The ORF75c tegument protein of murine gammaherpesvirus 68 (MHV68) promotes the degradation of the antiviral promyelocytic leukemia (PML) protein. Surprisingly, MHV68 expressing a degradation-deficient ORF75c replicated in cell culture and in mice similar to the wild-type virus. However, in cells infected with this mutant virus, PML formed novel track-like structures that are induced by ORF61, the viral ribonucleotide reductase large subunit. These findings may explain why ORF75c mutant viruses unable to degrade PML had no demonstrable phenotype after infection.
methods used for the ORF75c-mu viruses. The BAC sequences were removed by passage through Vero-cre cells as described previously (22). Following infection in 3T12 cells, the ORF75c-mu virus expressed the Δ648-659 ORF75c protein at levels similar to that of wild-type ORF75c, and it had a similar localization pattern in the nucleus but failed to induce the PML degradation (Fig. 1B and C). The marker-rescued virus regained the ability to induce PML degradation (Fig. 1B and C) and grew in mouse fibroblasts similarly to the wild-type viruses (data not shown). Because MHV68 replicates more robustly in fibroblasts lacking PML (12), a multistep growth curve experiment was performed to determine whether losing the ability to induce PML degradation had an effect on MHV68 replication. Surprisingly, ORF75c-mu grew similarly to ORF75c-MR in both PML-expressing (PML1) and nonexpressing (PML0) cells (Fig. 1D). ORF75c-mu was also tested for its ability to replicate and establish long-term infection in mice. Female wild-type 129S1/SvImJ mice (6 to 8 weeks old) were purchased from The Jackson Laboratory (Bar Harbor, ME). The animal research protocol was approved by the Institutional Animal Care and Use Committees at Baylor College of Medicine, Houston, TX. Preparation of virus stocks, infection of mice, and analysis of acute and latent infection was carried out as described previously (22). Acute replication in the lungs and spleens after infection with the ORF75c-mu virus was comparable to that with the wild-type (ORF75c-MR) virus, as was the development of splenomegaly (Fig. 2A and B). At 42 days postinfection, when replicating virus had been cleared, the frequency of reactivation from splenocytes and peritoneal cells was similar between mice infected with ORF75c-mu virus and those infected with ORF75c-MR virus (Fig. 2C). Mechanically disrupted cells were

FIG 1 Expression of a mutant ORF75c protein deficient for PML degradation has no effect on virus growth in cell culture. (A) Diagram of the cysteine-rich region in ORF75c aligned with ORF75a and ORF75b. The 12-amino-acid insertion between residues 648 and 659 (red) in ORF75c is indicated, and cysteine residues are shown in blue. The coding region for this segment was deleted using PCR (12). (B) Immunoblot analyses of 3T12 cells infected with MHV68 expressing wild-type ORF75c (WT), the Δ648-659 ORF75c (mu), or the marker-rescued virus (MR) at 4 h postinfection with a multiplicity of infection (MOI) of 5. Specific proteins detected are indicated to the right of each blot with antibodies that have been described previously (11). (C) Immunofluorescence analyses of PML1 cells, which express a Flag-tagged PML (12), infected with ORF75c-mu or ORF75c-MR 3 h postinfection. Cells were stained for ORF75c (red) using mouse anti-ORF75c antibody (BN-6C12) (34), PML (green) using rabbit anti-Flag antibody (Sigma), and DAPI (4′,6-diamidino-2-phenylindole) (blue). (D) Multistep growth curve of the average viral titers from PML1-infected (solid lines) or PML0-infected (dashed lines) cells. Cells were infected with ORF75c-mu (gray) or ORF75c-MR (black) with an MOI of 0.01. Virus was harvested from infected cells at 0, 24, 48, 72, and 96 h postinfection, and titers were determined as described previously (12). Error bars indicate standard deviations from three replicate experiments.
also plated in parallel as described previously (22), and no significant levels of preformed infectious virus were detected for either virus (data not shown).

MHV68 ribonucleotide reductase (RNR) large subunit homolog (ORF61) induces PML NBs to form track-like structures. Since MHV68 replicates better in cells lacking PML protein, it seemed inconsistent that the ORF75c-mu virus had no observable growth phenotype. To identify possible explanations for this observation, we looked for whether PML was affected at later time points postinfection. Interestingly, we observed that PML formed track-like structures in more than 80% of infected cells, starting at 4 h postinfection with the ORF75c-mu virus (Fig. 3). To determine whether formation of the track-like structures was potentially dependent on the synthesis of viral proteins, 3T12 cells were treated before and during infection with cycloheximide (CHX) or phosphonoacetic acid (PAA) to inhibit viral protein synthesis or viral DNA replication, respectively. Treatment with CHX but not PAA was able to block formation of the track-like PML structures, which is consistent with results of the time course experiment shown in Fig. 3, although the onset of track-like PML structures was slightly delayed in PAA-treated cells (Fig. 4A and B). Together, these results suggested that the protein responsible for this action was an immediate early or early viral protein (but not a tegument protein) or possibly a cellular protein induced during virus infection but before viral DNA replication.

To identify a potential viral protein candidate that could in-
produce PML track-like structures, we screened a library of MHV68 ORF expression plasmids by transient transfection in 3T12 cells, followed by detection of PML localization by immunofluorescence assays. We found that transient expression of a viral early gene (23), ORF61, which is a homolog of the ribonucleotide reductase small subunit ORF60 and the Δ648-659 ORF75c did not affect PML staining patterns (Fig. 5A). In the context of infection, ORF61 localized as perinuclear foci in the cytoplasm and as track-like structures in the nucleus that colocalized with PML (Fig. 5B). Compared to the transient overexpression of ORF61 shown in Fig. 5A, PML track-like structures were observed in greater than 80% of infected cells. Colocalization of ORF61 and PML in the nucleus of PML1 cells (12) infected with ORF75c-mu virus was also observed (Fig. 5B). These cells were used because the antibodies for endogenous ORF61 and PML are both mouse monoclonal antibodies, and PML1 cells express a Flag-tagged PML that can be detected with a variety of nonmouse antibodies. Finally, we used ORF61-specific small interfering RNAs (siRNAs) (Custom ON-TARGETplus SMARTpool, consisting of the following siRNAs: ATGAGATGTTTACCCGTTA, CAAAGCTGTTGGCGTGTAA, GGCAAGGTCCTGCGAGGTA, ACAGCAACCTCTAAGGAAT) to deplete ORF61 expression during infection to confirm that it was the main driver for the formation of PML track-like structures. Western blot analysis indicated that ORF61-specific siRNAs were able to reduce ORF61 expression by only 30 to 40% in infected cells (data not shown). However, we were able to track ORF61 expression on an individual cell basis. To determine the cells in which the ORF61-specific siRNAs successfully depleted ORF61 expression, we focused on cells that expressed the early gene ORF57 but not ORF61. Cells were also transfected with control siRNAs (Dharmacon ON-TARGETplus nontargeting siRNAs). Approximately 10% of infected cells transfected with ORF61-specific siRNAs had no detectable ORF61 expression while continuing to express ORF57. Within this subset of cells, PML track-like structures were never observed when ORF61 was not expressed (Fig. 5C). In contrast, all infected cells transfected with the control siRNAs coexpressed ORF57 and ORF61, and PML track-like structures were associated with ORF61 expression (data not shown).

**Summary.** MHV68 ORF75c induces the rapid degradation of the antiviral PML protein after infection. To investigate the significance of this activity, we generated a virus that expressed a mutant form of ORF75c that was unable to induce degradation of the PML protein. Surprisingly, this virus grew in tissue culture and in mice with kinetics similar to that of the wild-type virus (Fig. 1 and 2). A more detailed examination of infection with ORF75c-mu revealed that PML was induced to form novel track-like structures, which is mediated by the viral homolog of the large ribonucleotide reductase subunit (ORF61) (Fig. 3 to 5). These findings indicate that MHV68 encodes a second PML-modifying protein and may explain why viruses expressing a mutant form of ORF75c that is unable to induce PML degradation had no observable replication defect in tissue culture or infection in mice.

To generate an ORF75c protein unable to induce PML degradation, we identified a 12-amino-acid insertion (amino acid residues 648 to 659) within ORF75c that was critical for this function. Although ORF75c does not contain canonical RING finger or HECT domains, this insertion lies within a cysteine-rich region (Fig. 1A). However, it has been shown before that a cyst-rich domain is essential for mediating E3 ubiquitin ligase activity of another gammaherpesvirus protein, KSHV Rta (24). Therefore, the deletion within the cyst-rich region of ORF75c might be sufficient to disrupt its E3 ubiquitin ligase activity (11), which promotes PML poly-ubiquitination, leading to its degradation via the proteasome (12).
While the ORF75c-mu virus failed to initiate PML degradation, PML was clearly altered during infection with the mutant virus, as evidenced by its formation of track-like structures mediated by ORF61, as early as 4 h postinfection. ORF61, a viral homolog of the ribonucleotide reductase large subunit, has been shown previously to be important for infectious virion production in vitro and establishment of upper respiratory tract infection and virus transport from lungs to lymphoid organs in vivo (25). However, it is difficult to assess the impact of ORF61 on PML during infection from the previous studies because the viruses used still expressed a wild-type ORF75c, which presumably initiated the degradation of PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22). It is unclear why MHV68 evolved an additional protein to modulate PML. In addition, the effect on establishment and maintenance of latency or reactivation was not assessed in peritoneal cells from mice infected with the ORF61-null virus, where in a previous study by our laboratory, PML had a significant role in modulating these processes rather than during acute infection (22).

PML track-like structures have also been observed in adenovirus-infected cells and are induced by the E4 ORF3 protein (16). E4 ORF3 is a small protein (~13 kDa) that forms a nuclear polymer which disrupts large cellular protein complexes, including PML NBs, and this appears to facilitate adenovirus replication (16, 27–29). While ORF61 and E4 ORF3 do not share any recognizable sequence homology, it appears that ORF61 has evolved a similar mode of action to inactivate PML. Alpha- and gammaherpesviruses encode functional ribonucleotide reductase (RNR) enzymes composed of two subunits (R1 and R2) (30). Interestingly, betaherpesviruses encode only an R1 subunit and are catalytically inactive (30). Evidence is emerging that herpesvirus RNRs have evolved to carry out additional functions (30). For example, the R1 homologs in HSV (ICP10) and murine cytomegalovirus (M45) mediate anti-apoptotic functions by blocking tumor necrosis factor alpha (TNF-α)-induced apoptosis and interaction with receptor-interacting protein 1 (RIP1), respectively (30–33). Amino acid sequence alignment of MHV68 ORF61 with R1 proteins from HSV, EBV, and other rhadinoviruses, including KSHV and HVS, indicates that the GxGxxG nucleotide-binding site and cysteine and tyrosine residues that are important for catalytic function of R1 are conserved in MHV68 ORF61 (data not shown). This suggests that MHV 68 ORF61 possibly retains RNR function. Future studies will be needed to identify the novel domains within ORF61 that mediate the formation of PML track-like structures and identify candidate mutant proteins that can be expressed within the context of the ORF75-mu virus to fully assess the impact of PML on MHV68 replication in cell culture and in mice. These studies are also likely to yield insight into yet additional

FIG 4 PML track-like structures are induced by an immediate early or early viral gene. (A) 3T12 cells were treated with 50 μg/ml cycloheximide (CHX) for 1 h before and during infection with ORF75c-mu. Cells were stained for PML (green) using a mouse anti-PML antibody (Millipore), ORF57 (red) using rabbit anti-ORF57 antibody (11), and DAPI (blue) at 4, 8, and 24 h postinfection. Mock-infected cells are shown in the farthest right-hand panels. (B) 3T12 cells were infected with ORF75c-mu as described for panel A, but instead they were treated with 200 μg/ml phosphonoacetic acid (PAA). An inset shows an enlargement of PML track-like structures in the nucleus of the same infected cell shown at 8 h postinfection.

MHV68 Encodes a Second PML-Modifying Protein

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Adaptations that herpesviruses have made via acquisition of new functions through their RNR-encoded proteins.

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FIG 5 PML track-like structures are induced by ORF61. (A) Immunofluorescence analyses of 3T12 cells transfected with expression plasmids encoding hemagglutinin (HA)-tagged ORF61, ORF60, or the \( \Delta 648-659 \) ORF75c. Twenty-four hours posttransfection, cells were stained for PML (green) using mouse anti-PML antibody (Millipore), HA (red) using a rabbit anti-HA antibody (Millipore), and DAPI (blue). Cells expressing each of these proteins are indicated to the left of each panel. (B) Immunofluorescence analyses of PML1 cells infected with ORF75c-mu or ORF75c-WT virus. Twenty-four hours postinfection, cells were fixed and stained for PML (green) using a rabbit anti-Flag antibody (Sigma), ORF61 (red) using mouse anti-ORF61 antibody (BZ-5B2) (35), and DAPI (blue). Insets show an enlargement of track-like structures in the nuclei of the same infected cells. (C) Immunofluorescence analyses showing two different microscopic fields of PML1 cells transfected with a customized siRNA pool against MHV68 ORF61 for 24 h before being infected with ORF75c-mu. Cells were fixed 24 h postinfection and stained for PML (green) using rat anti-Flag antibody (Novus Biologicals), ORF61 (red) using mouse anti-ORF61 antibody (BZ-5B2), and ORF57 (blue) using rabbit anti-ORF57 antibody as described for Fig. 2 and 3. White arrows indicate infected cells in which ORF61 was effectively depleted.


