Murine Cytomegalovirus US22 Protein pM140 Protects Its Binding Partner, pM141, from Proteasome-Dependent but Ubiquitin-Independent Degradation

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Stable assembly of murine cytomegalovirus (MCMV) virions in differentiated macrophages is dependent upon the expression of US22 family gene M140. The M140 protein (pM140) exists in complex with products of neighboring US22 genes. Here we report that pM140 protects its binding partner, pM141, from ubiquitin-independent proteasomal degradation. Protection is conferred by a stabilization domain mapping to amino acids 306 to 380 within pM140, and this domain is functionally independent from the region that confers binding of pM140 to pM141. The M140 protein thus contains multiple domains that collectively confer a structure necessary to function in virion assembly in macrophages.

Murine cytomegalovirus (MCMV) US22 family genes M36, M139, M140, and M141 promote efficient replication of the virus in macrophages (1, 8, 12, 17). The M139, M140, and M141 genes are clustered within the MCMV genome and appear to function cooperatively (10, 12). During infection, the protein M140 (pM140) forms a stable complex with pM141, and one or more larger complexes are formed by the addition of M139 gene products (15). Although these complexes are evident in infected fibroblasts as well as macrophages, they are required for optimal MCMV replication selectively in macrophages (1, 17). In the absence of M140, virion assembly in macrophages is defective, likely due to the reduced levels of the major capsid protein and tegument protein M25 (11). pM140 also confers stability to its binding partner, pM141; in the absence of the M140 gene, the half-life of pM141 is reduced from 2 h to 1 h (12). Deletion of M141 compromises virus replication in macrophages (12), and pM141 directs pM140 to a perinuclear region of infected macrophages adjacent to an enlarged microtubule organizing center with characteristics of an aggresome (11, 15). Aggresomes are sites where proteins are targeted for degradation by either the proteasome or autophagy (3, 6, 19). We therefore hypothesized that complexing of pM141 to pM140 rescues pM141 from degradation by the proteasome and/or autophagy.

pM141 is degraded by the proteasome in the absence of M140. To determine whether pM141 is degraded by the proteasome, steady-state levels of pM141 were compared in cells infected with MCMV in which the M140 gene was deleted (RVΔ140) or with revertant (wild-type [WT]) virus (12) in the presence or absence of proteasome inhibitors. Murine fibroblasts (NIH 3T3) or differentiated macrophages (IC-21, TIB-186; American Type Culture Collection, Manassas, VA) were infected with 2 to 4 PFU/cell RVΔ140 or revertant virus for 4 h prior to the addition of 25 μM or 75 μM MG132 (Calbiochem, Gibbstown, NJ), 1 μM or 5 μM epoxomicin (Calbiochem), or vehicle (dimethyl sulfoxide [DMSO]) alone. The cells were harvested 18 h posttreatment, and Western blot analysis was performed as previously described (12) to detect steady-state levels of pM141 (rabbit polyclonal antiserum) (12) and actin (monoclonal anti-β-actin; Sigma-Aldrich, St. Louis, MO). Secondary antibodies conjugated to infrared fluorescent dyes were used for detection with the LI-COR (Lincoln, NE) Odyssey infrared imaging system. Representative results from infected fibroblasts treated with MG132, epoxomicin, or vehicle are shown in Fig. 1. Results were similar for infected and treated macrophages (data not shown). Both proteasome inhibitors conferred a dose-responsive increase in the steady-state levels of pM141 in RVΔ140-infected cells. The data indicate that pM141 is degraded, at least in part, by the proteasome in the absence of its binding partner, pM140.

Additional experiments indicated that pM141 was stabilized by much shorter MG132 treatments (Fig. 1C). NIH 3T3 cells were treated with 75 μM MG132 3 h after RVΔ140 infection and harvested every 3 h to determine the interval between pM141 expression and MG132-induced stability. The data indicate that pM141 was stabilized 3 to 6 h posttreatment, implicating a direct effect of the proteasome inhibitor on stability.

pM141 is not degraded by autophagy. Because neither proteasome inhibitor restored steady-state levels of pM141 to WT levels, we considered the possibility that this protein, when not in complex with pM140, may also be degraded by autophagy. Monomeric forms of pM141 may be degraded by the proteasome, while aggregated forms may be degraded by autophagy, as is the case with mutant forms of the cellular endoplasmic reticulum protein torsin A (7). Autophagy is induced in MCMV-infected fibroblasts by 2 h postinfection (unpublished data), as evidenced by the turnover of microtubule-
bule-associated protein 1 light chain 3 II (LC3-II) (16). Nevertheless, treatment of RVΔ140-infected cells with the autophagy inhibitor 3-methyladenine (3-MA; Sigma) did not consistently increase steady-state levels of pM141 in the absence of M140. While 1 mM 3-MA clearly inhibited virus-induced autophagy in fibroblasts and macrophages, even a 5 mM concentration had no effect on pM141 levels (data not shown).

Degradation of pM141 is predominantly ubiquitin independent. Proteins destined for destruction by the proteasome are most often polyubiquitinated; therefore, we examined the extent to which pM141 is ubiquitinated in the absence of M140. Initially, we transiently expressed ubiquitin as a hemagglutinin (HA)-tagged molecule from the pMT123 plasmid (18) (a gift from Lubbertus Mulder, The Rockefeller University, New York, NY) in RVΔ140-infected fibroblasts to detect ubiquitinated forms of pM141. Fibroblasts expressing HA-tagged ubiquitin were infected with RVΔ140 in the presence of 75 μM MG132. Cell lysates were immunoprecipitated with anti-pM141 antibody, and the precipitates were subjected to Western blotting with anti-HA antibody (Roche). However, no ubiquitinated forms of pM141 were evident (data not shown).

FIG. 1. Steady-state levels of pM141 in infected NIH 3T3 fibroblasts in the presence or absence of proteasome inhibitors. Fibroblasts were mock infected or infected with revertant (WT) or RVΔ140 MCMV and treated for 18 h with MG132 (A), epoxomicin (B), or vehicle (DMSO) as described in the text. The cells were harvested, counted, and normalized based on the cell number prior to lysis and detection of pM141 (141) or actin by Western blotting. (C) Cells were infected and treated with MG132 (+) as described in the text for the indicated times (in hours) posttreatment and infection (post tx/inf). Cells not receiving MG132 (−) were treated with DMSO.

FIG. 2. Steady-state levels of pM141 in WT MCMV- or RVΔ140-infected ts20 cells. ts20 cells were mock infected or infected with WT or RVΔ140 MCMV (2 PFU/cell) and incubated at the permissive (35°C) or nonpermissive (39°C) temperatures. After approximately 18 h, the cells were harvested, counted, and normalized based on the cell number prior to Western blot analysis for detection of pM141 (141), p53, or actin.

FIG. 3. Steady-state levels of pM141 in the presence of increasing amounts of pFLAG140FL. NIH 3T3 fibroblasts were untreated (Null) or transfected with the indicated quantities of FLAG140FL. Approximately 48 h later, the transfected cells were infected with MCMV RVΔ140 (2 PFU/cell). The cells were harvested, counted, and normalized to the cell number approximately 18 h postinfection. (A) Western blot analysis was performed using antibodies to pM141 (141), pM140 (140), or actin. (B) M141 protein levels were normalized to levels of actin using infrared imaging as described in the text.
As an alternative approach, we assessed pM141 stability in ts20 cells (provided by Harvey Ozer, University of Medicine and Dentistry of New Jersey [UMDNJ]-New Jersey Medical School, Newark, NJ). These cells express a temperature-sensitive form of the E1 ubiquitin (Ub)-activating enzyme (2) such that incubation at the nonpermissive temperature of 39°C renders the enzyme dysfunctional (2). At 39°C, steady-state levels of proteins, such as p53 that are regulated by Ub, are increased (Fig. 2). In contrast to p53, pM141 in RVΔ140-infected cells was undetectable at either the permissive or nonpermissive temperature. Replicate experiments using up to 68% of total infected-cell lysates failed to detect pM141. The results were not due to differences in the replicative capacity of the mutant and revertant viruses at either temperature in ts20 cells (data not shown). The fact that the levels of pM141 were below the level of detection even at the nonpermissive temperature suggests that pM141 is predominantly degraded in a Ub-independent manner. This is similar to HCMV UL21a, also required for production of infectious virus (4) and the Ub-independent degradation of cellular retinoblastoma (Rb) (14) and Daxx (13) by HCMV pp71 during infection. These findings suggest that alternative pathways of protein degradation dominate during infection, perhaps as a result of virally induced alterations in the cellular proteasome (5).

pM141 stability is dependent upon pM140. Experiments were designed to assess the extent to which pM140 is responsible for protecting pM141 from degradation. A plasmid expressing full-length pM140 with an N-terminal FLAG tag (pFLAG140FL) was constructed by digesting His140FL (15) with HindIII, blunting the HindIII site, and digesting the linearized plasmid with BamHI. The resulting insert was ligated into BamHI/SmaI-digested p3XFLAG-CMV-7 (Sigma). Vari-
ous concentrations of this plasmid were transfected into fibroblasts that were infected 48 h later with RVΔ140 (2 PFU/cell). A dose-dependent increase in steady-state levels of pM141 was observed with increasing concentrations of FLAG140FL (Fig. 3). These results supported our hypothesis that pM140 protects pM141 from degradation.

The domain within pM140 that confers stabilization to pM141 is dispensable for complex formation. To determine the region of pM140 required to protect pM141 from degradation, we generated plasmids to express C-terminally truncated pM140 products (Fig. 4A). Using His140FL (15) as template DNA, the M140 gene was amplified by PCR with a forward primer upstream of the M140 start codon and with reverse primers that introduced a stop codon at position 381, 307, or 181. Each of the truncated gene products was subsequently cloned into BamHI/ApaI-digested FLAG140FL plasmid generating FLAG140USAcidic stabilized pM141 (Fig. 4B to D). These results map the stabilization domain within M140 to amino acids (aa) 306 to 380.

We speculated that the pM140 stabilization domain would overlap with the pM140 binding domain required for complexing with pM141. Therefore, the same M140 truncation mutants were tested for their ability to bind pM141. Experiments were performed as described above, with the addition of transfected FLAG-tagged bacterial alkaline phosphatase as a control. The lysates were immunoprecipitated with anti-pM141 serum followed by Western blotting to assess steady-state levels of pM141, M140 products (12), the M142 protein as a control for infectivity (9), and actin. Although expression of each of the truncated proteins increased with increasing amounts of transfected plasmid, only FLAG140USAcidic stabilized pM141 (Fig. 4E). These results support our hypothesis that pM140 protects pM141, nevertheless retained the ability to bind pM141. Thus, the M140 stabilization domain is independent of the binding domain.

There are two likely explanations for the finding that binding of pM140 is not sufficient for stabilization of pM141. First, a third binding partner may be recruited to the pM140-pM141 complex through pM140, and this protein may stabilize pM141 directly. At present, we have identified two pM140 binding partners: products of MCMV M139 and an unknown protein of 98 kDa. Published data indicate that the M139 products do not confer stability to pM141 (12). Proteomic analyses by mass spectrometry have identified other potential binding partners, but these require verification. A second possibility is that stabilization of pM141 is dependent on the conformation of pM140. In this scenario, binding of the truncated pM140 protein to pM141 may result in an altered conformation of pM141, exposing a degradation signal. A candidate degradation signal is the unstructured PEST sequence identified within the C-terminal region of pM141 downstream of motif IV (aa 360 to 378) (10).

In conclusion, pM140 protects its binding partner, pM141, from degradation by the proteasome in a predominantly ubiquitin-independent manner. Such protection is conferred by a stabilization domain mapping to amino acids 306 to 380 within pM140, and this domain is functionally independent from the region that confers binding of pM140 to pM141. The M140 protein thus contains multiple domains that collectively confer structure necessary to function in virion assembly in macrophages.

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