also sensitive to MG132 in NB324K cells (Fig. 7A).

MG132 blocks the infection of CPV but not that of BPV

It has been previously shown that adeno-associated virus (AAV) infections are not disturbed by proteasome inhibitors. On the contrary, under proteasome blocking conditions, AAV nuclear transduction is enhanced. It has been shown that in cells with active proteasomes, a proportion of incoming AAV particles are ubiquitinated and degraded (Douar et al., 2001; Duan et al., 2000; Yan et al., 2002). We show, however, that MVM incoming capsids are neither ubiquitinated nor degraded and that proteasome inhibitors block the nuclear translocation of the virus. These observations reveal an obvious difference between the cytoplasmic trafficking of MVM and AAVs. To further study this intriguing discrepancy, we examined whether the sensitivity of MVM to proteasome inhibitors is an isolated case within the parvovirus family. In this way, we investigated the effect of proteasome inactivation on two other parvoviruses, canine parvovirus (CPV) and bovine parvovirus (BPV). We selected these parvoviruses because CPV is closely related to MVM, and its cytoplasmic trafficking resembles that of MVM in many aspects, while BPV is distantly related (Lukashov and Goudsmit, 2001). Interestingly, CPV infection was also sensitive to MG132. However, BPV

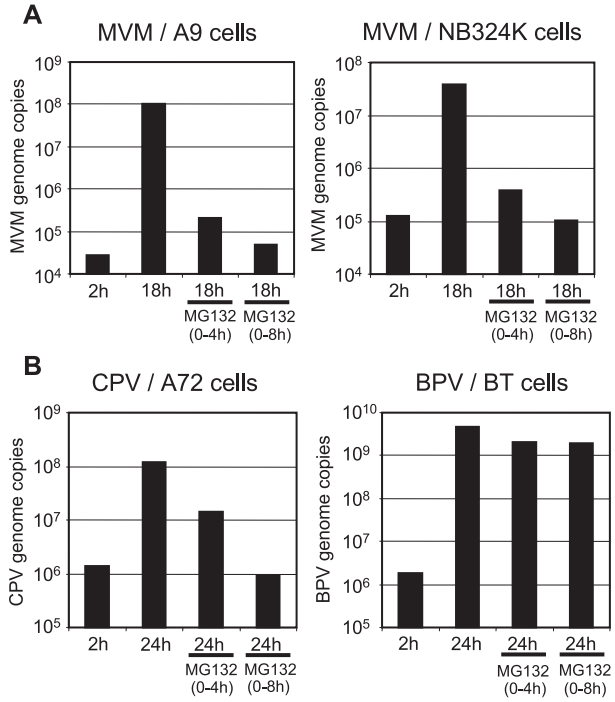

Fig. 7. Effect of proteasome inactivation on different cell lines and parvovirus infections. All cells were infected at an MOI of 1000 DNA-containing particles/cell in the presence or absence of 25 μM MG132. At 4 and 8 h post-infection, the cells were washed to remove the drug and further incubated until 18 h for MVM-infected cells or until 24 h for CPV and BPV-infected cells. Total DNA was extracted and MVM genome copies were detected and quantified by real-time PCR. (A) Proteasome inactivation disrupts MVM infection on A9 and NB324K cells. (B) CPV but not BPV is sensitive to MG132. Values represent the mean of three independent experiments.

infection was largely unaffected by treatments with MG132, regardless the time that the drug was present in the cells (Fig. 7B).

Discussion

Given the value of parvoviruses as gene delivering vectors for human therapy, it is crucial to identify the intracellular factors that enhance or disturb their nuclear translocation. The 26S proteasome is a large multi-subunit complex that selectively degrades intracellular proteins. It is involved in a wide variety of proteolytically mediated intracellular processes, such as cell cycle progression, apoptosis, metabolic regulation, and transcriptional control (Hilt and Wolf, 2000). An additional function of the proteasome is the processing of antigens generating peptides for the major histocompatibility complex (MHC) pathway (Hilt and Wolf, 2000; Niedermann, 2002; Rock and Goldberg, 1999). In this way, proteasomes would represent a degradative pathway and an obstacle for the infection, as it has been recently shown for adeno-associated viruses, in which a proportion of AAV-2 and -5 incoming viruses have been found to be ubiquitinated and degraded (Douar et al., 2001; Duan et al., 2000; Yan et al., 2002). In these studies, an enhanced AAV nuclear translocation was observed in the presence of MG132; however, the mechanisms of this enhancement were not elucidated. We have previously shown that differently to AAVs, MVM infection is not enhanced but disrupted in the presence of proteasome inhibitors (Ros et al., 2002).

In the present studies, we characterize various aspects of the MVM-proteasome interplay. We observed that MG132 was fully active against MVM when present at the beginning of the infection and a very limited effect was observed when present later after infection (Fig. 1). This observation indicated the involvement of the cytosolic proteasomes at the level of transport or trafficking of incoming particles toward the nuclei. This result was confirmed by observing that in cells with inactive proteasomes, MVM is unable to penetrate into the nuclei, remaining blocked in the perinuclear region (Figs. 3A and B), explaining the reason for the decreased or total absence of MVM-DNA or protein synthesis under proteasome blocking conditions. It was interesting to observe that in both treated and nontreated cells, the virus remained intact even 8 h after internalization (Fig. 4B). In case of MG132-treated cells, even at 24 h after internalization, the capsids remained intact in the perinuclear region (Fig. 5B). We know from our previous results that under similar conditions, viral DNA replication starts around 8 h after internalization in a population of asynchronized A9 cell and that both endosomal and proteasomal drugs are no longer effective if applied 8 h after internalization. This would indicate that for the bulk of MVM incoming particles, the endosomal and proteasomal pathways are concluded by that time. Therefore, we can assume that neither the endosomes nor the proteasomes are the sites of final uncoating and thus disassembling of the viral capsids should take place later, at the nuclear pore or inside the nuclei. This observation would be in agreement with the results obtained with CPV in which microinjection of an antibody that recognizes the intact CPV capsid into the cytoplasm of cells blocks the infection even when injected 8 h after virus inoculation (Vihinen-Ranta et al., 2002). It has also been shown that AAV-2 can efficiently enter intact nuclei purified from both permissive and less permissive cell types and that purified nuclei contain all of the machinery necessary for uncoating (Hansen et al., 2001).

The proteasomes are involved in the proteolytic degradation and processing of many proteins. Therefore, it was interesting to investigate the possible role of the proteasome in the proteolytic processing of VP2 to VP3, a natural cleavage observed during the internalization of MVM or in the externalization of the N-terminal of VP1. In the presence of MG132, VP2 was cleaved and the N-terminal of VP1 was externalized similarly to untreated cells. Thus, proteasome are not involved in these two events and their role in MVM infection should be different.

The majority of proteins that target the proteasome are tagged with polyubiquitin, which serves as a signal for their degradation. However, there are a limited number of examples of proteasome-dependent, ubiquitin-independent degradation of proteins (Asher et al., 2002; Kalejta and Shenk, 2003; Krappmann et al., 1996; Michalek et al., 1996; Miller and Pintel, 2001; Sheaff et al., 2000). A proportion of AAV-2 and -5 incoming viruses are ubiquitinated during the internalization process and degraded (Douar et al., 2001; Duan et al., 2000; Yan et al., 2002). Because ubiquitination is a signal to enter the degradative pathway of the proteasome, it is not surprising that MVM particles are not ubiquitinated because they are not degraded by the proteasome.

The differences observed among AAVs, BPV, CPV, and MVM regarding proteasome requirement and ubiquitination would suggest that parvoviruses follow divergent nuclear translocation strategies. For example, it is known that the cytoplasmic trafficking of AAVs is fast (Bartlett et al., 2000; Seisenberger et al., 2001) although that of MVM and other autonomous parvoviruses, like CPV, is slow (Ros et al., 2002; Vihinen-Ranta et al., 2002). Recently, several viruses were found to require active proteasomes. Retroviruses require the proteasome for budding from cells (Patnaik et al., 2000; Schubert et al., 2000; Strack et al., 2000), the human cytomegalovirus for inducing cell cycle progression (Kalejta and Shenk, 2003; Kalejta et al., 2003), the simian virus 5 and human parainfluenza virus type 2 for blocking IFN signaling (Andrejeva et al., 2002; Didcock et al., 1999), and Epstein–Barr virus and herpes simplex virus 1 for regulating viral latency (Dantuma and Masucci, 2003; Eom and Lehman, 2003). Adenovirus requires active proteasomes to promote late gene expression (Corbin-Lickfett and Bridge, 2003) and coxsackievirus for viral RNA trans-

cription and protein synthesis (Luo et al., 2003). The proteasome-MVM interplay described here has a unique feature, only shared with influenza virus, in that the requirement of ubiquitin–proteasome activity is at the early stages of the infection. It has been recently shown that influenza virus requires the proteasome for endosomal progression, and therefore, proteasome inhibitors block the infection inside endosomal vesicles (Khor et al., 2003). Although the blocked MVM incoming particles did not colocalize with EEA1 or Lamp2, we should however consider the possibility that MVM could be blocked inside an endosomal vesicle that is not recognized by the antibodies used in this study. Regarding this question, a more detailed characterization of the ring-like aggregates is anticipated. Interestingly, the exposure of the N-terminal of VP1, which is required for endosomal escape and nuclear penetration (Tullis et al., 1993; Vihinen-Ranta et al., 1997, 2002; Zadori et al., 2001), was not affected by proteasome inhibitors. Hence, there should be additional factor(s), besides the N-VP1 exposition, which are required for MVM nuclear translocation and which are dependent on the proteasome activity. The results obtained with other parvoviruses reveal divergent strategies of infection, where some parvoviruses like MVM and CPV require the activity of the ubiquitin–proteasome machinery, although other parvoviruses like AAV and BPV do not require such activity or it is even detrimental for the infection.

Materials and methods

Cells and viruses

Mouse A9 fibroblast (Littlefield, 1964; Tattersall and Bratton, 1983) and human NB324K cells (Shein and Enders, 1962) were propagated in Dulbecco's modified Eagle's medium (DMEM). Canine A72 cells were propagated in L-15 Leibovitz medium and bovine turbinate (BT) cells were maintained in minimal essential medium (MEM). The medium was supplemented with 10% of heat inactivated fetal calf serum (FCS), except for BT cells that were maintained with 10% horse serum. Virus stocks were titrated by using a standard $TCID_{50}$ technique and stored at −70 °C. MVM empty capsids, and DNA-full virions were prepared from MVM stocks as previously described (Hernando et al., 2000).

Chemicals and antibodies

MG132, epoxomicin, and aclarubicin were obtained from Calbiochem (La Jolla, CA), and α-chymotrypsin (TLCK-treated) was purchased from Sigma (St. Louis, MS). Rabbit anti-VPs (polyclonal against MVM structural proteins) and mouse anti-capsid (monoclonal against intact capsids, clone B7) were kindly provided by J.M. Almendral (Lombardo et al., 2000; Lopez-Bueno et al., 2003). Rabbit antibody against the N-terminal of VP1 was kindly provided by P. Tattersall (Cotmore et al., 1999). Mouse anti-giantin (monoclonal antibody against Golgi) was kindly provided by H.-P. Hauri. Mouse monoclonal against ubiquitin (P4D1) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse antibody against early endosomes (EEA1) was obtained from BD Transduction Laboratories (Lexington, KY). Rat antibody against late endosomes or lysosomes (Lamp2) was purchased from Calbiochem.

Real-time PCR

Quantitative PCR was used to detect and quantify viral DNA sequences. Primers for MVM-DNA amplification were forward: 5′-GACGCACAGAAAGAGAGTAACCAA-3′ (nucleotide position, 231–254) and reverse: 5′-CCAACCATCTGCTCCAGTAAACAT-3′ (nucleotide position, 709–732). Primers for CPV-DNA amplification were forward: 5′-CGCCAAAAAGCAAGTACAAAC-3′ (nucleotide position, 523–543) and reverse: 5′-ACCGAACAAAGAGTCACCAAC-3′ (nucleotide position, 745–765). Primers for BPV-DNA amplification were forward: 5′-CCATCTTG-GTCCTACGTCATC-3′ (nucleotide position, 390–410) and reverse: 5′-TGGCTCCAGCAACTAAATGTC-3′ (nucleotide position, 651–671).

Amplification and real-time detection of PCR products was performed on the DNA samples using the Lightcycler system (Roche Diagnostics, Rotkreuz, Switzerland) with SYBR Green (Roche). A melting curve analysis was performed for specific product identification. PCR was performed using the FastStart DNA SYBR Green kit (Roche) following the manufacturer's instructions. Cycling conditions consisted of a step at 95 °C for 10 min to activate the polymerase enzyme followed by 35 cycles with the following thermal profile: 94 °C/15 s, 66 °C (MVM-PCR); 61 °C (CPV and BPV-PCR)/5 s and 72 °C/30 s. As external standards, the PCR products generated by the above primers were cloned into the pGEM-T vector (Promega, Madison, WI) and used in 10-fold dilutions.

To asses the period of time after internalization at which the cellular proteasomes are required, A9 cells were infected with MVM and the effect of MG132, added at different intervals, was evaluated by real-time PCR. A9 cells were seeded in 12-well plates at 10^5 cells per well in Dulbecco's modified Eagle's medium supplemented with 10% FCS and incubated at 37 °C. Twenty-four hours later, cells were infected at an MOI of 1000 DNA-containing particles/cell in Dulbecco's modified Eagle's medium without FCS fetal calf serum for 30 min at 4 °C. The cells were subsequently washed to remove unbound virus and further incubated at 37 °C in the presence of MG132 (25 μM). At different intervals, as specified in Fig. 1, cells were washed to remove MG132 and further incubated until 18 h. At the end of the incubation period, cells were trypsinized and collected by low-speed centrifugation. Total DNA was extracted from the cell pellet by using the DNeasy tissue kit (Qiagen, Valencia,

CA). Control cells were similarly infected with MVM but did not receive any inhibitor, instead, they were treated with DMSO (the diluent used for MG132). Negative controls were included which were neither infected nor treated with any compound.

Immunofluorescence

A9 cells (10^5) were seeded onto coverslips inside 12-well plates. After 24 h, the cells were infected with MVM at 4 °C at an MOI of 5000 DNA-containing particles/cell. At different times, cells were washed with PBS and fixed in a solution of methanol–acetone (1:1) for 6 min at −20 °C. Cells were subsequently washed two times with PBS. A blocking solution of PBS–10% goat serum was added for 20 min and the cells were washed with PBS two times. Cells were incubated with antibodies in PBS containing 2% goat serum and incubated at RT for 30–45 min. The cells were subsequently washed several times in PBS and the secondary antibodies coupled with rhodamine or FITC were added for 30–45 min in PBS containing 2% goat serum. After final washings in PBS, the cells were rinsed in water and ethanol and mounted with mowiol (Calbiochem) containing 30 mg/ml of Dabco (Sigma), as antifading agent. The cells were examined by fluorescence microscopy. Images were captured with the proper filter sets and a 100× oil immersion objective.

Detection of viral structural proteins and ubiquitin

A9 cells (10^5) were infected and treated with drugs as specified in the figure legends. At various times after infection, cells were lysed in protein loading buffer. Proteins were resolved by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE). After transfer to a PVDF membrane, the blot was probed with a rabbit anti-VPs polyclonal antibody (1:2000 dilution, kindly provided by J. Almendral) or a mouse monoclonal against ubiquitin (P4D1, 1:200 dilution) followed by a horseradish peroxidase-conjugated secondary antibody (1:20 000 dilution). The proteins were visualized with a chemiluminescence system (Pierce, Rockford, IL).

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