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Recruitment and dynamics of proteasome association with rhTRIM5 α cytoplasmic complexes during HIV-1 infection

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Abstract

A variety of proteins have been identified that restrict infection by different viruses. One such restriction factor is the rhesus macaque variant of TRIM5 α (rhTRIM5 α), which potently blocks infection by HIV-1. The block to infection mediated by rhTRIM5 α occurs early after entry into the host cell, generally prior to reverse transcription. However, proteasome inhibitors reveal an intermediate step of restriction in which virus can complete reverse transcription but still fails to infect the cell. While proteasome inhibitors have been a useful tool in understanding how restriction takes place, the role of the proteasome itself during restriction has not yet been examined. Here, we characterize the interaction of rhTRIM5 α and incoming virions with the proteasome. We show that proteasomes localize to rhTRIM5 α cytoplasmic bodies, and this localization is more evident when the activity of the proteasome is inhibited pharmacologically. We also show that restricted virus associates with complexes of proteasomes and rhTRIM5 α , suggesting that rhTRIM5 α utilizes the proteasome during restriction. Finally, live cell imaging experiments reveal that virus associates with proteasomes, and proteasome inhibition affects the duration of association. Taken together, these studies implicate the proteasome as playing a functional role during rhTRIM5 α restriction of incoming virions.

Keywords

HIV; HIV-1; TRIM5; rhTRIM5 α ; restriction factor; cytoplasmic bodies; proteasome; live cell imaging; viral entry; IDL

Introduction

Members of the tripartite motif (TRIM) family(1) have been shown to block infection by a variety of viruses(2). Perhaps the most well characterized example of this is the resistance of rhesus macaques to infection by HIV-1, which is primarily mediated by a protein called TRIM5 α (3, 4). In addition to the characteristic RING finger, B-box, and coiled coil domains found in other TRIM family members, TRIM5 α also contains a C-terminal SPRY domain that provides the specificity for recognition of particular retroviruses(5–7). It is known that the rhesus macaque variant of TRIM5 α (rhTRIM5 α) restricts HIV-1 at an early step post-entry by interacting with the capsid core(3, 8, 9) soon after it has been deposited into the host cell cytoplasm. Recognition of the virus requires an intact capsid core(10, 11), but occurs regardless of whether entry into the host cell is mediated by the wild-type HIV-1 envelope or if the viral core has been pseudotyped with VSV-G(3). The interaction of the C-terminal SPRY domain of rhTRIM5 α with the capsid of HIV-1 usually results in a block prior to reverse transcription(3), as measured by quantitative PCR to examine late reverse

transcription products (Late RT products). However, carrying out infection in the presence of a pharmacological proteasome inhibitor such as MG132 reveals an intermediate stage of restriction in which virus is able to complete reverse transcription but is still unable to enter the nucleus and establish a productive infection(12, 13). A similar intermediate has been demonstrated with the use of chimeras containing TRIM5 sequences(14) as well as with mutations in the RING domain(15). This intermediate stage of restriction has been visualized within cells through the use of virus labeled with a fluorescent protein (mCherry-Vpr)(16, 17). In the presence of the proteasome inhibitor MG132, fluorescently labeled virions appear to be sequestered in accumulations of rhTRIM5 α known as cytoplasmic bodies(17, 18). Additionally, it has been shown that ubiquitin localizes to these rhTRIM5 α -containing cytoplasmic bodies(17), implicating the ubiquitin-proteasome system as playing a role during restriction. Proteasomes serve many important purposes in cells, such as degradation of misfolded proteins and regulation of short-live proteins(19). Several enzymes act in a coordinated fashion to attach ubiquitin to specific proteins to facilitate recognition and subsequent degradation by the proteasome. Several additional pieces of evidence connect rhTRIM5 α with the ubiquitin-proteasome pathway. The RING domain of rhTRIM5 α is able to act as an E3 ubiquitin ligase to conjugate ubiquitin to itself *in vitro*(20) and *in vivo*(15), and mutations that impair self-ubiquitination also lead to loss of restriction (3, 15). Furthermore, rhTRIM5 α undergoes accelerated proteasomal degradation in the presence of restricted virus(21), demonstrating that the action of the proteasome is altered during restriction, and again implicating self-ubiquitination of rhTRIM5 α in the mechanism of restriction. Recently it has been shown that the E3 ligase function of the African Green Monkey variant of TRIM5 α (TRIM5 α _{AGM}) is required for restriction of SIV_{mac}(22). How ubiquitination and proteasome function lead to restriction of the virus, however, is unknown. Before the discovery of rhTRIM5 α , *in vitro* purified 20S proteasomes were demonstrated to be capable of degrading incoming viral proteins(23). However, this process was not examined within intact cells, and the function of the proteasome with regard to incoming viral cores, especially in the context of rhTRIM5 α -mediated restriction, has remained largely unexplored. Therefore, we sought to characterize the interaction of rhTRIM5 α and incoming virions with the proteasome in order to provide a more complete picture of the involvement of the ubiquitin-proteasome pathway in rhTRIM5 α restriction.

Results

Proteasomes localize to rhTRIM5 α cytoplasmic bodies

To investigate the role of the proteasome in rhTRIM5 α restriction of HIV-1, we first determined where proteasomes localize within cells in relation to rhTRIM5 α during the intermediate stage of restriction that can be observed during proteasome inhibition(17). Proteasomes comprise many subunits (19) in both the 19S regulatory complex, which recognizes ubiquitinated cargo and begins to unfold it so that it can be fed through the central channel of the 20S catalytic core, which contains proteolytic active sites that degrade proteins into short polypeptides(24). To examine localization of proteasomes in cells expressing rhTRIM5 α during the intermediate stage of restriction, we performed antibody staining directed towards various proteasome subunits after treating cells with virus in the presence of proteasome inhibitor MG132. Staining with a limited panel of commercially available antibodies revealed that several 19S regulatory subunits, including Rpn10 and Rpt6, accumulated in rhTRIM5 α -containing cytoplasmic bodies (Figure 1).

Increased localization of proteasomes to rhTRIM5 α cytoplasmic bodies with proteasome inhibition

Our initial immunofluorescent studies did not identify an antibody to a 20S subunit that efficiently stained our cultured cells. Therefore, we utilized a fluorescent proteasome

construct, LMP2-GFP, which is a fluorescently tagged β subunit of the 20S catalytic core. This LMP2-GFP construct is incorporated into active endogenous proteasomes, and has been well characterized as representing functional proteasomes(25, 26). When expressed in cells containing HA-tagged rhTRIM5 α (3), the majority of LMP2-GFP is localized diffusely throughout the nucleus, but a subpopulation of these fluorescent proteasomes localize to puncta in the cytoplasm that overlap with rhTRIM5 α (Figure 2A). More relevant to restriction, however, is that these puncta containing fluorescent proteasomes and rhTRIM5 α can be observed in the presence of virus (Figure 2B). Moreover, inhibiting the proteasome with MG132, a drug that blocks the threonine residue of catalytic β subunits required for nucleophilic attack of peptide bonds during proteasomal degradation(27), induces a dramatic relocalization of LMP2-GFP to rhTRIM5 α cytoplasmic bodies (Figure 2C). Finally, during the intermediate stage of restriction that can be visualized in the presence of virus and MG132, fluorescently tagged proteasomes also exhibit extensive localization to rhTRIM5 α cytoplasmic bodies (Figure 2D) similar to that seen with untagged endogenous proteasomes (Figure 1). The presence of accumulations of proteasomes in cytoplasmic bodies under all conditions examined implies that some proteasomes are constitutively present in rhTRIM5 α cytoplasmic bodies, suggesting that there might be an interaction occurring between these components. As the bright puncta of proteasomes observed in these experiments certainly comprise many individual proteasomes, the presence of these complexes may indicate that rhTRIM5 α is responsible for recruiting multiple proteasomes to the site of restriction to more effectively prevent infection.

Images were analyzed using Imaris to measure the number of cytoplasmic complexes per cell containing concentrations of both LMP2-GFP and rhTRIM5 α . This quantification indicates that the greatest effect on relocalization is seen with proteasome inhibition alone or together with virus (Figure 2E). All experiments were imaged in a blinded fashion and analyzed with automated algorithms to prevent any potential bias. These results were similar when cells were treated with another proteasome inhibitor, epoxomicin(28, 29), suggesting that these changes in localization are induced by the inhibition of proteasome function rather than from off-target effects (Figure 2E and data not shown). The relocalization of additional proteasomes to cytoplasmic puncta upon proteasome inhibition mimics the change in localization of rhTRIM5 α that is induced by proteasome inhibitors(13), suggesting that the greater number of rhTRIM5 α cytoplasmic bodies present under these conditions also serves to recruit a greater concentration of proteasomes to these structures.

As proteasomes are involved in cellular stress responses(30, 31), their localization may be sensitive to cellular stresses such as transient transfection. To ensure that LMP2-GFP localization to cytoplasmic bodies (Figure 2) was not an artifact of transient transfection, these experiments were repeated in cell lines stably expressing LMP2-GFP. These experiments were also performed in a blinded fashion and analyzed using Imaris with automated algorithms (Supplemental Figure 1), and yielded results similar to those shown in Figure 2.

Virions associate with complexes containing proteasomes and rhTRIM5 α

Having seen that both endogenously expressed untagged (Figure 1) and fluorescently tagged proteasomes (Figure 2, Supplemental Figure 1) localize to rhTRIM5 α cytoplasmic bodies, we next asked whether virus might associate with proteasomes during the normal process of restriction or in the intermediate stage of restriction seen with proteasome inhibition. Cells expressing HA-tagged rhTRIM5 α were transfected with LMP2-GFP and either infected for four hours with mCherry-Vpr labeled virus(16, 32) alone or mCherry-Vpr labeled virus in the presence of proteasome inhibitor MG132 (Figure 3). The number of virions per cell associated with either LMP2-GFP, rhTRIM5 α , or both LMP2-GFP and rhTRIM5 α was manually counted in a blinded fashion, as was the total number of virions per cell (Table 1).

The programs we had previously used for other types of image analysis (softWoRx and Imaris) had only a limited ability to accurately identify small particles such as virions, so these experiments were quantified by manually counting each virion within each cell. As the manual analysis required for counting virions associated with cellular factors is rather laborious, we developed algorithms for Interactive Data Language (IDL) to enable automated quantification of association (see Materials and Methods). These image quantification methods are useful in that they minimize the time required for analysis, and furthermore any potential bias that could be introduced during manual analysis is automatically excluded using IDL. Results obtained from performing both types of analysis on the same dataset is shown in Table 1, and the numbers obtained with the automated IDL analysis are readily comparable to those obtained by manual counting.

Strikingly, almost all virions associated with rhTRIM5 α cytoplasmic bodies were also associated with concentrations of LMP2-GFP both in the absence of drug and under proteasome inhibition. A significantly greater proportion of virions associated with cytoplasmic complexes containing both LMP2-GFP and rhTRIM5 α was seen in the presence of MG132. This result is consistent with previous observations of virus sequestered within rhTRIM5 α cytoplasmic bodies during the intermediate stage of restriction seen during proteasome inhibition(17). The observation that nearly all virions associated with rhTRIM5 α are also associated with an accumulation of proteasomes is consistent with the model that rhTRIM5 α may utilize the proteasome during restriction.

A smaller proportion of virions were observed to be associated with LMP2-GFP in the absence of detectable HA-rhTRIM5 α signal. The percentage of virions associated with proteasomes in the absence of detectable HA-rhTRIM5 α signal did not change when the activity of the proteasome was inhibited with MG132. The presence of virions associated with proteasomes without a detectable level of rhTRIM5 α may indicate that proteasomes are recruited to viral complexes in the absence of rhTRIM5 α , albeit less efficiently.

To determine whether the observed complexes of rhTRIM5 α and proteasomes that associate with HIV represent restriction events, we performed similar experiments with SIV, which is not efficiently restricted by rhTRIM5 α (3, 10, 33, 34). Cells expressing HA-tagged rhTRIM5 α were infected with VSV-G pseudotyped HIV or SIV that had been fluorescently labeled with either GFP-Vpr (HIV) or GFP-Vpx (SIV), both in the presence of MG132, for four hours (Figure 4). As these virions were labeled with GFP, we were unable to use the fluorescently labeled LMP2-GFP to identify proteasomes in these cells as we did previously (Figure 3). Instead, cells were stained for endogenous proteasome subunit Rpt6, which we have shown exhibits a similar localization to LMP2-GFP (Figure 1). Images were analyzed both manually and using our newly developed algorithms for IDL to quantify the proportion of HIV or SIV virions associated with accumulations of proteasome subunit Rpt6, rhTRIM5 α , or both proteasome subunit Rpt6 and rhTRIM5 α . Also for these experiments, automated analysis using IDL performed very well compared to manual analysis, confirming the ability of these algorithms to accurately detect associations of virions with cellular proteins. Consistent with experiments using fluorescent proteasomes (Figure 3), a large proportion of HIV virions were observed to be associated with complexes containing both proteasomes (Rpt6) and rhTRIM5 α (Table 2). Analysis of GFP-Vpx labeled SIV virions, however, revealed only a small background level of association with such complexes (Table 2). These results support the involvement of complexes containing proteasomes and rhTRIM5 α in restriction.

In the case of both HIV and SIV, some virions were associated with proteasome subunits Rpt6 only, in the absence of detectable HA-rhTRIM5 α signal, as we observed with fluorescently tagged proteasomes. These results support the possibility that in the absence of

restriction, proteasomes are still capable of some level of nonspecific recognition of virions, but that this is insufficient to prevent infection. However, as we cannot determine which virions have completed fusion in these experiments, it is also possible that some of the virions associated with proteasomes alone are trapped in endosomes. Previous work has shown that only a small proportion of unfused virions associate with rhTRIM5 α cytoplasmic bodies(17), so it is likely that most of the virions associated with complexes of proteasomes and rhTRIM5 α observed in the present study have undergone fusion, but we cannot rule out this possibility for virions not associated with rhTRIM5 α . Finally, as fixed cell imaging can only provide a glimpse of what might be a very dynamic process, virions that appear to be associated with proteasomes alone could merely represent a transient, unproductive association.

Unfortunately the lack of available antibodies specific to rhTRIM5 α makes it impossible to determine whether endogenous rhTRIM5 α also localizes to such complexes. Because we are unable to visualize endogenous rhTRIM5 α , we instead chose to examine the localization of HIV in relation to endogenous proteasomes in primary rhesus macaque fibroblast cells. These cells express only endogenous rhTRIM5 α and are able to restrict HIV, thus allowing us to determine whether endogenous levels of rhTRIM5 α are sufficient for allowing proteasomes to associate with restricted virus. In these experiments, we treated primary rhesus macaque fibroblasts with interferon prior to infecting with VSV-G pseudotyped mCherry-Vpr labeled HIV for four hours either alone or in the presence of proteasome inhibitor MG132 for four hours (Figure 5). Although constitutively expressed, rhTRIM5 α is also upregulated in response to interferon(35, 36), a situation certainly relevant to infection. These experiments were imaged in a blinded fashion, and then quantified using IDL to determine the proportion of virions associated with endogenous proteasome subunit Rpt6. Consistent with our experiments in cells expressing tagged rhTRIM5 α , a significantly greater proportion of virions was observed to be associated with Rpt6 puncta with MG132 treatment compared to virus alone (Table 3). These results provide further support that the cytoplasmic complexes of virus and proteasomes we observed in the presence of tagged rhTRIM5 α (Figure 3 and Figure 4) are relevant to restriction.

Virus associates with proteasomes in living cells

The presence of virions in complexes containing proteasomes and rhTRIM5 α suggested that proteasomes may be capable of interacting with virus during restriction. To observe the dynamics of such interactions, live cell imaging was performed in cells stably expressing HA-tagged rhTRIM5 α and transiently expressing LMP2-GFP that had been infected with mCherry-Vpr labeled virus. An identical number of experiments was performed with virus alone as with virus in the presence of the proteasome inhibitor MG132. In analyzing these experiments, associations were only counted if the duration was at least one minute and both LMP2-GFP and mCherry-Vpr were in the same focal plane. Because we could only make these observations in two colors, it was not possible to ascertain if rhTRIM5 α was present in these complexes.

Notably, these live cell imaging experiments revealed that proteasomes and virus can associate with each other during early post-entry events occurring during restriction by rhTRIM5 α . A greater number of association events between virus and proteasome puncta were observed in experiments performed under proteasome inhibition compared to no treatment, with 89 events observed in the presence of MG132 and 26 events with no drug (Figure 6). The more frequent associations of virus with proteasomes seen with MG132 treatment are consistent with proteasome inhibition stabilizing the intermediate stage of restriction, and in line with previous studies examining the association of virions with rhTRIM5 α in living cells(17). This stabilization would allow virions to remain sequestered

in rhTRIM5 α cytoplasmic bodies(17), which we have shown are also enriched in proteasomes (Figure 1, Figure 2, Supplemental Figure 1, Figure 3, Figure 4).

In addition to revealing that virus can associate with proteasomes, these experiments also highlighted four types of associations that can occur between these two components. One type of association was long-term association between proteasomes and mCherry-Vpr labeled virus (Figure 7, Supplemental Movie 1). In this type of association, both the virus and the proteasome accumulation may be moving dynamically within the cell, but they remain associated with each other for some period of time (associations that endured for up to 30 minutes, the length of a typical imaging experiment, were observed). In other cases, a similar type of association was observed except the associated virus appeared to decrease in fluorescent intensity during its association with a concentration of proteasomes (Supplemental Movie 2). This decrease was quantified by measuring the maximal fluorescence intensity of the virus signal over the course of the association (Figure 8). As the decrease in virus fluorescence only occurs during some associations it is not likely to be due to photobleaching. Although the virus signal was not observed to disappear during imaging, the decrease in fluorescence intensity following proteasome association might suggest that the proteasome is acting on the virus to perturb its structural integrity. In the third type of association after several minutes of association with a proteasome accumulation, the virus was able to escape and continue to move throughout the cell during the course of imaging (Supplemental Movie 3). These brief associations might occur if the proteasomes are unable to act on the virus and instead move on to search for other cargo to degrade. However, it might also represent the virus and proteasome puncta passing through a small region of the cell without interacting, but appearing to be in a single complex because of the diffraction limit of fluorescent microscopy(37). The escape of virus from proteasomes after short associations was observed more frequently when the activity of the proteasome was inhibited with MG132 (Table 4). This observation suggests that the proteasome activity is important for maintaining a longer interaction with virus, which may represent the beginning of degradation of some component of the virus or of cytoplasmic bodies. Finally, a fourth type of association was observed in which the accumulation of proteasomes associated with a virus loses its punctate localization and becomes more diffuse. These types of events often left behind a virus that had decreased in fluorescence intensity during its association with the concentration of proteasomes. In more rare cases the diffusion of proteasomes occurred soon after the escape of a virus. The change in proteasome localization after viral association may suggest that after proteasomes have already acted on a virus a high concentration of proteasomes is no longer needed at this site. However, the loss of the punctate localization of proteasomes might also result from an unproductive association with the virus, for example, due to MG132 treatment.

To more quantitatively compare these different types of events and to assess whether the presence of a functional proteasome changes the way it interacts with virus, events were characterized by the total duration of association (Table 4, Figure 6). This quantitative analysis revealed that longer associations took place more frequently in the absence of drug. The average duration of associations seen with no drug was 14.0 minutes ($\pm$ SEM 2.1) compared to 6.5 minutes ($\pm$ SEM 0.7) for MG132 ($p < 0.01$ using a two-sample t-test with unequal variance). This observation of longer associations under conditions in which the proteasome is functional further supports the idea that the activity of the proteasome is stabilizing the interactions required to keep these two components localized together. Such interactions could involve proteasomal degradation of the virus or of virus-associated rhTRIM5 α .

Discussion

Despite the utility of proteasome inhibitors such as MG132 as experimental tools to elucidate the mechanism by which rhTRIM5 α restriction of HIV-1 occurs, the localization and dynamics of proteasomes in the context of restriction has not yet been explored within the cell. Here, we demonstrate that proteasomes localize to rhTRIM5 α cytoplasmic bodies and associate with virus.

First, we have shown that concentrations of proteasomes are present in rhTRIM5 α cytoplasmic bodies. The accumulation of proteasomes in rhTRIM5 α cytoplasmic bodies was observed for untagged endogenous 19S proteasome subunits (Figure 1) as well as for a fluorescently tagged 20S proteasome subunit expressed transiently (Figure 2) or stably (Supplemental Figure 1). Antibody staining revealed that several subunits of endogenous proteasomes (i.e. Rpn10 and Rpt6) localize to rhTRIM5 α cytoplasmic bodies (Figure 1), especially in the intermediate stage of restriction that can be visualized in the presence of virus and MG132. Such proteasome inhibitors reversibly block the activity of the proteasome(29) and will thus stabilize events that under normal conditions occur very quickly. The increased accumulation of proteasomes within rhTRIM5 α cytoplasmic bodies seen during proteasome inhibition could implicate the proteasome as transiently localizing to these structures during restriction. However, further analysis of proteasomal localization under different conditions revealed that a subpopulation of proteasomes localizes to rhTRIM5 α cytoplasmic bodies in all conditions examined, whether or not virus is present (Figure 2, Supplemental Figure 1). The vast majority of rhTRIM5 α cytoplasmic bodies contain a concentration of proteasomes, suggesting that proteasomes may be recruited to these cytoplasmic structures by rhTRIM5 α . These rhTRIM5 α -associated proteasomes would then be poised carry out proteasomal degradation of a viral or cellular component during restriction. Although some proteasomes always show an accumulation at rhTRIM5 α cytoplasmic bodies, treatment of the cells with a proteasome inhibitor increases the number of cytoplasmic puncta containing both proteasomes and rhTRIM5 α . Therefore, these data support a model in which rhTRIM5 α recruits proteasomes to cytoplasmic bodies to carry out the proteasome-dependent step of restriction.

Second, virus was frequently observed sequestered within cytoplasmic complexes containing both rhTRIM5 α and proteasomes (Figure 3). Nearly all virions associated with rhTRIM5 α are also associated with proteasome subunit LMP2-GFP (Table 1), implicating the proteasome as playing a role in the early events occurring during restriction of incoming virions. As previous work has shown that the presence of restricted virus induces proteasomal degradation of rhTRIM5 α (21), the presence of proteasomes at these sites is relevant to restriction. A smaller proportion of virions can also be detected associated with LMP2-GFP puncta that do not contain detectable levels rhTRIM5 α , suggesting that proteasomes may be capable of recruiting the virus in the absence of rhTRIM5 α . However, as the proportion of virions associated with proteasomes alone does not change with proteasome inhibition, these may merely be snapshots of transient interactions that will not lead to a functional degradation event. It is also possible that proteasomes are recruited to incoming viral complexes in a manner that is relevant, but not necessarily restrictive, to infection.

To further explore these questions, we performed experiments examining the localization of an unrestricted virus, SIV. A small proportion of SIV virions labeled with GFP-Vpx were observed to associate with proteasomes alone, similar to what we observed with HIV (Table 2). Importantly, however, SIV virions did not associate with complexes containing both rhTRIM5 α and proteasomes (Figure 4, Table 2), supporting the idea that such complexes are relevant to restriction.

Although there are no antibodies currently available that specifically recognize rhTRIM5 α , we performed experiments in primary rhesus macaque fibroblast cells to explore the localization of virus and proteasomes in a more physiologically relevant context. These experiments revealed that virus associates with cytoplasmic proteasome puncta even when only endogenous rhTRIM5 α is present (Figure 5, Table 3). Similar to what we observed in cells expressing tagged rhTRIM5 α (Figure 3, Table 1, Figure 4, Table 2), inhibiting the function of the proteasome pharmacologically led to a significantly greater proportion of virions associated with these proteasome puncta.

In addition to our observations regarding the association of incoming virions with cytoplasmic complexes of proteasomes and rhTRIM5 α , we also present here novel image analysis algorithms we have developed to use IDL to quantify the subcellular localization of viral particles. The development of this tool advances the efficiency of image quantification by allowing the use of larger datasets, and should prove useful in future studies examining the localization of virions within cells.

Third, concentrations of proteasomes are able to associate with HIV in living cells. Both proteasomes and virus were observed to move dynamically about the cell, but frequently remained stably associated with each other for up to 30 minutes (Supplemental Movie 1, Figure 7). Restriction of incoming virions by rhTRIM5 α occurs quickly, and durations of the length observed in these experiments may be sufficient to restrict the virus from infecting the cell. Independent of duration, more association events were observed in the presence of MG132 (Figure 6), which again is consistent with proteasome inhibitors stabilizing a transient intermediate stage of restriction. However, the majority of events in cells treated with MG132 were of a shorter duration (Table 4), implying that longer interactions depend on a functional proteasome. Characterizing the associations between virus and proteasome puncta revealed that several types of associations can be observed. One type of association was a long-term association (Supplemental Movie 1, Figure 7), and in some cases this was accompanied by a decrease in the fluorescent signal of the associated virus (Supplemental Movie 2, Figure 8). A greater proportion of these events were seen in experiments without drug (Table 4), suggesting that the activity of the proteasome is important to allow these types of associations. For example, the proteasome may act to degrade a component of the virus itself or a virus-associated cytoplasmic body protein. Either of these scenarios could serve to accelerate uncoating, which has been suggested as a mechanism of rhTRIM5 α restriction(9, 38), and such a structural change could be sufficient to explain the decrease of virus fluorescence. While some association events endured until the end of the movie, in other cases the virus was able to escape after associating with an accumulation of proteasomes (Supplemental Movie 3). These shorter associations that ended with the escape of virus were observed more frequently when the activity of the proteasome was inhibited by MG132 (Table 4), again suggesting that longer events may represent the beginning of proteasome-mediated degradation. Further live cell studies of an unrestricted virus would help to define which of these types of associations are specific to restriction.

A complication in the interpretation of our results is a lack of understanding of the localization of endogenously expressed rhTRIM5 α , because antibodies with the appropriate specificity to detect untagged protein have yet to be developed. The inability to study the localization and dynamic nature of endogenous rhTRIM5 α is a present issue in the field because of a lack of specific antibodies. But we believe that our observations are not artifacts of overexpression for several reasons. First, to date no function of rhTRIM5 α or any other TRIM family proteins has been shown to be due to overexpression. Using YFP-tagged rhTRIM5 α , diffuse cytoplasmic pools of protein have been shown to exist alongside cytoplasmic bodies, and the rhTRIM5 α protein is continuously cycling between these two pools, as we have previously reported(18). Furthermore, the rhTRIM5 α protein in both the

free cytoplasmic pool and in cytoplasmic bodies can be recruited to incoming virions, and in both cases the viral capsid becomes associated with a large number of rhTRIM5 α proteins. Finally, we observed that only restricted virus was able to associate with proteasomes and rhTRIM5 α (Figure 4, Table 2), and also observed virus associated with cytoplasmic proteasome puncta in primary rhesus macaque cells only expressing endogenous rhTRIM5 α (Figure 5, Table 3). Therefore, the association of proteasomes with rhTRIM5 α -virus complexes reported here is likely directly relevant to restriction mediated by endogenously expressed protein.

Taken together, these studies implicate the proteasomal destruction of a viral or cellular component of rhTRIM5 α cytoplasmic bodies as playing a role in restriction of incoming virions. Although a small proportion of virions appeared to be associated with proteasomes in the absence of a detectable concentration of rhTRIM5 α (Figure 3, Table 1), and this is also seen for a similar virus, SIV, that is not efficiently restricted by rhTRIM5 α (Figure 4, Table 2), the presence of rhTRIM5 α likely facilitates viral recruitment by proteasomes, leading to destruction of the integrity of the viral core and a successful block to infection. rhTRIM5 α is known to multimerize(39–41), and this multimerization is important for capsid binding and restriction(42–44). Recent studies show that a chimera of rhTRIM5 α and human TRIM21 (TRIM5-21R) that also restricts HIV-1 forms a hexagonal lattice larger than the capsid lattice, and that capsids promote the assembly of these TRIM5 α lattices(45). If rhTRIM5 α forms a lattice surrounding a restricted viral core, then proteasomal degradation of these rhTRIM5 α proteins could perturb the capsid coat on the virus and lead to premature uncoating, which would negatively impact infection(46). Our observation of a decrease in mCherry-Vpr signal during some associations with LMP2-GFP labeled proteasomes is consistent with this model in which the proteasome acts indirectly on the viral proteins of the reverse transcription complex. In this model as rhTRIM5 α is pulled into the proteasome for destruction, the force would lead to a premature disassembly of the higher order structure of the capsid protein. Future studies should help to delineate the mechanistic details of the proteasome's role during restriction, and future studies visualizing the fate of these proteasome-associated complexes would provide a more complete picture of how restriction takes place, which types of associations lead to restriction, and the fate of rhTRIM5 α -associated virions.

Materials and methods

Cells and pharmaceuticals

293T or HeLa cells (American Type Culture Collection) or HeLa cells stably expressing HA-tagged rhTRIM5 α were cultured in Dulbecco's modified Eagle's medium (HyClone) or DMEM/High Modified medium without phenol red (HyClone) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 292 μ g/mL μ -glutamine (Gibco). Primary rhesus macaque fibroblast cells, originally obtained from skin punch biopsy by Dr. Ronald Desrosiers, were cultured in DMEM containing 20% fetal bovine serum, 100 U/mL penicillin, 100 μ g/mL streptomycin, 292 μ g/mL μ -glutamine (Gibco), and 10 mM HEPES. MG132 (Sigma-Aldrich) was prepared in ethanol and stored at -20°C , and used at a final concentration of 1 μ g/mL. Epoxomicin (Sigma-Aldrich) was prepared in DMSO and stored at -20°C , and used at a final concentration of 3 μ M. DEAE-dextran (Sigma-Aldrich) was prepared in water, sterile filtered, and stored at 4°C , and used at a final concentration of 10 μ g/mL. Polybrene (Sigma-Aldrich) was prepared in water and stored at 4°C , and used at a final concentration of (10 μ g/mL). Universal Type I Interferon (PBL InterferonSource) was used at 1000 U/mL.

Transfection

HeLa cells stably expressing HA-tagged rhTRIM5 α were seeded onto coverslips (Fisherbrand) in 24-well plates (Falcon) after treating with fibronectin (Sigma-Aldrich), and the following day each well was transfected with 0.2 μ g of a construct encoding a fluorescently tagged β subunit of the proteasome, LMP2-GFP, using Effectene (Qiagen). Experiments were continued 24 hours post-transfection. For cells expressing LMP2-GFP, transfected cells were selected in 0.5 mg/mL G418 (Cellgro) and individual colonies were expanded.

Virus production

VSV-G pseudotyped virus was produced by polyethylenimine (PEI, Polysciences) transfection of 293T cells with an HIV-1 proviral plasmid (R7 Δ envGFP(47)) and the vesicular stomatitis virus envelope (VSV-G). Fluorescently labeled HIV was made by additional cotransfection of a plasmid encoding mCherry-Vpr or GFP-Vpr. For experiments using SIV, VSV-G pseudotyped SIV was produced by transfection of 293T cells with plasmids SIV Cherry HiC, VSV-G, SIV3+, DM121, and GFP-Vpx. Virus was harvested approximately 48 hours post-transfection, purified through 0.22 μ m filters, and stored at -80°C before use. Viruses were spun onto glass coverslips in the presence of polybrene (10 μ g/mL) and stained with monoclonal antibody AG3.0 (1:250 dilution) to detect HIV or SIV particles. Input virus for experiments using both HIV and SIV was normalized on the basis of the number of GFP-positive and AG3.0-positive particles detected on glass for 40 images per virus prep, and these numbers were quantified using IDL analysis. All virus preps used had high levels of incorporation of fluorescent proteins into viral particles, minimizing the detection of false positives. For experiments using labeled HIV only, cells were incubated with 200 ng/well VSV-G pseudotyped HIV-1 and 10 μ g/mL DEAE-dextran at 37°C for 4 hours either alone or in the presence of MG132. For experiments using primary rhesus fibroblasts, cells were treated with 1000 U/mL interferon for approximately 20 hours before adding virus.

Microscopy

Cells were fixed in 3.7% formaldehyde (Polysciences) in PIPES buffer. Coverslips were stained with rabbit-anti-HA (Sigma-Aldrich) at a 1:300 dilution followed by a fluorescently conjugated secondary antibody (Jackson ImmunoResearch) at a 1:400 dilution (AMCA donkey anti-rabbit) or a 1:1000 dilution (RedX donkey-anti-rabbit) prior to mounting onto slides (VWR) with Fluoro-Gel (Electron Microscopy Sciences). In some experiments cells were also stained with an antibody directed towards the Rpn10 (1:400 dilution) or Rpt6 (1:250 dilution) subunits of the proteasome (Enzo/Biomol) followed by a fluorescently conjugated secondary antibody at a 1:1000 dilution (Cy5 donkey-anti-mouse). Image stacks containing 20 sections in the Z plane, 0.2 μ m apart, were acquired and deconvolved using softWoRx software (Applied Precision) on a DeltaVision microscope.

Live cell imaging

HeLa cells stably expressing HA-tagged rhTRIM5 α were seeded onto delta T dishes (Fisher Scientific) and transfected with LMP2-GFP(25) as with fixed cell imaging. 24 hours after transfection, cells were incubated with VSV-G pseudotyped HIV-1 at 17°C for 1–2 hours in the presence of 10 μ g/mL DEAE-dextran. Unbound virus was removed and warm DMEM/High Modified medium was added to allow virus entry. Cells were immediately imaged on a DeltaVision deconvolution microscope equipped with an environmentally-controlled chamber to maintain cells at 37°C and 5% CO_2 .

Image quantification

Images were quantified using either softWoRx (Applied Precision) or Imaris (Bitplane) software, or algorithms we have written for Interactive Data Language (IDL). Prior to all types of analysis, images were shifted in the Z-plane to correct for chromatic aberration, according to values obtained after imaging virus on glass on the same microscope with the same parameters. Analysis using Imaris software (i.e. quantifying the number of cytoplasmic puncta containing LMP2-GFP and rhTRIM5 α) was performed in an automated fashion using the Colocalization module and Surface Finder function of the Surpass module, with the same algorithms for all images within an experiment. Algorithms included the following variables: surface grain size, diameter of largest sphere, manual threshold value, region growing estimated diameter, region growing background subtraction, and quality. All manual analysis using softWoRx (i.e. counting virions associated with LMP2-GFP or rhTRIM5 α) was done using identical display intensities for each experiment, such that only accumulations of rhTRIM5 α or proteasomes were visible during analysis, and the diffuse pool of protein was excluded from the analysis.

IDL analysis

Automated 3D Z-stack analysis using IDL was performed using algorithms written to quantify associations of particles. First, masks were manually outlined in the X-Y plane around cell membranes for inclusion in analysis, and around nuclei for exclusion. Nuclei were excluded from IDL analysis, to prevent the intense signal of nuclear proteasomes from being falsely identified as associated with virions located above the nucleus. Next, thresholds for each channel were chosen to be similar to those used in manual analysis, such that virus could be discriminated from background, and only punctate accumulations of proteasomes and rhTRIM5 α were included in the analysis. Finally, virions were identified by thresholding the appropriate channel (TRITC for mCherry-Vpr, or FITC for GFP-Vpr and GFP-Vpx). Each virion was assigned a unique ID, and the remaining channels were thresholded to determine whether each virion was also positive for proteasomes (FITC for LMP2-GFP or Cy5 for Rpt6) or rhTRIM5 α in the case of tagged rhTRIM5 α (DAPI for HA-rhTRIM5 α staining). The algorithm yields statistics on a per-virion and per-image basis for further statistical analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A. Rpn10 **B. Rpt6** **Figure 1. Endogenous proteasomes localize to rhTRIM5 α cytoplasmic bodies**

Cells stably expressing HA-tagged rhTRIM5 α were treated with virus and MG132 before fixing and staining for 19S proteasome subunits Rpn10 or Rpt6 (green) and HA (red).

Figure 2. Fluorescently tagged proteasomes localize to rhTRIM5 α cytoplasmic bodies
A. A subpopulation of fluorescently tagged proteasomes (LMP2-GFP) localize to rhTRIM5 α cytoplasmic bodies constitutively and **B.** in the presence of virus, and a greater proportion are recruited to cytoplasmic bodies **C.** with proteasome inhibitor treatment (MG132) alone or **D.** with proteasome inhibitor and virus treatment. **E.** A greater number of cytoplasmic puncta containing both fluorescently tagged proteasomes (LMP2-GFP) and rhTRIM5 α are detected after proteasome inhibition (MG132 or epoxomicin) alone or after proteasome inhibition in the presence of virus. Three independent experiments were performed, and values shown are average $\pm$ SEM from a representative experiment (N=40)

images for each condition). Bars indicate $p < 0.01$ between indicated treatments using a paired t-test.

Figure 3. Virions are frequently associated with complexes containing rhTRIM5 α and LMP2-GFP

mCherry-Vpr labeled HIV can be observed to be associated with complexes containing rhTRIM5 α and LMP2-GFP with **A.** no drug or **B.** MG132 treatment.

Figure 4. Unrestricted virions do not associate with complexes containing rhTRIM5 α and proteasome subunit Rpt6

A. GFP-Vpr labeled HIV can be seen associated with complexes containing rhTRIM5 α and 19S proteasome subunit Rpt6, but **B.** GFP-Vpx labeled SIV does not localize to these complexes.

Figure 5. Virions associate with endogenous proteasomes in primary rhesus macaque cells
mCherry-Vpr labeled HIV can be seen associated with puncta of endogenous proteasomes (Rpt6) with **A.** no drug or **B.** MG132 treatment.

Figure 6. Durations and types of associations observed between virus and proteasomes in living cells

Fluorescently labeled virus (mCherry-Vpr) and fluorescently tagged proteasomes (LMP2-GFP) were observed in live cells expressing HA-tagged rhTRIM5 α . An identical number of experiments were performed with no drug and with MG132 treatment to inhibit proteasome activity. More frequent associations were observed with proteasome inhibition (N=89 for MG132 compared to N=26 for no drug), but a greater proportion of events were of shorter duration and ended with escape of the virus.

Figure 7. Proteasomes and virus associate in living cells

Time-lapse images from a live cell imaging experiment in which cells expressing HA-tagged rhTRIM5 α were transfected with LMP2-GFP and infected with mCherry-Vpr labeled virus. Virus can be seen associated with an accumulation of proteasomes for the duration of the movie (30 minutes).

Figure 8. Decrease in fluorescence intensity of virus occurs during some associations with proteasomes in living cells

In this experiment, one association between virus and proteasomes is accompanied by a decrease in virus fluorescence (black triangles), while another association within the same cell is not (yellow squares).

Table 1

Virions are frequently associated with complexes containing rhTRIM5α and LMP2-GFP.

Manual analysis:		NO DRUG	MG132
LMP2-GFP and rhTRIM5α	12.11 (±1.25) [†]		25.28 (±1.30) [†]
rhTRIM5α	1.48 (±0.48)		2.95 (±0.51)
LMP2-GFP	6.95 (±1.18)		8.04 (±1.25)
IDL analysis:		NO DRUG	MG132
LMP2-GFP and rhTRIM5α	10.36 (±1.33) [†]		20.28 (±1.17) [†]
rhTRIM5α	1.46(±0.35)		4.65 (±0.90) [†]
LMP2-GFP	12.27 (±1.94)		12.28 (±1.89)

Numbers listed are the percent of total virions per cell associated with indicated protein(s).

Numbers do not add to 100% because not all virions are associated with these protein(s).

Three independent experiments were performed, and values shown are average ±SEM from a representative experiment (N=40 cells per condition).

The total number of virions counted by hand was 3,929 for no drug and 7,347 for MG132.

The total number of virions identified by IDL was 4,532 for no drug and 8,011 for MG132.

[†]p<0.01 between no drug and MG132 using a paired t-test.

*p<0.01 between indicated proteins using a two-sample homoscedastic t-test.

Table 2

Unrestricted virus does not associate with complexes containing rhTRIM5α and proteasomes.

Manual analysis:	HIV+MG132		SIV+MG132	
Rpt6 and rhTRIM5α	18.02 (±0.87) [†]		4.32 (±1.00) [†]	
rhTRIM5α	4.80 (±0.38) [†]		1.37 (±0.45) [†]	
	2.74 (±0.33)		5.92 (±1.18)	
Rpt6				
IDL analysis:				
	HIV + MG132		SIV + MG132	
Rpt6 and rhTRIM5α	16.67 (±0.76) [†]		2.85(±0.70) [†]	
rhTRIM5α	7.41 (±0.50) [†]		2.45 (±0.64) [†]	
	5.19 (±0.41)		5.92 (±1.09)	
Rpt6				

Numbers listed are the percent of total virions per cell associated with indicated protein(s).

Numbers do not add to 100% because not all virions are associated with these protein(s).

Three independent experiments were performed, and values shown are average ±SEM from a representative experiment (N=40 cells per condition).

The total number of virions counted by hand was 7,506 for HIV and 556 for SIV.

The total number of virions identified by IDL was 8,456 for HIV and 846 for SIV.

[†]p<0.01 between HIV and SIV using a paired t-test.

*p<0.01 between indicated proteins using a two-sample homoscedastic t-test.

Table 3

Virions are frequently associated with endogenous proteasome puncta in primary rhesus macaque fibroblasts.

IDL analysis:	NO DRUG	MG132
Rpt6	8.72 (± 0.64) [†]	17.92 (± 0.84) [†]

Numbers listed are the percent of total virions per cell associated with Rpt6 puncta.

Three independent experiments were performed, and values shown are average $\pm$ SEM from a representative experiment (N=40 cells per condition).

The total number of virions identified by IDL was 2,001 for no drug and 6,600 for MG132.

[†]p<0.01 between no drug and MG132 using a paired t-test.

Table 4

Associations between mCherry-vpr labeled virus and fluorescently tagged proteasomes (LMP2-GFP) in living cells expressing rhTRIM5 α .

A.	<u>NO DRUG</u>	<u>MG132</u>
1–10 minutes	46.15%	83.15%
10–20 minutes	23.08%	8.99%
20–30 minutes	30.77%	7.87%
B.	<u>NO DRUG</u>	<u>MG132</u>
Long-term association	28.57%	15.22%
Long-term + virus dim	39.29%	9.78%
Escape of virus	21.43%	71.74%
Diffuse proteasomes	10.71%	3.26%

Proportion of associations between virus and proteasomes represented **A.** by duration of association and **B.** by type of association.